Université de Lille 1 – Sciences et Technologies

Mémoire présenté par

Sébastien FLAJOLLET

En vue de l'obtention du

Diplôme National
d'Habilitation à Diriger des Recherches

Caractérisation et Etude du Réseau d'Interaction des Facteurs de Transcription TMPRSS2:ETS dans les Cellules Cancéreuses de Prostate

Soutenance le 10 décembre 2013

Jury :

Président	Pr Xuefen Le Bourhis, Université de Lille 1
Rapporteurs	Dr Olivier Cuvillier, Université de Toulouse
	Pr Lluis Fajas, Université de Lausanne
	Dr Virginie Vlaeminck-Guillem, Université de Lyon 1
Examinateur	Dr Yvan de Launoit, Institut de Biologie de Lille
Garant de l'HDR	Dr Martine Duterque-Coquillaud, Institut de Biologie de Lille

Remerciements

Je commencerai par remercier

Monsieur Jean François Pauwels, Vice président de l'Université de Lille 1 chargé de la Recherche et de la Valorisation et des Etudes Doctorales, ainsi que les membres du Conseil Scientifique de l'Université de Lille 1 pour avoir accepté la soutenance de cette Habilitation à Diriger des Recherches dans leur établissement ;

les Professeurs Xuefen Le Bourhis, Olivier Cuvillier, Lluis Fajas et Virginie Vlaeminck-Guillem pour avoir accepté spontanément d'endosser les rôles de rapporteurs de ce mémoire et de participer à ce jury de HDR ;

les Professeurs Yvan de Launoit, Pierre Formstecher, Edgardo D. Carosella pour m'avoir accueilli au sein de leur laboratoire respectif ;

les docteurs Philippe Lefebvre et Philippe Moreau pour m'avoir fait confiance, pour nos échanges fructueux et pour vos conseils ;

et le docteur Martine Duterque pour t'être portée Garant de cette HDR, pour ton investissement, ton soutien et le dynamisme que tu sais impulser à chacun des membres de l'équipe.

Un grand merci à toutes les personnes de l'IBL, de J&K, du SRHI, de l'U459 et des différentes plateformes que j'ai côtoyées au cours de ces années. Impossible de les nommer toutes, la liste pourrait être longue et je risque d'en oublier. Je garde à chaque fois des collègues, des amis et un excellent souvenir de ces différents moments qui ont marqué mes débuts de chercheur doctorant et post-doctorant. Je les remercie donc toutes ici.

Néanmoins un clin d'œil

à Nath, Anne et Carine pour votre motivation, votre « positive attitude » et cette ambiance qui fait que venir au labo n'est pas que mener un projet scientifique,

aux étudiants: Linda, Vanessa, Céline, Gwendoline, Damien, Marcel…et Céline,

à Claude et Pierre-Marie pour mes premiers pas dans l'enseignement,

à Céline et Isa, mes paires de mains supplémentaires,

à Ludo et Hervé, pour votre enthousiasme dans la mise en place de nouvelles choses,

à Mademoiselle Laurissa et Rami, mes colocataires de bureau,

à Bin' et Tonze Mentor, pour votre amitié,

à mes amis…et les géants.

Enfin, je terminerai par les personnes qui me sont les plus chères :

Séverine pour ton soutien sans faille de tous les jours, je ne pensais pas écrire tout ça si vite,

Benjamin, Sylvain et Baptiste, pour tout ce que vous m'apportez…

Mes parents, pour m'avoir soutenu dans ce que j'entreprenais,

mes « petites » sœurs, ma grand-mère, et mes beaux-parents pour vos encouragements.

« Un chef d'orchestre a son utilité. C'est quand il arrête de remuer les bras que les sourds savent qu'ils peuvent applaudir. », Philippe Geluck

Sommaire

Curriculum vitae

Sébastien FLAJOLLET

Né le 29 juin 1978 à Grande-Synthe
Marié, 3 enfants

Situation actuelle : Chercheur Post-doctorant à l'UMR8161/CNRS/Institut de Biologie de Lille
Equipe : Protéines Ets, Régulation transcriptionnelle et Pathologies associées
Tel.: +33 (0) 3.20.87.12.33 sebastien.flajollet@ibl.fr

Le Cursus : Parcours académique et professionnel

Parcours académique

1999-2000 **Licence de Biologie mention Biologie Cellulaire et Physiologie**
Université des Sciences et Technologies de Lille 1
Mention assez bien

2000-2001 **Maîtrise de Biologie Cellulaire et Physiologie mention Génétique Moléculaire et Cellulaire**
Université des Sciences et Technologies de Lille 1
Mention assez bien

2001-2002 **DEA/Master Biologie et Santé**
Ecole doctorale de Biologie et Santé de Lille
Mention assez bien

2002-2006 **Doctorat en Sciences de la Vie et de la Santé**
Ecole doctorale de Biologie et Santé de Lille, Université de Lille 2
Mention très honorable avec les félicitations du jury

2009 **Formation à l'Expérimentation Animale – Niveau 1**
« De la modification génétique à l'exploration fonctionnelle et comportementale », Institut Pasteur de Lille

Parcours professionnel

2001-2002 **Stagiaire Diplôme d'Etudes Approfondies DEA/Master**
Unité de Recherche INSERM U459, « Signaux, Récepteurs nucléaires et Différenciation cellulaire »
Sujet : Régulation de la réponse transcriptionnelle dans les cellules tumorales humaines en réponse aux rétinoïdes.
Responsable scientifique : Dr Christophe Rachez
Financement : Bourse de DEA

2002-2006 **Chercheur doctorant**
Unité de Recherche INSERM U459, « Signaux, Récepteurs nucléaires et Différenciation cellulaire »
Thématique de recherche : Etude du mécanisme de la régulation de la transcription en réponse aux rétinoïdes.
Responsable scientifique : Dr Philippe Lefebvre
Financement : Allocation de recherche cofinancée par l'INSERM et la Région

Nord-Pas-de-Calais, 4ème année financée par la Ligue Nationale contre le Cancer

2007-2008 **Chercheur post-doctorant**
Service de Recherches en Hémato-Immunologie au CEA, Hôpital Saint Louis, Paris.
Thématique de recherche : Mécanismes épigénétiques et facteurs de transcription de la régulation de l'expression de la molécule immuno-régulatrice HLA-G.
Responsable scientifique : Dr Philippe Moreau
Financement : Contrat ingénieur postdoctorant, CEA

2008-2010 **Chercheur post-doctorant**
Unité de Recherche CNRS UMR 8161, « Approches génétiques, fonctionnelles et structurales des cancers »
Thématique de recherche : Identification des gènes régulés par le facteur transcriptionnel ERG et impliqués dans la chondrogenèse.
Responsable scientifique : Dr Martine Duterque-Coquillaud
Financement : Contrat ingénieur de recherche ANR géré par le CNRS

2011 **Chercheur post-doctorant**
Unité de recherche INSERM U1011, « Récepteurs nucléaires, maladies cardiovasculaires et diabète »
Thématique de recherche : Etude des gènes régulés par le glucose
Responsable scientifique : Dr Philippe Lefebvre
Financement : Contrat post-doctoral géré par Adrinord

Depuis 2012 **Chercheur post-doctorant**
Unité de Recherche CNRS UMR 8161, « Approches génétiques, fonctionnelles et structurales des cancers »
Thématique de recherche : Rôle des gènes de fusion TMPRSS2-ETS dans la formation des métastases osseuses dérivées du cancer de la prostate
Responsable scientifique : Dr Martine Duterque-Coquillaud
Financement : Contrat ingénieur de recherche INCA géré par le CNRS

Activité en matière d'enseignement et d'encadrement

Enseignement

2008-2009 - TD en Master 1 Biologie et Santé, Université de Lille 2, module de Biologie cellulaire – UE méthodes d'Etudes en biologie cellulaire : sensibiliser les étudiants en médecine dès la fin du 1er cycle, former les futurs médecins hospitaliers et hospitalo-universitaires à la recherche médicale
Responsables : Pierre Formstecher et Claude Mereau

2009-2011 - Cours et TD en Master 1 Biologie et Santé, Université de Lille 2, module de Biologie cellulaire – UE méthodes d'Etudes en biologie cellulaire : analyses d'articles scientifiques et encadrement ; participation aux jurys d'examen
- TD en Master 1 Biologie et Santé, Université de Lille 2, module de Biologie cellulaire – UE Différenciation et Oncogenèse : analyses d'articles scientifiques
Responsables : Claude Preudhomme et Pierre-Marie Danze

- Journée thématique en Master 2 de Recherche Biologie Santé. Spécialité C « Génétique et Microbiologie » : étude de la fonction biologique d'un gène par inactivation ou RNAi.
Responsable : Martine Duterque

Encadrement

Juin 2003 - Accueil de lycéen en Stage de découverte professionnelle (1 semaine) avec l'accord de Philippe Lefebvre et Gilbert Briand (Service spectrométrie de masse)

2003-2004 - Co-encadrement d'une étudiante en Diplôme Universitaire Sciences du médicament et Sciences Biologiques (Institut pharmaceutique de Lille) avec l'accord de Philippe Lefebvre : Linda Cambula
« Etude de différents coactivateurs par la technique d'ARN interférence »

Juin 2004 - Accueil de collégiens en Stage de découverte professionnelle (1 semaine) avec l'accord de Philippe Lefebvre

2004-2005 - Encadrement d'une étudiante en Diplôme Universitaire Sciences du médicament et Sciences Biologiques (Institut pharmaceutique de Lille) avec l'accord de Philippe Lefebvre : Vanessa Petit
« Etude de l'interaction du complexe coactivateur SWI/SNF dans le mécanisme d'activation de la transcription de différents récepteurs nucléaires »

2005 - Encadrement d'une étudiante en Maîtrise de biologie (Université de Lille1/Lille2) avec l'accord de Philippe Lefebvre: Céline Cudejko
« Etude fonctionnelle de BAF60 et BAF250 »

2007 - Encadrement d'une étudiante en Licence professionnelle (Ecole Supérieure des Techniques de Biologie Appliquée, Paris) avec l'accord de Philippe Moreau : Gwendoline Dubois
« Etude de l'implication de deux facteurs de transcription HIF1α et RREB1» dans la régulation de HLA-G »

2008 - Encadrement d'un étudiant en 1ère année de BTS en biophysique (Ecole Technique Supérieure de Laboratoire, Paris 13): Damien Durand
« Etude de l'implication du complexe CtBP via son recrutement par RREB1 dans la régulation de l'expression du gène HLA-G »

2011 - Encadrement d'un étudiant chirurgien dentaire en Master 1 Recherche : Marcel Decrequy

2013 - Encadrement de deux stagiaires (3ème et 1ère) dans le cadre d'un projet « Apprentis chercheurs » : Ombeline Charles et Antoine Lefebvre.

Qualification - CNU 64- Biologie moléculaire, obtenue en 01/2007
- CNU 65-Biologie cellulaire, N°10265178299, obtenue le 21/01/2010

Concours - MCF N°0252, CNU 87, 2009, Président : Pr Sophie Lestavel, classé
- MCF N°0714, CNU 64-65, chaire d'excellence, 2010, Président : Pr Lansiaux Amélie, classé
- MCF N°0473, CNU 65, 2011, Président : Pr Pierre Hardouin, classé 2nd.

Publications scientifiques

Publications originales dans des revues à comité de lecture		**12**
En 1er auteur	6	
En co-auteur	6	
Publications de revue dans des revues à comité de lecture		**4**
En 1er auteur	2	
En co-auteur	2	
Publications effectuées dans le cadre de congrès internationaux		**18**

Publications

Publications originales dans des revues à comité de lecture.

1. **Distinct roles of the steroid receptor coactivator 1 and of MED1 in retinoid-induced transcription and cellular differentiation.**
   Flajollet S., Lefebvre B., Rachez C., and Lefebvre P., J. Biol. Chem., 2006

2. **Down-regulation of the tumor suppressor gene RARβ2 through the PI3K/Akt signaling pathway.**
   Lefebvre B., Brand C., Flajollet S., and Lefebvre P., Mol. Endocrinol., 2006

3. **The core component of the mammalian SWI/SNF complex SMARCD3/BAF60 is a coactivator for the nuclear retinoic acid receptor.**
   Flajollet S., Lefebvre B., Cudejko C., Staels B., and Lefebvre P., Mol. Cell., Endocrinol., 2007

4. **Coadministration of Coenzyme Q prevents Rosiglitazone-induced adipogenesis in ob/ob mice.**
   Carmona M. C., Lefebvre P., Lefebvre B., Galinier A., Benani A., Jeanson Y., Louche K., Flajollet S., Ktorza A., Dacquet C., Pénicaud L. and Casteilla L., Int. J. Obes. (Lond.), 2009

5. **RREB-1 is a transcriptional repressor of HLA-G.**
   Flajollet S., Poras I., Carosella E.D., Moreau P., Journal of Immunology., 2009

6. **Increased Soluble HLA-G Levels in Peripheral Blood from Climbers on Mount Everest.**
   Bourguigon M., Yaghi L., Flajollet S., Radanne-Krawice I., Rouas-Freiss N., Lugrin D., Richalet J.-P., Carosella E.D., Moreau P., Hum Immunol., 2010

7. **Abnormal Expression of the ERG Transcription Factor in Prostate Cancer Cells Activates Osteopontin.**
   Flajollet S., Tian T., Flourens A., Tomavo N., Villers A., Bonnelye E., Aubert S., Leroy X., Duterque-Coquillaud M., Mol. Cancer Research, 2011

8. **Increased adipogenesis in cultured embryonic chondrocytes and in adult bone marrow of dominant negative Erg transgenic mice.**
   Flajollet S., Tian T., Huot L., Tomavo N., Flourens A., Holder-Espinasse M., Le Jeune M., Dumont P., Hot D., Mallein-Gerin F., Duterque-Coquillaud M., Plos One, 2012

9. **Human Leukocyte Antigen-G Is Frequently Expressed in a Multicentric Study on Glioblastoma and May Be Induced in Vitro by Combined 5-aza-2'-deoxycytidine and Interferon-gamma Treatments.**
   Wastowski I.J., Simões R.T., Yaghi L., Donadi E.A., Pancoto J.T., Poras I., Lechapt-Zalcman E., Bernaudin M., Valable S., Carlotti C.G. Jr, Flajollet S., Jensen S.S., Ferrone S., Carosella E.D., Kristensen B.W., Moreau P., Am J Pathol., 2012

10. **Targeting the DNA-binding activity of the human ERG transcription factor using new heterocyclic dithiophene diamidines.**
    Nhili R., Peixoto P., Depauw S., Flajollet S., Dezitter X., Munde M.M., Ismail M.A., Kumar A., Farahat A.A., Stephens C.E., Duterque-Coquillaud M., David Wilson W., Boykin D.W., David-Cordonnier M.H., Nucleic Acids Res., 2013

11. **Identification of novel TMPRSS2:ERG mechanisms in prostate cancer progression: involvement of MMP9 and PLXNA2**
    Tian T., Tomavo N., Huot L., Flourens A., Bonnelye E., Flajollet S., Hot D., Leroy X., de Launoit Y., Duterque-Coquillaud M., Oncogene, 2013

12. **The elongation complex components BRD4 and MLLT3/AF9 are transcriptional coactivators of nuclear retinoid receptors.**
    Flajollet S., Rachez C. , Ploton M. , Schutz C. , Gallais R. , Metivier R. , Pawlak M., Leray A., Issulahi A.A., Heliot L., Staels B., Salbert G., Lefebvre P., PlosOne, 2013

Publications de revue dans des revues à comité de lecture.

13. **Transcriptional activities of retinoic Acid receptors.**
    Lefebvre P., Martin P.J., Flajollet S., Dedieu S., Billaut X., Lefebvre B., Vitam. Horm., 2005

14. **Non classical transcriptional regulation of HLA-G : An update.**
    Moreau P.*, Flajollet S.*, Carosella E. D., J. Cell. Mol. Med., 2009
    (* Contribution équivalente des 2 auteurs à ce travail)

15. **Deciphering signaling mechanisms controling chondrocyte differentiation: Application to tissue engineering of cartilage: The ANR-TecSan PROMOCART project**
    Claus S., Aubert-Foucher E., Perrier-Groult E., Bougault C., Ronziere M.C.., Freyria A.M., Legendre F., Ollitrault D., Boumediene K., Demoor M., Galera P., Tian T., Flajollet S., Duterque-Coquillaud M., Damour O., Chajra H., Mallein-Gerin F., IRBM, 2011

16. **Retinoids and nuclear retinoid receptors in white and brown adipose tissues: physio-pathologic aspects**
    Flajollet S., Staels B., Lefebvre P., HMBCI, 2013

Communications

Communications effectuées dans le cadre de congrès internationaux

1. **Analysis of transcriptional coregulators recruited by the retinoic acid receptor RARα**
   Rachez C., Melotte P., Flajollet S., Belaiche D., Lefebvre P.
   EMBO Conference “Biology of Nuclear Receptors”, Villefranche-sur-Mer (France), 4-7 juin 2003, Poster

2. **Mécanisme de régulation de la réponse transcriptionnelle aux rétinoïdes**
   Flajollet S., Lefebvre B., Cambula L., Rachez C, Lefebvre P.
   XXIVème Forum de Cancérologie/Eurocancer 2004, Paris (France), 29 juin 2004, Poster
   Acte dans Bulletin du Cancer. Volume 91, Numéro 6, 471-568, Juin 2004, XXIVe Forum de Cancérologie

3. **Mécanisme de régulation de la réponse transcriptionnelle aux rétinoïdes**
   Flajollet S., Lefebvre B., Cambula L., Rachez C, Lefebvre P.
   3ème Journée André Verbert, Colloque Annuel des Doctorants de l'Ecole Doctorale Biologie Santé de Lille, Lille (France), septembre 2004, Poster

4. **Functional importance of some coactivators in the mechanism of transcriptionnal regulation by nuclear retinoid receptors**
   Flajollet S., Lefebvre B., Cambula L., Rachez C, Lefebvre P.
   Nuclear Receptors 2004, Stockholm (Suède), 10-13 octobre 2004, Poster

5. **Recruitment of chromatin remodeling coactivators during retinoid-induced response**
   Flajollet S., Lefebvre B., Rachez C., Lefebvre P.
   Gene regulation: from chromatin remodelling to transcription, Paris (France), 24-25 novembre 2005, Poster

6. **Crucial role of adipose Coenzyme Q content in Obesity**
   Carmona MC., Lefebvre P., Lefebvre B., Galinier A., Benani A., Jeanson Y., Louche K., Flajollet S., Nybbelink M., Fernandez Y., De Fanti B., Ktorza A., Dacquet C., Pénicaud L. and Casteilla L.
   15th European Congress on Obesity, Budapest (Hongrie), 2007, Poster
   Acte dans Int J Obes (Lond). 2007 May;31 Suppl 1:S1-225.

7. **Epigenetic and regulation of HLA-G expression in U251MG glioblastoma cell line**
   Simoes R., Flajollet S., Sangrouber D., Ferrone S., Carosella E., Moreau P.
   Colloque Cancer Genome et epigénome-INCA, Paris (France), 13-14 décembre 2007, Poster

8. **Micro-environnement et facteurs de transcription impliqués dans l'expression de HLA-G**
   Flajollet S., Rousseau P., Dubois G., Carosella E.D., Moreau P.
   XXVIIIème Forum de Cancérologie/Eurocancer 2008, Paris (France), 24-26 juin 2008, Poster
   Acte dans Bulletin du Cancer. Volume 95, Numéro 6, Juin 2008, XXVIIIe Forum de Cancérologie

9. **L'expression du gène GRP, codant 4 nouvelles protéines Gla, est finement régulée dans le cartilage**
   Flajollet S., Tian T., Legeai-Mallet L., Tomavo N., Flourens A., Camuzeaux B., Holder-Espinasse M., Galera P., Mallein-Gerin F., Le Jeune M., Duterque-Coquillaud M.
   12èmes Journées Françaises de Biologie des Tissus Minéralisés, Saint-Etienne (France), 9-11 juin 2010, Poster

10. **Activation of the OSTEOPONTIN gene, a bone metastatic signature, by transcription factor ERG in prostatic cancer cells**
    Flajollet S., Tian T.V., Flourens A., Tomavo N., Bonnelye E., Leroy X., Duterque-Coquillaud M.

10th International Conference “Cancer-Induced Bone Disease”, Sheffield (Angleterre), 22–25 septembre 2010, Poster
Abstract in Bone, Volume 48, Issue 1, Pages S1-S56, 1-166 (1 January 2011)

11. **The expression of the *GRP* gene, encoding four new Gla-rich protein isoforms, is finely regulated in cartilage**
Flajollet S., Tian V.T., Legeai-Mallet L., Tomavo N., Flourens A., Camuzeaux B., Holder-Espinasse M., Galera P., Mallein-Gerin F., Le Jeune M., Duterque-Coquillaud M.
ASBMR 2010 Annual meeting, Toronto (Canada), 15-19 octobre 2010, Poster
Abstract in the electronic supplement to the Journal of Bone and Mineral Research

12. **The expression of the *GRP* gene, encoding four new Gla-rich protein isoforms, is finely regulated in cartilage**
Le Jeune M., Flajollet S., Tian V.T., Legeai-Mallet L., Tomavo N., Flourens A., Holder-Espinasse M., Galera P., Mallein-Gerin F., Duterque-Coquillaud M.
1st IOF-ESCEO European Congress on Osteoporosis and Osteoarthritis Pre-clinical Symposium, Valencia (Espagne), 22 mars 2011, Poster

13. **Etude du gène de fusion TMPRSS2-ERG dans les métastases osseuses dérivées du cancer de la prostate**
Tian T.V., Flajollet S., Flourens A., Tomavo N., Villers A., Bonnelye E., Aubert S., Leroy X., Duterque-Coquillaud M.
4èmes journées scientifiques du cancéropôle Nord-ouest, Deauville (France), 5 et 6 mai 2011, Poster

14. **ASPORIN, a potential biomarker of prostate cancer progression and bone metastasis**
Tian TV, Flajollet S, Marquez M, Flourens A, Tomavo N, Wernert N, de Launoit Y, Leroy X, Bonnelye E, Duterque-Coquillaud M
11th International Conference “Cancer-Induced Bone Disease”, Chicago (U.S.), 30–3 décembre 2011, Poster

15. **Targeting ERG :DNA complex by a small selective DNA ligand: importance of the sequence**
Nihil R, Depauw S, Flajollet S., Dezitter X, Duterque-Coquillaud M, Boykin D., Wilson D., David-Cordonnier M.-H.
International Congress on Targeted Anticancer Therapies, Amsterdam (Pays-Bas), 8 et 10 mars 2012, Poster,
Abstract in Annals of Oncology 23 (Supplement 1)

16. **Inhibition du complexe ERG :ADN par un ligand séquence – spécifique de l’ADN : importance de la séquence**
Nihil R, Depauw S, Flajollet S., Dezitter X, Duterque-Coquillaud M, Boykin D., Wilson D., David-Cordonnier M.-H.
5èmes journées scientifiques du Cancéropôle Nord-ouest, Deauville (France), 10 et 11 mai 2012, Poster

17. **Le gène de Fusion TMPRSS2-ERG et les métastases osseuses du cancer de la prostate**
Tian T., Fradet A., Flajollet S, Flourens A, Tomavo N, Wernert N, de Launoit Y, Leroy X, Bonnelye E, Duterque-Coquillaud M
14èmes Journées Françaises de Biologie des Tissus Minéralisés, Bordeaux (France), 9-11 juin 2012, Oral

18. **L'expression d'un Dominant-Négatif du facteur de transcription ERG augmente l'adipogenèse dans les chondrocytes embryonnaires en culture et dans la moelle osseuse des souris transgéniques adultes**
Flajollet S., Tian T., Huot L., Tomavo N., Flourens A., Holder-Espinasse M., Le Jeune M., Dumont P., Hot D., Mallein-Gerin F., Duterque-Coquillaud M.
15èmes Journées Françaises de Biologie des Tissus Minéralisés, Poitiers (France), 30 mai-1er juin 2013, Poster

Intervention orale

1. **Distinct roles of BAF60, SRC1 and MED1 in retinoid-induced transcription and cellular differentiation**
Juin 2006, Institut Universitaire d'Hématologie, Paris

Mémoires

D.E.A. de Biologie et santé

Titre du rapport : Régulation de la réponse transcriptionnelle dans les cellules tumorales humaines en réponse aux rétinoïdes.

Tuteur : Christophe Rachez

Thèse de doctorat d'Université

Titre de la thèse : Etude du mécanisme d'activation de la transcription en réponse aux rétinoïdes.

Thèse soutenue le 17 novembre 2006 à la Faculté de Médecine de Lille

Composition du jury :
Pierre Formstecher (Président)
Philippe Lefebvre (Directeur de thèse)
Cécile Rochette-Egly(Rapporteur)
Lluis Fajas (Rapporteur)
Martine Duterque-Coquillaud (Examinatrice)

Discipline de rattachement : Sciences de la Vie et de la Santé (Biochimie, biologie cellulaire et moléculaire)

Collaborations scientifiques

Dr L. Casteilla
INSERM UMR5241 UPS-CNRS, CHU Rangueil, Toulouse

Dr Eduardo.A Donadi
Faculté de médecine de Ribeirao Preto, Université de Sao Paulo, Ribeirao Preto (Brésil)

Dr Frédéric Mallein-Gerin
FR3302 –IBCP Institut de Biologie et chimie des protéines, Biologie et ingénierie du Cartilage, Lyon

Dr Marie-Hélène David
INSERM U837 - Centre de Recherches Jean-Pierre Aubert (JPARC), Equipe 4 "Ciblage Moléculaire et Cellulaire dans le Traitement des Cancers", Lille

Dr Edith Bonnelye
INSERM U1033, Lyon

Bourses et Contrat de Recherche

- Allocation de recherche cofinancée par l'INSERM et la Région Nord-Pas-de-Calais (2002-2005)
- Bourse de la Ligue Nationale contre le Cancer (2006)
- Bourse du CEA (2007-2008)

Activités d'animation et de rayonnement

En matière d'administration et autres responsabilités collectives

- Représentant des étudiants/postdoctorants au sein du Conseil de laboratoire
- Correspondant informatique de l'équipe (2002-2006)
- Demande de financement (4ème année de thèse, postdoctorant)
- Sauveteur-secouriste du Travail (2002-2007)
- Responsable de salles de travail et machines de l'unité

Evaluation d'articles

- Evaluation d'articles pour différentes revues internationales avec comité de lecture internationale :
    - Biochemical Genetics
    - PlosONE
    - Molecular Carcinogenesis

Parcours scientifique

Au cours de mes travaux de recherche doctoraux et post-doctoraux, j'ai mené plusieurs projets de recherche liés aux domaines de la cancérologie, de l'immunité, du développement et du métabolisme. Le fil conducteur est l'étude des mécanismes de régulation transcriptionnelle.

A la suite de ma thèse (de 2002 à 2006), au cours de laquelle je me suis intéressé aux mécanismes de régulation transcriptionnelle dirigée par les récepteurs à l'acide rétinoïque, j'ai rejoint le Service de Recherches en Hémato-Immunologie (CEA, hôpital St Louis). Pendant ce premier stage post-doctoral (2007 à 2008), j'ai étudié les mécanismes de régulation transcriptionnelle de l'expression de la molécule HLA-G. J'ai ensuite intégré l'équipe actuelle au sein de l'UMR8161, dans le cadre d'un projet ANR-PROMOCART, afin d'étudier le rôle du facteur ERG dans la chondrogenèse. Je me suis ensuite intéressé à la régulation transcriptionnelle médiée par le facteur de transcription CHREBP et j'ai poursuivi des travaux initiés pendant ma thèse visant à montrer le processus d'élongation RAR et au sein de l'unité INSERM 1011. Depuis janvier 2012, dans le cadre d'un projet INCA, j'étudie l'implication de ERG dans les processus de métastases osseuses du cancer de la prostate.

Pour expliquer la régulation de l'expression des gènes par les facteurs de transcription, j'aime utiliser la comparaison à un grand orchestre symphonique. *Un ensemble musical composé de musiciens instrumentistes* (les facteurs de transcription et cofacteurs) *dont la composition dépend du répertoire abordé* (domaine de la cancérologie, immunité, métabolisme,…). *Chaque musicien doit exécuter sa partition* (séquence d'ADN). *Tout est parfaitement organisé jusque dans les moindres détails afin que chacun joue sa partie de façon ordonnée, précise et en parfaite harmonie. Un orchestre est donc une machine parfaite* (machinerie transcriptionnelle), *qui exige de ses membres de l'attention, de la discipline* (mécanisme mis en jeu). *Le chef d'orchestre* (le facteur de transcription étudié) *est le musicien qui dirige l'orchestre et qui veille sur l'interprétation juste de l'œuvre musicale* (l'expression du gène). *De son podium* (élément régulateur/site de fixation), *il coordonne, par les gestes de sa main et de sa baguette* (modifications post-traductionnelles, interaction avec les cofacteurs), *le jeu de ses musiciens. Il indique à chaque groupe d'instrumentistes le tempo et les nuances* (modification des histones). *Si l'on considère le nombre important de musiciens qui composent un orchestre symphonique, on comprend aisément que chaque chose, même la plus simple, doit être prévue et réglée afin d'éliminer la moindre possibilité de dissonance* (dérégulation génique, pathologie). *Enfin chaque chef d'orchestre a son*

propre style, son propre tempérament, qui détermine le caractère de l'exécution et imprime sa marque à l'œuvre musicale que percevra l'auditeur (le chercheur).

La présentation de mes travaux de recherche commencera donc par un medley de mes travaux antérieurs avant de développer le projet de recherche que je souhaite mettre en œuvre dans l'équipe actuelle. Mon objectif est d'établir le casting et de déchiffrer *le conducteur du chef d'orchestre* : le réseau transcriptionnel du facteur TMPRSS2 :ERG.

Medley des travaux antérieurs

I. Etude des mécanismes de régulation transcriptionnelle dirigée par les récepteurs à l'acide rétinoïque

Cette étude a été effectuée au cours de ma thèse (de 2002 à 2006) dans l'unité de recherche INSERM U459, *« Signaux, Récepteurs nucléaires et Différenciation cellulaire »*, ainsi que durant mon stage post-doctoral (en 2011) au sein de l'unité de recherche INSERM U1011 « *Récepteurs nucléaires, Maladies Cardiovasculaires et Diabète* ».

Je ne ferai ici qu'un bref résumé de ces travaux déjà développés dans mon mémoire de thèse :
(https://www.researchgate.net/publication/234053692_Etude_du_mcanisme_de_la_rgulation_de_la_transcription_en_rponse_aux_rtinodes?ev=prf_pub).

A. Résumé des travaux de thèse

1. Contexte scientifique

Les rétinoïdes sont des molécules impliquées dans un grand nombre de processus biologiques fondamentaux et utilisés en thérapie, notamment anticancéreuse. Le mécanisme d'action de l'acide rétinoïque et de ses dérivés synthétiques sont transmis dans la cellule par des protéines intracellulaires appartenant à la famille des récepteurs nucléaires (RNs), les hétérodimères RAR et RXR (Retinoic Acid Receptor) qui régulent la transcription par plusieurs mécanismes distincts. Ils peuvent activer ou réprimer la transcription par liaison directe aux éléments de réponse (REs) mais également interférer avec l'activité d'autres facteurs de transcription tels que AP-1 ou encore NF-KB, expliquant leur effet anti-prolifératif et anti-inflammatoire. La régulation de la transcription par les hétérodimères RAR/RXR met en jeu une pléthore de protéines corégulatrices qui sont soit des coactivateurs, soit des corépresseurs (Lefebvre et al. 2005). Un grand nombre de cofacteurs ont été identifiés sur la base de leur interaction physique et/ou fonctionnelle avec les récepteurs aux rétinoïdes. Cependant la contribution fonctionnelle ainsi que la coordination du recrutement des différents protagonistes lors de la transactivation régulée par les récepteurs aux rétinoïdes restent peu caractérisées. Afin de disséquer le mécanisme transcriptionnel mis en jeu, nous avons étudié l'activité transcriptionnelle du promoteur du gène suppresseur de tumeur *Rarβ2* dans la lignée cellulaire P19 de térato-carcino-embryonnaire de souris, sensible aux rétinoïdes. L'expression du gène *Rarβ2* est précocement induite par les rétinoïdes et est directement corrélée avec les effets antiprolifératifs et apoptotiques des rétinoïdes.

2. Résultats obtenus

Plusieurs points essentiels ressortent de ma thèse.

1. Au niveau épigénétique, nous avons montré une hyperacétylation constitutive des histones H3 et H4 associés au promoteur du gène R*arβ2*. Les mécanismes à l'origine de cette hyperacétylation sont liés à une absence de recrutement des complexes corépresseurs au niveau de ce promoteur régulé par plusieurs voies de signalisation cellulaire, et en particulier par la voie PI3K/Akt (Lefebvre et al. 2006).Cette voie de signalisation est associée à la survie cellulaire et est fréquemment suractivée dans les cancers épithéliaux. Dans des lignées cellulaires sensibles aux rétinoïdes, nous avons mis en évidence que l'hyperactivation de la voie PI3K/Akt entraine la déacétylation du promoteur du gène *Rarβ2* et l'absence d'expression de ce gène aux doses physiologiques en rétinoïdes. En effet l'activation de la sérine/thréonine kinase Akt a pour conséquence la phosphorylation directe des corépresseurs tels que SMRT, et la stabilisation de leur interaction avec l'hétérodimère RAR/RXR. La suractivation de la voie de signalisation PI3K/Akt peut représenter un mécanisme par lequel les cellules cancéreuses acquièrent une résistance aux rétinoïdes.

2. Afin de clarifier le recrutement chronologique des complexes p160 (SRC) et Médiateur (Med, encore appelé DRIP ou TRAP) dans le mécanisme d'activation de la transcription dirigé par les RARs, nous avons utilisé une technique d'ARN interférence par shRNA (pour *short hairpin RNA*) pour générer des lignées cellulaires P19 déplétées par chacun des coactivateurs (Flajollet et al. 2006). Nous avons démontré le rôle majeur du coactivateur SRC1 dans le mécanisme d'activation de la transcription. En parallèle, l'étude de la sous-unité Med1 (DRIP205) du complexe Med par cette même stratégie s'est révélée plus surprenante et intéressante. Le complexe Med qui interagit avec le récepteur RAR en présence d'atRA joue un rôle modulateur dans la réponse aux rétinoïdes dans les cellules P19. En effet, Med1 affecte négativement le cycle de transcription de l'ARN polymérase, sur le promoteur des gènes cibles du RAR, en perturbant le recrutement des facteurs généraux de la transcription tels que TFIIH, cdk8. L'étude détaillée du recrutement des différents intervenants lors du mécanisme transcriptionnel (récepteur, coactivateurs et machinerie transcriptionnelle), des modifications post-traductionnelles de l'ARN polymérase et du contexte de la chromatine au niveau de la région promotrice du gène *Rarβ2* m'a permis de proposer un modèle du mécanisme mis en jeu lors de l'activation de la transcription en réponse aux rétinoïdes. L'altération de l'activation de la transcription par les rétinoïdes se répercute dans les réponses biologiques à long terme, telles que la différenciation cellulaire et l'apoptose induites par les rétinoïdes.

3. Il avait été établi dans le laboratoire que les différences structurales des rétinoïdes (rétinoïdes de synthèse) sont associées à des changements de conformations distinctes du récepteur, attribuables au positionnement différent du ligand dans le site de liaison (Lefebvre et al. 1998). Ces différences de propriétés structurales affectent l'interaction de l'hétérodimère RAR-RXR avec les coactivateurs (Mouchon et al. 1999; Flajollet et al. 2006). Les résultats obtenus *in vitro* suggèrent que la diminution d'expression d'un coactivateur donné, consécutive à une pathologie par exemple, est susceptible d'entrainer des changements dramatiques dans la sensibilité des réponses aux ligands.

4. Le recrutement du complexe de remodelage de la chromatine ATP dépendant, SWI-SNF, par l'hétérodimère RAR-RXR a été décrit (Flajollet et al. 2007). Une sous-unité du complexe SWI/SNF, l'isoforme BAF60c, interagit physiquement et fonctionnellement avec le récepteur RARα de manière ligand dépendante. D'autre part nous avons montré que le complexe SWI/SNF agit en synergie avec deux membres de la famille p160 (SRC1 et SRC2) sans pour autant interagir directement avec ceux-ci.

Publications relatives à ce projet

1. **Transcriptional activities of retinoic Acid receptors.**
   Lefebvre P., Martin P.J., Flajollet S., Dedieu S., Billaut X., Lefebvre B., Vitam. Horm., 2005
2. **Distinct roles of the steroid receptor coactivator 1 and of MED1 in retinoid-induced transcription and cellular differentiation.**
   Flajollet S., Lefebvre B., Rachez C., and Lefebvre P., J. Biol. Chem., 2006
3. **Down-regulation of the tumor suppressor gene RARβ2 through the PI3K/Akt signaling pathway.**
   Lefebvre B., Brand C., Flajollet S., and Lefebvre P., Mol. Endocrinol., 2006
4. **The core component of the mammalian SWI/SNF complex SMARCD3/BAF60 is a coactivator for the nuclear retinoic acid receptor.**
   Flajollet S., Lefebvre B., Cudejko C., Staels B., and Lefebvre P., Mol. Cell., Endocrinol., 2007

Publication issue de travaux de collaboration

5. **Coadministration of Coenzyme Q prevents Rosiglitazone-induced adipogenesis in ob/ob mice.**
   Carmona M. C., Lefebvre P., Lefebvre B., Galinier A., Benani A., Jeanson Y., Louche K., Flajollet S., Ktorza A., Dacquet C., Pénicaud L. and Casteilla L., Int. J. Obes. (Lond.), 2009

3. Encadrement

Au cours de ma thèse, j'ai encadré 2 étudiantes en Diplôme Universitaire sciences du médicament et sciences biologiques (Institut de Chimie Pharmaceutique Albert Lespagnol – Université de Lille2) :

- **Linda Cambula** (2003-2004) : « Etude fonctionnelle des coactivateurs des récepteurs nucléaires, Application au modèle du récepteur des rétinoïdes », avec pour objectif de mettre en place une banque de shRNA dirigés contre les coactivateurs étudiés dans le cadre de mon projet et de celui de l'équipe.

- **Vanessa Petit** (2004-2005) : « Etude de l'interaction fonctionnelle du complexe coactivateur SWI/SNF dans le mécanisme d'activation de la transcription par les récepteurs nucléaires », avec l'étude des interactions de certaines sous-unités du complexe SWI-SNF et les récepteurs nucléaires RAR, GR et PPARγ.

Ainsi qu'une étudiante en maîtrise :

- **Céline Cudejko** (2005) : « Etude fonctionnelle de BAF60 et BAF250 ».

Mon rôle a été : - de définir et réaliser le projet
- d'enseigner les outils technologiques nécessaires à la réalisation du projet.
- de définir les objectifs journaliers
- d'aider à la rédaction du mémoire et à la préparation de l'oral

B. Stage postdoctoral (2011)

1. Recherche de nouveaux coactivateurs transcriptionnels de RAR et le mécanisme de l'élongation

Un projet initié avec mon tuteur de master le docteur Christophe Rachez avait pour but de rechercher de nouvelles protéines interagissant avec le domaine fonctionnel AF1 de RARα qui possède une activité transactivatrice indépendante de la fixation du ligand. Six partenaires protéiques ont ainsi été isolés par la technique *in vitro* de co-rétention protéique GST-pulldown, et identifiés par spectrométrie de masse : le récepteur RARγ, AF-9/MLLT3 (pour *All1 fused gene from chromosome 9*), PAK6 (pour *P21-activated kinase-6*), NAP1L2 (pour *Nucleosome associated protein 1-like2*), BTF2 /TFIIHp62 (pour p62 subunit of TFIIH) et BRD4/Hunk1 (pour *Bromodomain-containing protein 4*). Compte tenu des données publiées entre 2006 et 2011 concernant l'importance du domaine situé en N-terminal (NTD) du RAR (Lalevee et al. 2011) et les travaux menés sur le complexe de super élongation (complexe SEC) au cours de l'activation transcriptionnelle (Yang et al. 2005; Iankova et al. 2006; Dawson et al. 2011; Luo et al. 2012), j'ai poursuivi l'étude du recrutement de AF9/MLLT3 et BRD4/Hunk1 dans la régulation transcriptionnelle dirigée par le RAR, au cours de mon stage postdoctoral en 2011 (Flajollet et al. 2013b). Nous avons montré que les récepteurs nucléaires RAR établissent des interactions physiques et fonctionnelles avec la machinerie d'élongation transcriptionnelle p-TEFb (pour positive-transcription elongation factor b). Dans le cas de la régulation de l'expression du gène archétype *Rarβ2* induit par les rétinoïdes, nous mettons en évidence que l'activité de P-TEFb/cycline T/CDK9 est

important, et que RARα est retrouvée dans les régions transcrites de façon dépendante à CDK9 et colocalise avec RNApolII phosphorylée sur la sérine 5. L'interaction de RARα avec AF-9/MLLT3 et BRD4/HUNK1 est indépendante du ligand. L'analyse fonctionnelle du recrutement des protéines AF-9 et BRD4 par le RAR activé par son ligand révèle une régulation différentielle de l'expression des gènes. En effet l'analyse transcriptomique des cellules P19 déplétées en AF9 et/ou BRD4 en présence de rétinoïdes, permet de distinguer des clusters de gènes dont l'expression est dépendante de l'une et/ou l'autre de ces protéines suggérant des rôles distincts dans la régulation transcriptionnelle des gènes et dans la différentiation neuronale induite en réponse aux rétinoïdes.

Publication relative à ce projet

1. **The elongation complex components BRD4 and MLLT3/AF9 are transcriptional coactivators of nuclear retinoid receptors.**
   Flajollet S., Rachez C., Ploton M., Schutz C., Gallais R., Metivier R., Pawlak M., Leray A., Issulahi A.A., Heliot L., Staels B., Salbert G., Lefebvre P., PlosOne, 2013

La dissection de la régulation transcriptionnelle en réponse aux rétinoïdes met en évidence un mécanisme finement orchestré par le récepteur nucléaire de l'acide rétinoïque RAR mettant en jeu un certain nombre de cofacteurs dont la composition et la disposition dépendent du promoteur.

2. Les rétinoïdes et leurs récepteurs dans les tissus adipeux : Aspects physiopathologiques

Suite à une invitation à écrire une revue bibliographique dans le journal « Hormone Molecular Biology and Clinical Investigation » (HMBCI), je me suis intéressé au rôle des rétinoïdes et de leurs récepteurs nucléaires RAR et RXR dans les tissus adipeux (Flajollet et al. 2013a). Cet aspect n'avait pas été développé au cours de ma thèse. Le travail bibliographique que j'ai réalisé a permis de réaliser une synthèse des travaux récents sur l'action des rétinoïdes, de leur métabolisme et de leurs récepteurs dans la physiologie des adipocytes et des tissus adipeux. Puisque le tissu adipeux joue un rôle important dans le métabolisme et le stockage des rétinoïdes, l'impact des désordres métaboliques a été évalué. De même l'impact des rétinoïdes utilisés dans des thérapies (en particulier en oncologie et en dermatologie) sur le métabolisme lipidique a été abordé. Enfin l'utilisation des rétinoïdes et rexinoïdes dans des stratégies thérapeutiques des maladies métaboliques (telles que le diabète de type 2 ou l'obésité) a été discutée.

Publication relative à ce projet

1. **Retinoids and nuclear retinoid receptors in white and brown adipose tissues: physio-pathologic aspects**
   Flajollet S., Staels B., Lefebvre P., HMBCI, 2013

II. Mécanismes épigénétiques et facteurs de transcription de la régulation de l'expression de la molécule immuno-régulatrice HLA-G

Ces travaux ont été effectués au cours de mon premier post-doctorat (2007-2008) dans le Service de Recherches en Hémato-Immunologie du CEA, Hôpital Saint Louis à Paris.

A. Recherche de nouveaux acteurs impliqués dans la régulation de l'expression de HLA-G

La molécule HLA-G appartient à la famille des molécules HLA de classe I dite « non-classique ». Sa participation dans les mécanismes de tolérance immunitaire, particulièrement dans la tolérance fœto-maternelle, en cancérologie et en transplantation est étudiée depuis plusieurs années. Il a été montré que l'expression de HLA-G par les cellules tumorales constitue un mécanisme d'échappement efficace à la réponse immunitaire anti-tumorale exprimant HLA-G. Cette protéine se distingue des autres molécules HLA de classe I par son faible polymorphisme, son expression tissulaire très restreinte et son activité exclusivement inhibitrice. Alors que les fonctions immunologiques de HLA-G sont désormais bien établies, il est nécessaire de décrire davantage les mécanismes moléculaires de régulation de l'expression de la molécule HLA-G afin d'inhiber spécifiquement son expression dans la cellule tumorale ou d'induire son expression lors d'une transplantation.

L'expression de HLA-G est en partie régulée au niveau transcriptionnel. Un certain nombre de facteurs environnementaux (hormones, cytokines, stress [thermique, toxique]) et de mécanismes épigénétiques sont décrits pour jouer un rôle dans l'induction de l'expression de HLA-G. Ceci a fait l'objet d'une revue bibliographique (Moreau et al. 2009).

L'objectif de mon postdoctorat était d'identifier des nouveaux acteurs de la régulation de l'expression de HLA-G. Pour cela j'ai utilisé une approche de promoteur-pulldown couplée à une analyse protéomique différentielle. Des extraits nucléaires de cellules exprimant ou non HLA-G sont mis en présence d'un fragment de promoteur du gène *HLA-G*. Les protéines retenues sont alors séparées sur gel bidimensionnel, et identifiées par spectrométrie de masse. Cette stratégie nous a permis d'identifier plusieurs facteurs de transcription candidats. Parmi ceux-ci, l'un d'entre eux s'est révélé être particulièrement intéressant : RREB1 (pour *Ras Responsible Element Binding Protein 1*). Puisque ce facteur a été isolé dans les cellules négatives pour l'expression de *HLA-G.*

L'étude fonctionnelle de RREB1 nous a conduits à la conclusion que cette protéine joue un rôle répresseur dans l'expression de HLA-G (Flajollet et al. 2009). Des expériences d'interaction protéine-protéine nous ont permis de montrer que la protéine RREB1 participe au recrutement du complexe CtBP riche en histones déacétylases et histones méthyl-transférases favorisant un environnement chromatinien répressif. Cette interaction RREB1-

complexe CtBP a d'ailleurs été observée exclusivement dans les cellules n'exprimant pas HLA-G.

Publications relatives à ce projet

1. **Non classical transcriptional regulation of HLA-G : An update.**
   Moreau P.*, Flajollet S.*, Carosella E. D., J. Cell. Mol. Med., 2009
   (* Contribution équivalente des 2 auteurs à ce travail)
2. **RREB-1 is a transcriptional repressor of HLA-G.**
   Flajollet S., Poras I., Carosella E.D., Moreau P., Journal of Immunology., 2009

B. L'hypoxie dans la régulation de l'expression de HLA-G

Au cours de mon post-doctorat, nous avons étudié l'expression de HLA-G en condition hypoxique, microenvironnement pertinent retrouvé aussi bien au cours du premier trimestre de la grossesse, de la transplantation et de la tumorogénèse. La modulation transcriptionnelle et protéique de HLA-G en réponse à un stress hypoxique avait été démontrée dans plusieurs modèles cellulaires (Mouillot et al. 2007). Nous avons mis en évidence l'implication du facteur de transcription HIF-1, facteur clé de la réponse au stress hypoxique, et sa fixation sur son élément de réponse présent au niveau du promoteur de HLA-G par ChIP (résultats non publiés).

Afin d'évaluer *in vivo* l'effet de l'hypoxie sur l'expression de HLA-G, nous avons dosé par ELISA l'expression de HLA-G sous forme soluble (HLA-G1s et HLA-G5) dans le plasma d'individus exposés à une hypoxie d'altitude (avant, pendant et après exposition) au cours d'une expédition sur l'Everest (Bourguignon et al. 2010). Les résultats obtenus ont confirmé nos hypothèses, à savoir :

- une augmentation du taux de HLA-G circulant des individus placés en altitude comparativement aux taux présents en condition normale d'oxygénation
- une acclimatation congénitale des natifs de haute altitude (les sherpas) qui présentent des valeurs normales sans modulation.
- HLA-G doit être prise en compte dans les mécanismes qui participent à l'adaptation aux altitudes élevées puisque l'hypoxie est un facteur important dans la régulation de l'expression de HLA-G.

Publication relative à ce projet

1. **Increased Soluble HLA-G Levels in Peripheral Blood from Climbers on Mount Everest.**
   Bourguigon M., Yaghi L., Flajollet S., Radanne-Krawice I., Rouas-Freiss N., Lugrin D., Richalet J.-P., Carosella E.D., Moreau P., Hum Immunol., 2010

C. Implication fonctionnelle de HLA-G dans l'échappement immunitaire tumoral du carcinome rénal

L'expression ectopique de HLA-G au niveau des cellules tumorales est aujourd'hui largement documentée montrant que celle-ci est retrouvée dans divers types de cancers et en proportion variable. De ce fait, HLA-G a été proposée comme mécanisme d'échappement immunitaire des cellules tumorales HLA-G+. D'autre part, le contexte microenvironnemental et l'utilisation de certaines drogues anticancéreuses peuvent moduler l'expression du gène de la molécule HLA-G à la surface cellulaire et favoriser également l'échappement immunitaire tumoral.

Au cours d'une vaste étude réalisée sur des échantillons de patients atteints de glioblastome (collectés en France, Danemark et Brésil), l'expression de HLA-G a été dosée quantitativement et qualitativement. L'absence d'expression d'HLA-G est associée à un bon pronostic (Wastowski et al. 2013). Des études utilisant la 5-aza-2'-deoxycitidine, une molécule administrée en thérapie anticancéreuse et qui affecte l'environnement chromatinien, nous a permis de mettre en évidence une augmentation de l'expression de HLA-G associée au remodelage de la chromatine au niveau de son promoteur.

D'où l'importance de considérer l'expression de HLA-G dans les pathologies cancéreuses et lors de la conception des stratégies thérapeutiques.

Publication relative à ce projet

1. **Human Leukocyte Antigen-G Is Frequently Expressed in a Multicentric Study on Glioblastoma and May Be Induced in Vitro by Combined 5-aza-2'-deoxycytidine and Interferon-gamma Treatments.**
   Wastowski I.J., Simões R.T., Yaghi L., Donadi E.A., Pancoto J.T., Poras I., Lechapt-Zalcman E., Bernaudin M., Valable S., Carlotti C.G. Jr, Flajollet S., Jensen S.S., Ferrone S., Carosella E.D., Kristensen B.W., Moreau P., Am J Pathol., 2012

D. Encadrement

Ce travail a été mené avec l'aide technique de Céline Marcou (Technicienne de l'équipe, 2007) et Isabelle Poras (Ingénieur de l'équipe, 2008).

Au cours de cette période, j'ai encadré 2 étudiants

- **Gwendoline Dubois** (2007) : étudiante en Licence professionnelle (Ecole Supérieure des Techniques de Biologie Appliquée, Paris) « Etude de l'implication de deux facteurs de transcription HIF1α et RREB1 dans la régulation de HLA-G »
- **Damien Durand** (6 semaines, 2008) étudiant en 1ère année de BTS en biophysique (Ecole Technique Supérieure de Laboratoire, Paris 13) : « Etude de l'implication du complexe CtBP via son recrutement par RREB dans la régulation de l'expression du gène HLA-G »

Mon rôle a été : - de définir le sujet

- d'enseigner les bonnes pratiques de laboratoire
- d'enseigner les outils technologiques nécessaires à la réalisation du projet.
- de définir les objectifs journaliers
- d'aider à la rédaction du mémoire et à la préparation de l'oral

III. Le facteur de transcription ChREBP dans la réponse au glucose

Pour des raisons de confidentialité, je ne développerai pas les résultats obtenus pendant cette période dans le cadre d'un projet mené avec un laboratoire pharmaceutique. Néanmoins je décrirai ici le facteur de transcription ChREBP *(*pour *Carbohydrate-Responsive Element-Binding Protein),* un acteur majeur dans la réponse au glucose.

Le facteur de transcription ChREBP, appartient à la famille des protéines bHLH/LZ. Il est exprimé majoritairement dans le foie, les tissus adipeux blanc et brun et de façon plus modérée dans l'intestin, le pancréas au niveau des ilots de Langerhans, le muscle squelettique, le rein et localement dans le cerveau. En concentration faible de glucose, ChREBP est localisé dans le cytoplasme (Dentin et al. 2006). Un régime riche en glucose entraine sont activation : ChREBP est déphosphorylé permettant sa translocation dans le noyau.

ChREBP, associé à un partenaire d'hétérodimérisation appelé Mlx (pour *Max-like protein X*), forme un complexe tétramèrique et se lie sur des séquences consensus ChoRE en réponse au glucose (Ma et al. 2005). Un élément de réponse ChoRE (pour *Carbohydrate Response Element)* est formé de la répétition de (CACGTG) séparée par 5 paires de bases, situées dans la région promotrice des gènes cibles de ChREBP. Le facteur ChREBP joue un rôle déterminant dans la régulation de l'expression des gènes du métabolisme gluco-lipidique. L'inhibition spécifique de l'expression de ChREBP entraine une diminution de l'expression des gènes de la glycolyse et de la lipogenèse par le glucose (tels que la *L-PK, ACC1, FAS, SCD1, DGAT2, PNPLA3*). ChREBP joue également un rôle dans la différenciation adipocytaire (He et al. 2004). Une dramatique augmentation au niveau transcriptomique et protéomique a été observée au cours de la différenciation de préadipocytes 3T3L1 (résultats non publiés) et de préadipocytes primaires (Hurtado del Pozo et al. 2011). Enfin l'expression de ChREBP augmente dans le foie mais diminue dans le tissu adipeux de patients obèses (Résultats personnels et (Hurtado del Pozo et al. 2011).

La régulation du facteur ChREBP se fait au niveau transcriptionnel et au niveau post-traductionnel. L'expression de ChREBP est contrôlée en effet par les conditions nutritionnelles : un régime hyperglucidique augmente son expression, tandis que le jeûne et un régime riche en acide gras polyinsaturés la diminuent. L'activité du facteur ChREBP est également régulée par les modifications post-traductionnelles, telles que les phosphorylation/déphosphorylation (phosphorylation par la PKA en présence de glucagon sur le serine 196 et thréonine 666 et par l'AMPK en présence d'acide gras saturés), les glycosylations et acétylations. L'enzyme glucose 6-phosphate (G6P) jouerait un rôle

important dans l'expression, l'activation et la translocation nucléaire de ChREBP (Li et al. 2010; Dentin et al. 2012).

Un certain nombre de récepteurs nucléaires sont impliqués dans la régulation du métabolisme (pour revue, (Francis et al. 2003)). Plusieurs études ont décrit l'implication de certains d'entre eux (HNF4α (pour *Hepatic nuclear factor 4α)*, LXR (pour *Liver X Receptor*), FXR (pour *Farnesoid X Receptor*), les récepteurs aux hormones thyroïdiennes (TR), COUP-TFII (pour *Chicken Ovoalbumin Upstream Promoter transcription Factor II*) dans la régulation de la réponse au glucose en interférant sur l'expression et l'activité de ChREBP. De même, il a été montré que le facteur de transcription c-Myc, qui est mieux connu pour ses propriétés oncogéniques mais qui est aussi impliqué dans la régulation du métabolisme du glucose (Meyer and Penn 2008), a été identifié comme nécessaire pour le recrutement de ChREBP au niveau du promoteur de certains gènes dont l'expression est induite en réponse au glucose (Collier et al. 2007; Sloan and Ayer 2010; Zhang et al. 2010b). D'ailleurs l'inhibition de l'activité C-MYC par une petite molécule inhibitrice (10058-F4/1RH) empêche le recrutement de ChREBP au niveau du promoteur et abolit la réponse au glucose (Zhang et al. 2010b).

Encadrement

Au cours de cette période, j'ai encadré un étudiant

- **Marcel Decréquy** (2011) : étudiant en Master 1 Recherche – Biologie et Santé, Université de Lille 2 « Etude de la réponse au glucose dans les cellules pré-adipocytaires »

Mon rôle a été : - de définir le projet

- d'enseigner les outils technologiques nécessaires à la réalisation du projet.
- de définir les objectifs journaliers
- d'aider à la rédaction du mémoire et à la préparation de l'oral

IV. Généralités sur le facteur de transcription ERG

A. Généalogie du facteur de transcription ERG : la famille ETS

Les produits du gène ERG sont des facteurs de transcription. Ce gène appartient à la famille ETS (pour *E Twenty-Six)*, constituée d'une trentaine de membres. Les protéines ETS sont conservés au cours de l'évolution, et sont répartis en 13 groupes en fonction des homologies structurales et fonctionnelles (Laudet et al. 1999; Hollenhorst et al. 2011). L'ancêtre de cette famille des gènes ETS a été défini sur la base de son identité de séquence avec l'oncogène viral *v-ets* transduit par le rétrovirus aviaire E26 (d'où ETS pour *E Twenty-Six*) capable d'induire des leucémies myéloblastiques et érythroblastiques chez le poulet (Leprince et al. 1983; Nunn et al. 1983). La filiation « *ets* » réside dans la présence d'un domaine de fixation à l'ADN de type « hélice ailée », le domaine ETS. Ce domaine, d'environ 85 acides aminés et relativement bien conservé, permet la liaison à l'ADN sur une séquence d'environ 10 paires de bases riches en purines, et centrée sur le motif consensus 5'-GGA(A/T) nommé EBS (pour *ETS binding site*) (Boulukos et al. 1989; Karim et al. 1990; Nye et al. 1992; Wasylyk et al. 1993; Graves and Petersen 1998; Wei et al. 2010). Les facteurs ETS sont donc capables de se lier sur des séquences similaires, même si les nucléotides flanquant la séquence centrale assurent la spécificité de fixation (Wang et al. 1992; Graves and Petersen 1998; Sharrocks 2001). Une certaine superposition des gènes cibles des facteurs ETS peut être constatée (Hollenhorst et al. 2011). Cependant malgré cette redondance fonctionnelle, il existe une spécificité d'action biologique des protéines ETS liée à la présence de domaines fonctionnels qui confèrent des propriétés moléculaires propres, à des modifications post-traductionnelles et/ou au recrutement de partenaires protéiques corégulateurs (Verger and Duterque-Coquillaud 2002; Buchwalter et al. 2004; Charlot et al. 2010). Le partenariat avec d'autres protéines permet aux facteurs ETS d'accentuer leur activité régulatrice puisqu'ils sont décrits pour avoir une activité transactivatrice globalement faible (Buchwalter et al. 2004).

Les facteurs ETS interviennent, par la régulation de l'expression d'un grand nombre de gènes, dans de nombreux mécanismes cellulaires et moléculaires importants au cours du développement embryonnaire, des processus physiologiques et de l'évolution pathologique (Oikawa and Yamada 2003; Seth and Watson 2005; Gallant and Gilkeson 2006; Randi et al. 2009).

B. Le gène ERG et ses protéines

Le facteur de transcription ERG est codé par le gène *ERG* (tableau 1) qui présente de fortes homologies dans le domaine ETS avec FLI1 (pour *Friend Leukemia Integration 1*) et FEV (pour *Fifth ets Variant*), avec lesquels il compose le groupe ERG (Laudet et al. 1999).

Le gène *ERG* a été identifié en 1987 par criblage d'une banque de cellules d'adénocarcinome colique humain (Rao et al. 1987; Reddy et al. 1987).

	Gène ERG humain	Gène *ERG* murin
Gène ID	2078	13876
Chromosome	21	16
Locus	q22.3	16qC4
Taille	294,53 kb	227,43 kb
Nbre promoteurs	3	2
Nbre Exons	17	13
Transcrits	>19	>7
Proteines	>5	>5

Tableau 1: Tableau des caractéristiques des gènes ERG humain et murin.

Le gène *ERG* produit plusieurs isoformes protéiques résultats de la présence de multi sites d'initiation de la transcription et de l'épissage alternatif d'exons en particulier de l'exon noté A81 et A72 dans la région centrale. Les principales isoformes décrites à ce jour sont : ERG-1, ERG-2, $P38^{ERG}$, $p33^{ERG}$, $p49^{ERG}$, ERG-3.

C. Organisation fonctionnelle des protéines ERG

Les protéines ERG se différencient par leur extrémité N-terminale et la présence ou non de ces exons alternatifs A81 et A72. Elles possèdent donc les domaines fonctionnels suivants que l'on retrouve dans l'isoforme ERG^{p55} dite pleine longueur (figure 1) :

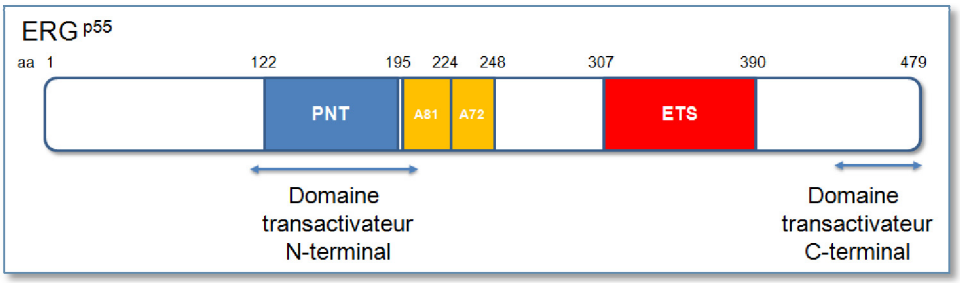


Figure 1: Organisation fonctionnelle du facteur de transcription ERG. L'isoforme ERGp55 est utilisée pour cette représentation.

- Le domaine ETS

Il est encore appelé DBD (pour *DNA Binding Domain*) caractéristique de la famille Ets. En plus de permettre la fixation à l'ADN sur le motif consensus 5'-GGA(A/T), ce domaine est une interface protéique privilégiée dans les interactions protéine-protéine (Verger and Duterque-Coquillaud 2002). Il est associé à la dimérisation des facteurs ETS ainsi que dans le recrutement des partenaires protéiques.

- Le domaine Sterile α module/Pointed (SAM/PNT)

Ce domaine, retrouvé dans les protéines du groupe ERG en amont du site ETS, présente de fortes homologies de séquence (sur 70 aa) avec la protéine issue du gène *Pointed* chez la

drosophile (Klambt 1993; Graves and Petersen 1998; Kim and Bowie 2003). Il n'est pas encore associé à une fonction précise. Sa ressemblance avec une structure de type hélice-boucle-hélice suppose une région d'interactions protéine-protéine, protéine/ARN ou une région servant à l'organisation de complexes protéiques au sein de la cellule. D'ailleurs il a été montré intervenir dans l'homodimérisation ou l'héterodimérisation des protéines ERG (Carrere et al. 1998; Mackereth et al. 2004).

- Les domaines transrégulateurs

Retrouvés de part et d'autre du domaine ETS, les domaines transrégulateurs sont responsables de l'activité intrinsèque activatrice ou répressive de l'expression des gènes (Siddique et al. 1993). Le domaine en position N-terminal, composé de 32 acides aminés et englobant le domaine pointed, est nommé domaine activateur (AD). Le second, en position carboxy-terminale, recouvre les 60 derniers acides aminés. Ils agissent en synergie au cours du processus de transcription, comme par exemple l'assemblage du complexe d'initiation de la transcription ou du passage à l'élongation. Leur délétion respective entraine une diminution de plus de 50% de l'activité transactivatrice des isoformes ERG^{P55}, ERG-1 et ERG-2 (Siddique et al. 1993; Carrere et al. 1998).

- Une région modulatrice de transactivation

La région située entre l'exon alternatif A72 et le domaine ETS jouerait un rôle inhibiteur sur la transactivation puisque sa délétion augmente de 25% la transcription d'un gène rapporteur (Carrere et al. 1998).

En sus pour certaines isoformes, sont décrits :

- Les exons alternatifs A81 et A72

  Leur fonction n'est pas encore connue, mais ils interviendraient dans l'activation transcriptionnelle

- La région d'interaction avec la protéine ESET (pour *Erg Associated Protein with Set Domain*) qui est délimitée par les acides aminés 1 et 114 est homologue à 92% avec la protéine SETDB1 (pour *Set domain bifurcated 1*) (Yang et al. 2002). Cette région est impliquée dans un mécanisme de répression transcriptionnelle (Yang et al. 2003).

Deux mécanismes sont connus pour être impliqués dans la régulation de l'activité du facteur de transcription ERG : les modifications post-traductionnelles et les interactions avec des partenaires protéiques

D. Les interactions protéiques de ERG

Malgré leurs domaines transrégulateurs, l'activité transcriptionnelle des protéines ERG est globalement faible. ERG interagit donc avec d'autres facteurs de transcription.

Les protéines ERG sont capables de former des homodimères avec elles-mêmes, ainsi qu'avec les autres membres de la famille ETS tels que FLI1, ETS2, ER81 et PU1 via les domaines ETS et pointed (Carrere et al. 1998; Verger and Duterque-Coquillaud 2002).

ERG coopére avec les protéines de la famille AP1 (pour *Activator Protein 1*), C-jun (pour *Ju-Nana*) et c-fos (Buttice et al. 1996; Verger et al. 2001; Camuzeaux et al. 2005). Des expériences de mutation ont permis de montrer l'importance de certains résidus, et en particulier de la tyrosine 351 (Verger et al. 2001). Il a été montré par coimmunoprécipitation que ERG coopère avec KLF2 (pour *Krüppel-like factor*) au niveau du promoteur du gène *Flk1* (pour *Fetal liver kinase 1*) au cours du développement vasculaire (Meadows et al. 2009). Une étude combinatoire dans les cellules souches hématopoïétiques a permis de mettre en évidence une interaction de ERG avec RUNX1 (Wilson et al. 2010).

ERG est capable d'interagir avec les RNs ER, RAR et TR (Vlaeminck-Guillem et al. 2003b). L'interaction entre ERG et ERα a pour conséquence une répression mutuelle de l'activité fonctionnelle de ces facteurs de transcription et requiert les 2 domaines transactivateurs de ERG et le domaine AF2 de ERα via probablement la compétition pour un même coactivateur. Une interaction physique a également été mise en évidence entre le récepteur AR et ERG dans le cancer de la porstate (Rickman et al. 2010). Nous reviendrons sur cette interaction dans le paragraphe VI.D.3.

ERG interagit avec la protéine ESET qui est essentiel dans le contrôle de la différenciation des chondrocytes hypertrophiques (Yang et al. 2013). Cette protéine possède une activité méthyltransférase sur la lysine 9 des histones H3 (Yang et al. 2002) et est capable de s'associer aux HDAC1 et HDAC2 (pour *Histone Deacetylase*), ainsi qu'aux co-répresseurs transcriptionnels mSin3A et mSin3B (pour *mammalian Switch Independent*) (Yang et al. 2003).

Enfin, une interaction directe a été révélée entre ERG et HSP90 par crible double hybride utilisant une banque d'ADNc de Xénope (Deramaudt et al. 2001), ainsi qu'entre ERG et la tubuline dans le cancer de la prostate (Galletti et al. 2013).

E. Rôle physiologique de ERG

L'étude du profil d'expression du gène *ERG* au cours de l'embryogénèse du Xénope (Baltzinger et al. 1999), du poulet (Dhordain et al. 1995; Iwamoto et al. 2000) et de la souris (Vlaeminck-Guillem et al. 2000; Iwamoto et al. 2007) a révélé une expression très précise dans le temps et dans l'espace (tableau 2).

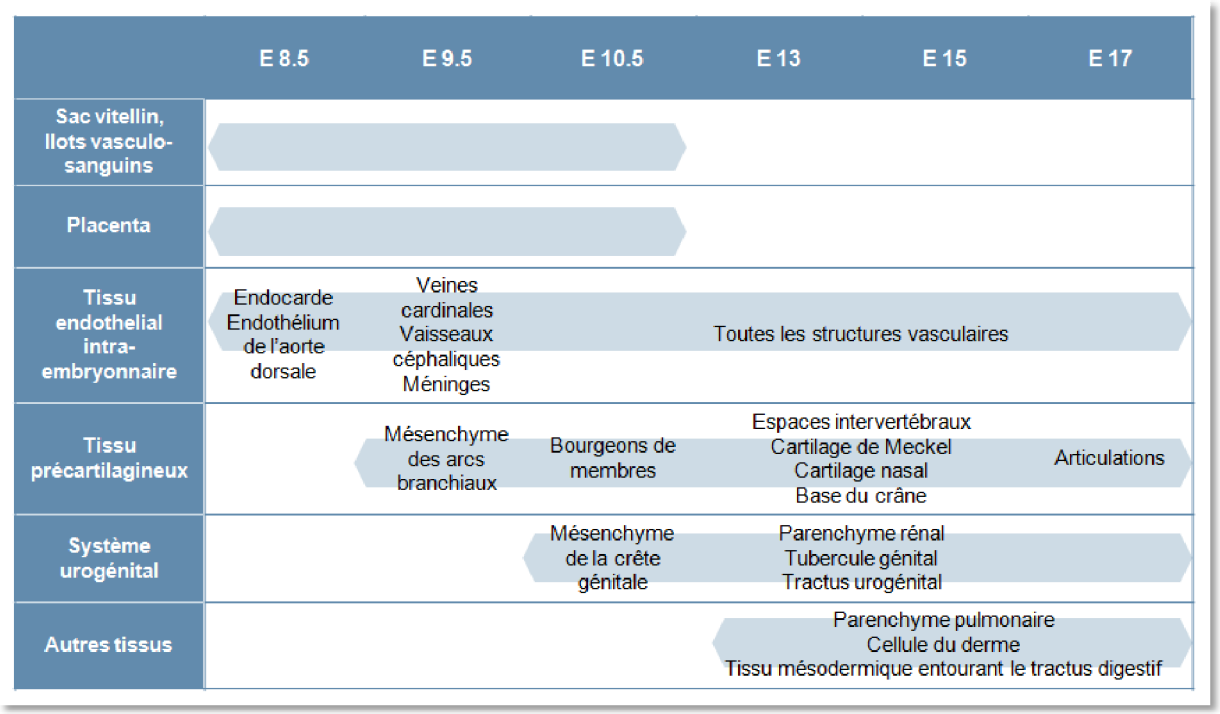


Tableau 2: Topographie de l'expression du gène *Erg* au cours de l'embryogenèse de la souris (d'après Vlaeminck-Guillem et al.2000).

Le gène *Erg* est exprimé dans les structures endothéliales en formation (à la fois intra-embryonnaires et annexes placentaires), les cellules en migration issues des crêtes neurales, ainsi que dans les structures d'origine mésenchymateuses telles que le tissus précartilagineux, le parenchyme rénal et pulmonaire, autour du tube neural et autour du tractus digestif), ainsi qu'au niveau du tractus urogénital et du tubercule urogénital (Dhordain et al. 1995; Vlaeminck-Guillem et al. 2000).

Le facteur de transcription ERG joue un rôle clé au cours de l'angiogenèse et de la vasculogenèse (Weber et al. 2005; Ellett et al. 2009) ainsi que dans la mise en place du cartilage et au cours de la squelettogenèse (Iwamoto et al. 2000; Iwamoto et al. 2007).

Récemment, l'expression du gène *ERG* a été montrée comme jouant un rôle essentiel dans l'hématopoïèse chez l'embryon et dans la régulation de la fonction des cellules souches hématopoïétiques chez l'adulte (Loughran et al. 2008). Enfin un rôle anti-inflammatoire a été décrit via l'inactivation génique (Yuan et al. 2009). Dans les cellules endothéliales, il a été montré que ERG réprime directement l'expression de l'interleukine 8 qui assure le recrutement des neutrophiles.

Chez l'homme, le profil d'expression au cours du développement embryonnaire n'est pas connu. L'expression de *ERG* est restreinte chez l'adulte au thymus (Watson et al. 1992; Anderson et al. 1999).

F. Expression pathologique de *ERG*

ERG est décrit comme étant un proto-oncogène. En effet, son expression ectopique dans la lignée cellulaire fibroblastique murine NIH3T3 est un événement suffisant dans l'oncogenèse (Hart et al. 1995). Son expression anormale est aujourd'hui associée à un développement pathologique. *ERG* est retrouvé impliqué dans des leucémies (leucémies myéloïdes chroniques, ainsi que les leucémies aiguë lymphoblastique de type T) (Shimizu et al. 1993; Martens 2011), des sarcomes d'Ewing (Sorensen et al. 1994; Wang et al. 2012), ainsi que dans les cancers de la prostate (Petrovics et al. 2005).

V. Etude de l'implication du facteur de transcription Erg dans la chondrogenèse

A. Genèse des souris transgéniques DN-Erg et caractérisation de leur phénotype

Afin de caractériser le rôle du facteur de transcription Erg dans la mise en place du cartilage, des modèles de souris transgéniques qui expriment une protéine Erg tronquée ont été générés dans notre équipe.

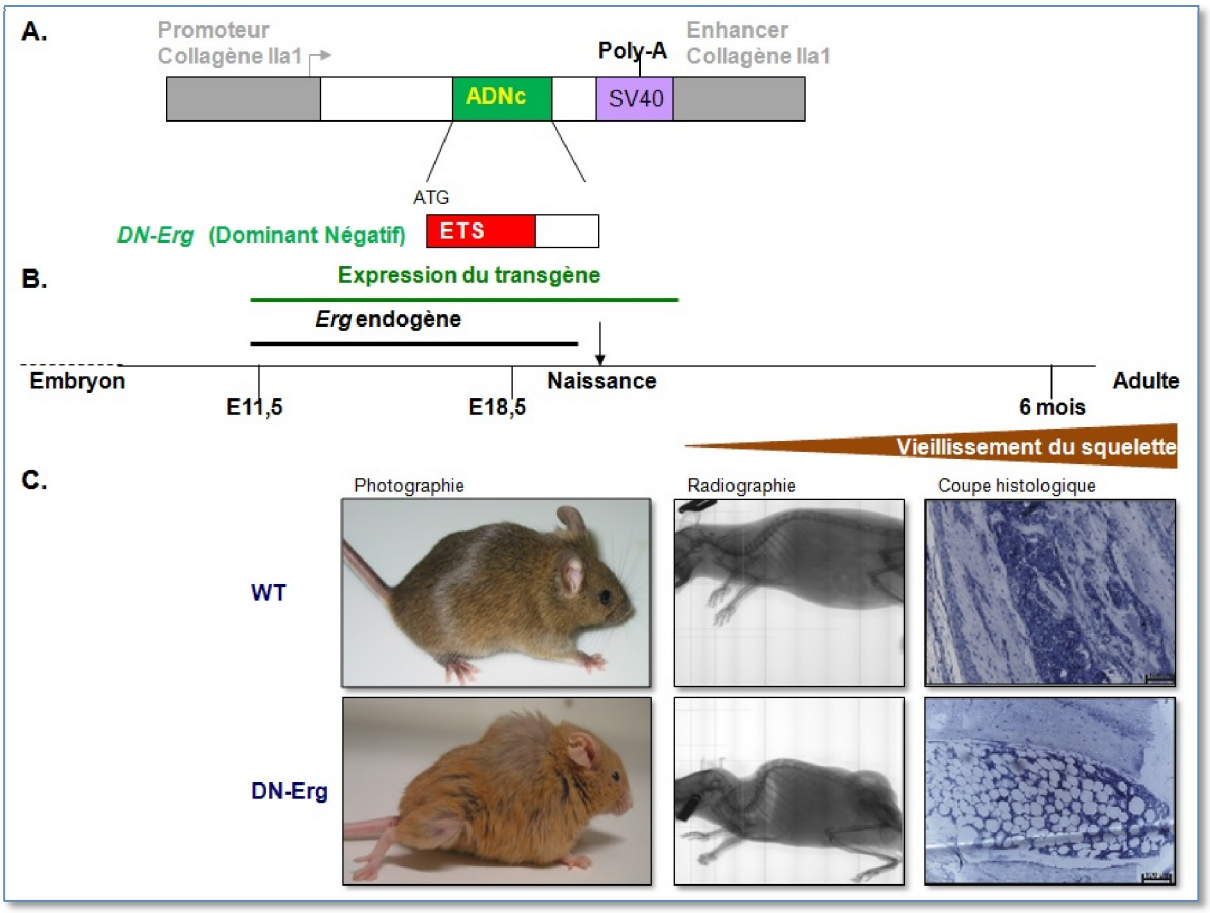


Figure 2: Modèle murin transgénique DN-Erg surexprimant le *trans*-Dominant négatif des protéines Erg dans le cartilage embryonnaire. Transgenèse ciblée au niveau du cartilage en formation chez la souris (A). Chronologie de l'expression de gène *Erg* et du transgène *DN-Erg* (B). Phénotype observé (photographie, radiographie et coupe histologique) (C).

Les données obtenues lors de l'étude du profil d'expression de *Erg* au cours du développement embryonnaire murin (Vlaeminck-Guillem 2000). Le promoteur/enhancer du Collagène2a1 (exprimé spécifiquement dans les étapes précoces de différenciation du cartilage) a été utilisé dans le vecteur d'expression du transgène pour permettre une expression précoce et ciblée de la protéine Erg tronquée (Figure 2A et 2B). Cette protéine Erg tronquée possède un effet transdominant négatif (DN-Erg, pour *Dominant-Negative Erg*) sur les protéines endogènes, puisqu'elle est réduite au domaine ETS responsable de la fixation à l'ADN. Elle perturbe en conséquence, par compétition avec les facteurs Ets

endogènes se fixant sur les sites EBS, l'expression des gènes régulés par ces facteurs de transcription.

Le phénotype des souris transgéniques obtenues sont « normaux » à la naissance et au cours des premiers mois de la vie, par contre des anomalies phénotypiques surviennent à partir de 6 mois de vie, telles que des troubles du déplacement qui apparaissent normalement bien plus tard chez la souris sauvage (Figure 2C). Les analyses radiologiques et histologiques ont confirmé l'apparition de signes associés au vieillissement précoce du squelette associant un aspect clinique arthrosique et hyperlordose (Holder-Espinasse 2006). Le phénotype observé pourrait être secondaire à une anomalie de la mise en place du cartilage causée par l'expression de la protéine DN-Erg au cours du développement embryonnaire

B. Hypothèses de travail

Le modèle des souris transgéniques DN-Erg est le modèle que nous avons utilisé pour l'étude du rôle des protéines ERG dans la mise en place du cartilage. En effet l'identification des gènes dont l'expression est perturbée par le transgène et la compréhension des mécanismes responsables du phénotype observé chez ces souris transgéniques permettront d'apporter des éléments de réponse au rôle du facteur de transcription Erg dans la mise en place du cartilage et de l'os.

i) Nous avons donc comparé le transcriptome des chondrocytes fraichement isolés d'embryons de souris DN-Erg à celui des chondrocytes d'embryons de souris sauvages. Nous avons choisi d'étudier les transcriptomes des chondrocytes issus de la cage thoracique d'embryons à 18,5 jours de développement correspondant à l'expression du transgène (Etude initiée par Marion Le Jeune au cours de sa thèse et poursuivie à mon arrivée dans l'équipe dans le cadre de mon contrat postdoctoral pour le projet PROMOCART).

ii) Par ailleurs, il est aujourd'hui bien établi que les chondrocytes primaires mis en culture en monocouche ont une tendance intrinsèque à perdre en quelques jours leur état différencié au cours de leur expansion et à acquérir un phénotype de type fibroblastique. Les événements moléculaires responsables de ce processus restent à découvrir. La mise en culture des chondrocytes primaires prélevés au niveau des cages thoraciques d'embryon de 18,5 jours et cultivés selon le protocole largement utilisé dans la littérature, nous a permis de remarquer que les chondrocytes présentent, comme attendu, une dédifférenciation mais que celle-ci est plus rapide pour les chondrocytes isolés des souris transgéniques par rapport à ceux des souris sauvages. D'autre part, nous avons noté que les chondrocytes DN-Erg mis en culture acquièrent rapidement un phénotype de type adipocytaire. Nous avons donc cherché à comprendre l'influence du facteur Erg dans ce processus de dédifférenciation. Afin de déterminer les gènes dérégulés au cours de leur mise en culture et

favorables à la dédifférenciation des chondrocytes et/ou favorables à l'émergence du phénotype de type adipocytaire dans les chondrocytes DN-Erg, nous avons réalisé une étude comparative du transcriptome des cellules wt et DN-Erg mises en culture pendant 20 jours.

C. Résultats obtenus

i) Les ARN issus des chondrocytes d'embryon de souris sauvages (wt) et transgéniques fraichement isolés ont été prélevés et utilisés afin de réaliser une étude comparative des transcriptomes utilisant des micro-arrays pan-génomiques murins (puces Agilent de 25000 gènes). Cette étude nous a permis de montrer la perturbation de l'expression de nombreux gènes dont les produits protéiques sont impliqués dans l'homéostasie osseuse, suggérant une modification de la matrice extra-cellulaire. Parmi ces gènes, nous pouvons citer l'*Ostéopontine* (*Opn*), l'*Ostéoprotégérine* (*Opg*), l'*Ostéoglycine* (*Ogn*), et l'*Ostéomoduline* (*Omd*). Après avoir confirmé la différence d'expression pour plusieurs d'entre eux par RT-PCR quantitative, nous avons entrepris des études plus approfondies afin de mettre en évidence leur régulation par le facteur de transcription Erg. Ces résultats obtenus pour l'un d'entre eux, l'étude de l'expression du gène *Opn* a servi de base pour les travaux de recherche menés dans le cadre du projet développé dans l'étude de l'implication du facteur ERG dans les métastases osseuses du cancer de la prostate. Les mécanismes précis associant la perturbation de ces gènes et le phénotype observé chez ces souris transgéniques restent cependant à déterminer.

ii) Afin d'explorer le rôle potentiel de Erg dans la maintenance du phénotype chondrocytaire, nous avons mis en culture en monocouche pendant 20 jours les chondrocytes primaires isolés des cages thoraciques d'embryons de souris exprimant ou non le transgène DN-Erg. Nous avons très vite remarqué une accumulation de gouttelettes lipidiques dans le cytoplasme des chondrocytes DN-Erg, suggérant l'émergence d'un phénotype de type adipocytaire. Afin de confirmer cette observation, nous avons réalisé une analyse transcriptomique, validée par RT-PCR quantitative. Les résultats obtenus révèlent un différentiel d'expression dans les chondrocytes DN-Erg cultivés, marqué par une diminution des marqueurs chondrogéniques, tels que *Collagène de type 2a1, Collagène de type 10a1, Sox9, Runx2, Scinderine*, plusieurs métalloproteinases, et des cathepsines (*CTS C et S*), et en parallèle une augmentation des marqueurs adipogéniques tels que *Adiponectine, Pparγ* (pour *Peroxisome proliferator-activated receptor γ*), *Peripline et Fabp4*. L'ensemble de ces résultats nous a ainsi permis de mettre en évidence l'implication de Erg soit dans le maintien du phénotype chondrogénique *in vitro*, soit dans l'orientation adipocytaire des chondrocytes mis en culture (Flajollet et al. 2012).

Suite à ces résultats obtenus *in vitro*, nous avons étudié le squelette des souris wt et

transgéniques, et en particulier la présence d'adipocytes dans la moelle osseuse (moelle osseuse jaune). Les études histologiques ont révélé une augmentation significative du nombre d'adipocytes dans la moelle osseuse des souris DN-Erg adultes par rapport à celle des souris wt, alors qu'aucune différence n'avait été constatée chez l'embryon.

L'ensemble de ces travaux réalisés dans le cadre du projet ANR TecSan-PROMOCART, a permis de mettre en évidence la contribution des membres de la famille Ets dans l'équilibre homéostatique de la plasticité cellulaire squelettique. Une des applications de ce travail concerne l'amélioration de la mise en culture de chondrocytes dans le cadre de prévention ou de thérapie de certaines pathologies dégénératives du cartilage telle que l'arthrose (Claus et al. 2011). Enfin, au cours de cette étude, nous avons identifié une liste de gènes dont la régulation est dépendante du facteur Erg et susceptibles d'être associés au phénotype arthrosique. Parmi ces gènes cibles, de nombreux marqueurs osseux, mais aussi des gènes associés au développement des métastases osseuses et/ou participant à l'ostéomimétisme des cellules tumorales dérivées de la prostate.

Publications relatives à ce projet

1. **Increased adipogenesis in cultured embryonic chondrocytes and in adult bone marrow of dominant negative Erg transgenic mice.**
   Flajollet S., Tian V. T., Huot L., Tomavo N., Flourens A., Holder-Espinasse M., Le Jeune M., Dumont P., Hot D., Mallein-Gerin F., Duterque-Coquillaud M., Plos One, 2012
2. **Deciphering signaling mechanisms controling chondrocyte differentiation: Application to tissue engineering of cartilage: The ANR-TecSan PROMOCART project**
   Claus S., Aubert-Foucher E., Perrier-Groult E., Bougault C., Ronziere M.C.., Freyria A.M., Legendre F., Ollitrault D., Boumediene K., Demoor M., Galera P., Tian T., Flajollet S., Duterque-Coquillaud M., Damour O., Chajra H., Mallein-Gerin F., IRBM, 2011

D. Formations

Ce projet m'a amené à travailler sur un modèle animal, la souris. J'ai donc complété ma formation en 2009, par un niveau 1 d'expérimentation animale, suivi au sein de l'Institut Pasteur de Lille.

D'autre part ce projet a nécessité l'analyse de listes de gènes et donc l'utilisation de différents logiciels bioinformatiques dont Ingenuity Pathway Analysis, Adgo2.0, David auxquels je me suis formé.

VI. ERG et le Cancer de la Prostate

A. Epidémiologie du cancer de la prostate

Au cours de ces trente dernières années, le nombre de cancers a explosé en France: +107,6 % pour les hommes et +111,4 % pour les femmes. Pour la seule année 2012, le nombre de nouveaux cas de cancers en France métropolitaine est estimé à 355 000 dont 200 000 pour les hommes et 155 000 pour les femmes. Notons que depuis trente ans, la population française a non seulement augmenté mais elle a aussi vieilli. Or, le cancer est une maladie dont l'incidence est la conséquence de l'effet combiné du vieillissement de la population et des pratiques diagnostiques.

Avec 71 220 nouveaux cas estimés en 2011, le cancer de la prostate (CaP) est le plus fréquent des cancers de l'homme et le troisième en mortalité avec 8 700 décès estimés (derrière le cancer du poumon, 21000 cas ; et du cancer colorectal, 9200 cas) (HAS 2012). Les CaP sont de plus en plus diagnostiqués précocement avec la généralisation du dosage biologique du PSA comme test de dépistage. Il n'y a pas de preuve que ce dépistage diminue la mortalité et donc qu'il y ait un bénéfice supérieur aux conséquences physiques et psychologiques. Néanmoins, compte-tenu de l'évolution démographique, et malgré l'augmentation du taux d'incidence (+115%) des CaP en France entre 1995 et 2005, la mortalité spécifique par CaP en France a reculé, mais n'a reculé que de 2,5% (Belot et al. 2008).

B. Les métastases osseuses du cancer de la prostate

1. La prostate

La prostate est une glande de l'appareil génital masculin située dans le bassin, sous le col vésical et en avant du rectum. La prostate constitue un véritable carrefour urogénital. En effet, elle entoure l'urètre sur quelques centimètres (urètre prostatique) à la sortie de la vessie, et c'est à ce niveau que les deux canaux éjaculateurs en provenance des vésicules séminales viennent se jeter dans l'urètre (Figure 3A).

Sa fonction principale est de secréter et de stocker une partie du liquide séminal (30% du volume de l'éjaculat) entrant dans la composition du sperme. La prostate est constituée d'une trentaine à une cinquantaine de glandes tubulo-alvéolaires ramifiées s'ouvrant par une vingtaine de petites ouvertures (« ductuli ») dans le segment distal de l'urètre prostatique. Les glandes sont enrobées dans un stroma fibro-myo-élastique, qui sert à l'expulsion des sécrétions prostatiques. Les cellules stromales entretiennent également un dialogue crucial avec les cellules de l'épithélium prostatique au cours du développement embryonnaire ainsi que dans l'homéostasie prostatique (Cunha 2008). L'activité de la prostate est en effet finement régulée par les hormones stéroïdiennes (androgènes principalement) et facteurs de

croissance (EGF, pour *Epidermal Growth Factor* ; FGF, pour *Fibroblast Growth Factor* ; TGFβ, pour *Transforming Growth Factor*) (Schauer and Rowley 2011).

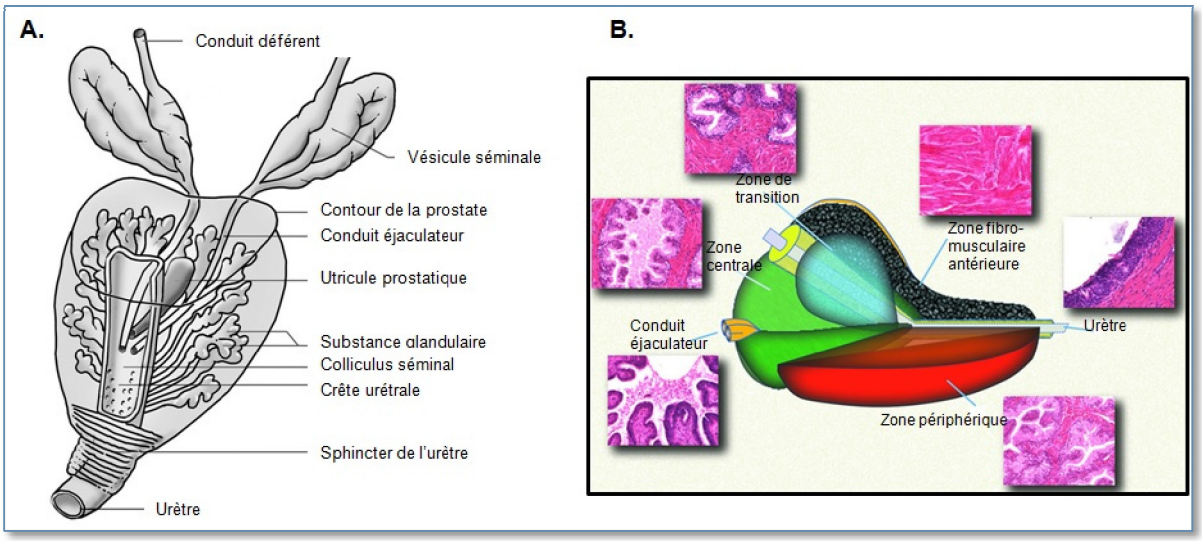


Figure 3: Représentations de la prostate. Schéma anatomique de la prostate (A). Représentation zonale de la prostate selon Mac Neal (d'après De Marzo et al. 2007) (B).

En forme de châtaigne, elle ne pèse pas plus de 20 grammes. Avec l'âge, le volume de la prostate tend à augmenter, l'adénome de la prostate, encore appelée hypertrophie bénigne de la prostate (HBP) qui résulte d'une croissance excessive du stroma et des glandes. Les hyperplasies de la prostate surviennent dans les zones antéromédiale de transition et dans la zone péri-urétrale (figure 3B). Elles provoquent une compression de l'urètre, et se traduisent par une rétention urinaire.

2. Le cancer de la prostate

Les CaP surviennent indépendamment de l'HBP, ce sont pour la majorité des adénocarcinomes. Ils se développent à partir des tissus d'origine épithéliale glandulaire de la prostate, dans la zone périphérique (70% des cas), raison pour laquelle le toucher rectal est nécessaire au diagnostic. Les foyers de cellules cancéreuses, initialement des lésions intraglandulaires appelées néoplasies intraépithéliales (PIN) apparaissent généralement à différents endroits et évoluent chacun plus ou moins rapidement. La mise en place des PIN se traduit par une désorganisation des structures prostatiques. Ces foyers, microscopiques, peuvent ne pas être détectés par les tests de dépistage actuels (dosage PSA, imagerie ou toucher rectal), il s'agit de la première phase du cancer, occulte ou latent.

Le CaP est avant tout une maladie qui survient tardivement et qui se caractérise par son évolution très lente sur plusieurs années. Longtemps asymptomatique, confiné à la prostate (deuxième phase), le cancer s'il est diagnostiqué (par dosage sérique de la PSA, l'imagerie, l'examen clinique et biologique) peut être soigné. Lorsque le cancer évolue, il s'étend en

dehors de la prostate par envahissement direct des tissus et des organes situés près de la prostate avec notamment les vésicules séminales, la vessie, le rectum. Le stade ultime des tumeurs prostatiques est la formation des métastases. Les CaP ont en effet un important pouvoir métastatique par voie hématogène et lymphatique, et vont rapidement coloniser os, ganglions lymphatiques, poumon, foie, cerveau… (Tantivejkul et al. 2004).

L'apparition des signes cliniques, tels que la rétention urinaire, l'hématurie, l'impuissance sexuelle, le dysfonctionnement d'autres organes ou encore des douleurs osseuses, pousse le patient à consulter et traduit un stade déjà relativement avancé avec ou sans métastases. Pour définir l'étendue du cancer au moment du diagnostic et de son évolution potentielle, les CaP sont classés en fonction de la valeur du PSA, de leur degré d'extension (classification TNM, pour Primary *Tumor (T), Regional lymph nodes (N), Distant Metastasis (M)*), de l'agressivité de la tumeur (degré de différenciation de la tumeur, score de Gleason), de leur risque d'évolution (Classification de d'Amico). L'enjeu de la prise en charge est d'évaluer pour chaque patient sa situation dans le parcours des différentes étapes de l'histoire du CaP, mais aussi l'agressivité de la tumeur. La connaissance de l'histoire naturelle du CaP est donc indispensable dans l'orientation et la prise de décision thérapeutique. Traiter ou surveiller avec traitement différé est une alternative envisageable en cas de diagnostic de CaP cliniquement localisé et à très faible risque de progression. La prise en charge thérapeutique des CaP avancés est variée (chirurgie, radiothérapie, curiethérapie, hormonothérapie, seule ou combinée) et évolue avec les progrès techniques et scientifiques (Audenet and Roupret 2011).

3. Les métastases osseuses du CaP

L'os est le site privilégié de développement de métastases des cancers du sein (avec une incidence de 73%) et du cancer de la prostate (68 %) (Coleman 2006). Les métastases osseuses constituent un problème de santé publique majeur car elles sont bien souvent incurables, détériorent la qualité de vie, et engagent le pronostic vital. Divers symptômes, telles que violentes douleurs, fractures osseuses, compression médullaire et perturbations du métabolisme phospho-calcique peuvent parfois être l'origine du motif de consultation lorsqu'aucun cancer n'est encore diagnostiqué. Les stratégies thérapeutiques actuelles engagées contre les métastases osseuses (souvent les biphosphonates pour le CaP) ne consisteront qu'à ralentir transitoirement les différentes étapes de l'histoire du CaP et améliorer la qualité de vie du patient. Il n'existe à ce stade aucun traitement curateur.

Les métastases osseuses sont de deux types : ostéolytiques marquées par une résorption osseuse intense ; et ostéoblastiques ou ostéocondensantes caractérisées par un déséquilibre du remodelage osseux en faveur d'une production osseuse composée de

collagène mal organisé. Dans le cas du CaP, les métastases osseuses sont de type ostéocondensantes, ou mixtes alliant la destruction et la production de l'os (Guise et al. 2006). L'activité ostéolytique qui résulte de l'activité des ostéoclastes, semble être une condition nécessaire à l'ostéocondensation. Les métastases osseuses se localisent principalement au niveau des os du bassin, des vertèbres et du sternum.

C. Les bases moléculaires

1. De la cancérogenèse prostatique

Les causes et l'évolution de la maladie sont complexes et encore imparfaitement comprises. En effet, le développement du CaP est multifactoriel, car il est lié à la prédisposition et/ou à la survenue d'événements génétiques, et de l'interaction des facteurs environnementaux (Hughes et al. 2005).

Un grand nombre de travaux ont été effectués et ont permis de déterminer des gènes de prédispositions, et de mettre en évidence des modifications d'expression de gènes comme l'activation d'oncogènes ou à l'inverse l'inactivation de gènes suppresseurs de tumeurs et de gènes gardiens de l'intégrité du génome. Parmi les gènes que l'on retrouve être associés dans la survenue du CaP, on peut distinguer deux catégories de gènes : ceux qui confèrent un caractère héréditaire à la pathologie (correspond à 10% des CaP) (*PcaP* pour *predisposing for prostate cancer, HPCX, HPC20, PG1* pour *prostate cancer susceptibility gene*, *BTLN2, BRCA2*) et ceux qui sont fréquemment mutés ou dérégulés (p53, AR, PTEN, *SRD5A2* pour *5-α-reductase, NKX3.1,…*) par des mécanismes qui restent souvent à décrire (pour revue : (Cussenot and Cancel-Tassin 2004)). Les altérations chromosomiques les plus fréquemment retrouvées aujourd'hui dans les tumeurs prostatiques concernent les gènes codant le récepteur aux androgènes et le gène de fusion TMPRSS2 :ERG.

Un certain nombre de facteurs sont considérés à risque pour avoir un impact dans le CaP. On peut citer l'âge, les antécédents familiaux, l'ethnie, le statut hormonal androgénique, l'inflammation, les agents environnementaux (le style de vie, le régime alimentaire, l'exposition aux pesticides et perturbateurs endocriniens) (Whittemore et al. 1995; De Marzo et al. 2007; McCracken et al. 2007; Belpomme et al. 2009). Cependant on ne sait pas à ce jour comment ces différents facteurs interagissent, s'ils se cumulent, d'où la difficulté de construire un modèle moléculaire pertinent de cancérogenèse prostatique. Une meilleure compréhension des mécanismes biologiques et génétiques déterminant pourquoi certains CaP restent cliniquement silencieux alors que d'autres sont agressifs, est nécessaire.

2. Du processus métastatique dans le CaP

L'apparition d'une métastase osseuse à partir d'une tumeur prostatique primaire est un processus composé de la succession d'événements au cours desquels les interactions des cellules tumorales avec les autres cellules ou avec l'environnement jouent un rôle crucial (tableau 3). Toutes les cellules tumorales n'ont pas la capacité à métastaser. Seules quelques cellules (1 à 2%) seront capables de franchir les différents obstacles que sont les carences en oxygène et nutriments, le déplacement, la survie, l'échappement au système de défense, et l'adaptation aux nouveaux environnements (Luzzi et al. 1998; Rosol et al. 2003). Les cellules cancéreuses qui survivent peuvent soit proliférer pour donner des métastases, soit entrer dans un état quiescent, dit dormant, qui peut se maintenir des années avant que la cellule ne se remette à proliférer et être à l'origine de récurrence métastatique. Le processus métastatique qui s'inscrit dans l'histoire du cancer comporte donc une sélection positive de sous-clones cellulaires à capacité métastasante.

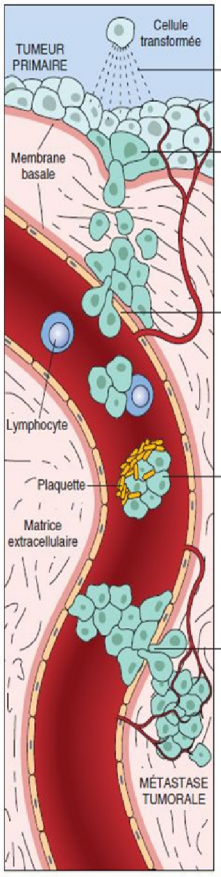
Obstacles		Etapes	Facteurs Mis en jeu	Références
Besoin en oxygène et nutriments, Déplacement		Etablissement d'un réseau vasculaire	Synthèse des facteurs VEGF, bFGF, PDGF	Bergers et Hanahan, 2008
Confinement dans l'organe, tumeur primaire		Perte d'adhésion locale (entre les cellules et à la matrice extra cellulaire)	Perte d'expression des molécules d'adhésion cellulaire (CAM) et en particulier des cadhérines épithéliales	Day, Zhao et al 1999 Rhodes et al 2003
Accès à la circulation Stroma		Invasion et dégradation de la matrice extra cellulaire	Synthèse des MMPs (pour Matrix metalloprotéinases) tels que MMP1, MMP9 Secrétion de la protéine uPA, Cathepsine (B, K et D)	Lichtinghagen, Wood, Fudge et al, 1997 Castellano et al, 2008 Hoosein, Boyd et al 1991 Xing et Rabbani 1999
Pénétration dans le réseau vasculaire		Intravasion	Perméabilisation de la membrane basale	van Zijl et al, 2011
		Transport via la circulation		
Résistance au système immunitaire(natural killer, Lymphocyte T) à l'agression mécanique , pression sanguine, élongation, friction dans les capillaires		Survie dans la circulation	Echappement immunitaire Agrégation Interaction avec les cellules plaquettaires	
Sortie du réseau vasculaire		Extravasion	Adhésion à l'endothélium Rétractation des cellules endothéaliales	Lehr et Pienta, 1998
Survie et prolifération au nouvel ecosytème		Invasion du nouveau territoire	Expression de molécules d'adhésion pour ancrage Facteurs chimiotactiques (chimiokines et récepteurs) Facteurs de croissance Echappement à la réponse immunitaire locale Néovascularisation	

Tableau 3: Principales étapes de la formation d'une métastase par voir hématogène. (Schéma d'après Collège Français des Pathologistes).

La compréhension du processus métastatique est donc impérative pour développer des nouveaux traitements afin de combattre efficacement ces lésions cancéreuses secondaires et d'envisager des perspectives de guérison.

3. Du ciblage osseux des cellules métastatiques du CaP

L'os est le site privilégié pour le développement des métastases des CaP. Probablement, les paramètres anatomiques, physiques et mécaniques du plexus vasculaire vont aider au tropisme osseux des cellules tumorales circulantes (CTC) (Weiss et al. 1989; Arya et al. 2006). Néanmoins des expériences *in vitro* ont montré que les cellules cancéreuses prostatiques ont une affinité pour l'endothélium recouvrant la moelle osseuse (Lehr and Pienta 1998) suggérant qu'il existe bien une compatibilité entre « the seed and the soil » (Pajet 1889).

En outre, Koeneman et al. ont formulé l'hypothèse que les cellules CaP qui métastasent à l'os, mimeraient les cellules normales de l'os en exprimant des gènes du phénotype d'une cellule osseuse et en codant des molécules de signalisation et d'échange entre les cellules osseuses et la matrice (Koeneman et al. 1999). Ce mimétisme osseux, appelé ostéomimétisme, favorise le tropisme des cellules cancéreuses circulantes à l'os et représente un avantage de survie dans le tissu hôte.

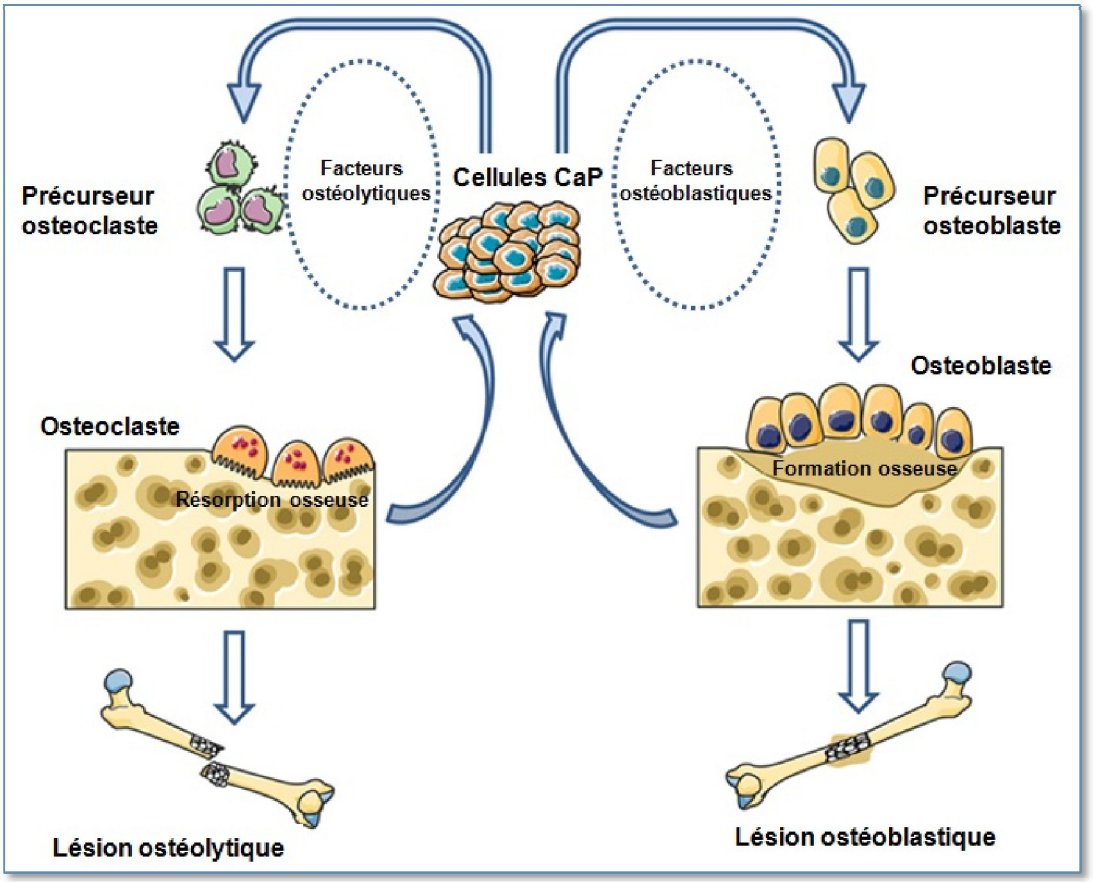


Figure 4: Représentation très simplifiée du développement de métastases osseuses dans le cancer de la prostate. Un cercle vicieux s'établit entre les cellules malignes et les cellules osseuses.

Echange cellules CaP --> cellules Os			Echange cellules Os --> Cellules CaP		
Facteurs chimiotactiques	MMP	Jacob et al, 1999 Tsingotjidou et al, 2001	Facteurs chimiotactiques	Osteonectine TGFbeta1	Jacob et al, 1999 festuccia et al, 1999
	CXCR4	Sun et Wang, 2003 Taichman et al, 2002		EGF IGF1 et IGF2 CXCL12	Rajan et al, 1996 Ritchie et al, 1997 Sun et Wang, 2003 Taichman et al, 2002
Ostéomimétisme	Runt-related transcription factor 2 (RUNX2) Sialo-protéine osseuse (BSP) Osteopontine (OPN) Osteonectine (ON)	Cher et al,2001 Edlung sung 2004 Clezardin et Teti, 2007			
Contact physique		Jun Wang et al, 2006	Contact physique		Shiirevnyamba et al, 2011
Environnement	Hypoxie Calcium Sensing Receptor (CaSR)		Environnement	Hypoxie (5% O2) Acidité Ca++ extra cellulaire (8-40 mM)	Kingsley et al, 2007
Dégradation de la matrice osseuse	Insulin-like Growth Factor Binding Protein (IGFBP) Parathyroid-hormone-related peptide (PTHrP) MMP urokinase type Plasmonogen activator (uPA)	 Kanoh et al, 2002	Croissance tumorale/facteurs prolifératifs	Insulin-like Growth Factor (IGF) Transforming Growth Factor (TGFb) Fibroblast Growth Factor (FGF) Bone Morphogenetic Protein (BMP) Bone Morphogenetic Protein (BMP)	Ritchie et al, 1997 Logothetis et lin, 2005 Ritchie et al, 1997 Juarez et Guise, 2011 Logothetis et lin, 2005 Ye et al, 2011
Facteurs ostéoclastiques	RANKL Osteoprotégerine (OPG) Parathyroid-hormone-related peptide (PTHrP) IL-6 MMP	Brown et al, 2001 Deftos et al, 2005 Siegall et al, 1990 Nemeth et al, 2002			
Inhibiteur activité ostéoclastique	Prostate specific antigen (PSA) Endotheline-1 (ET-1)				
Facteurs ostéoblastiques	Prostate specific antigen (PSA) urokinase type Plasmonogen activator (uPA) Endotheline-1 (ET-1) Sialo-protéine osseuse (BSP) TGFb Fibroblast Growth Factor 8 (FGF-8) VEGF Bone Morphogenetic Protein 2 (BMP2) Platelet-derived Growth Factor (PDGF) Parathyroid-hormone-related peptide (PTHrP)	Nadiminty et al, 2006 Rabbani et al, 1990 Koutsilieris et al, 1994 Guise et mohammed 2006 Eaton et Coleman 2003 Logothetis et lin 2005 Valta et al, 2008 Dai et al, 2004 Autzen et al, 1998 Lee et al 2003 Chott et al, 1999 Mehrotra et al, 2004 Cramer et al, 1996			

Tableau 4: Les interactions entres les cellules tumorales prostatiques et les cellules osseuses. (Liste non exhaustive).

Il est maintenant admis que la formation des métastases osseuses des CaP est un cercle vicieux (figure 4 et tableau 4) mettant en jeu les cellules tumorales prostatiques, les cellules osseuses et le microenvironnement, c'est-à-dire la matrice osseuse et des cellules présentes telles que les cellules immunitaires, et les adipocytes (figure 5). Les études menées ont permis de mettre en évidence la complexité de ce processus mettant en jeu un grand nombre d'interactions physiques et chimiques entre les cellules, des facteurs environnementaux tels que l'hypoxie, la calcémie, le pH, la pression physique. L'ensemble de ces échanges entraine un environnement dynamique favorable à la désorganisation de l'os et la multiplication des cellules tumorales.

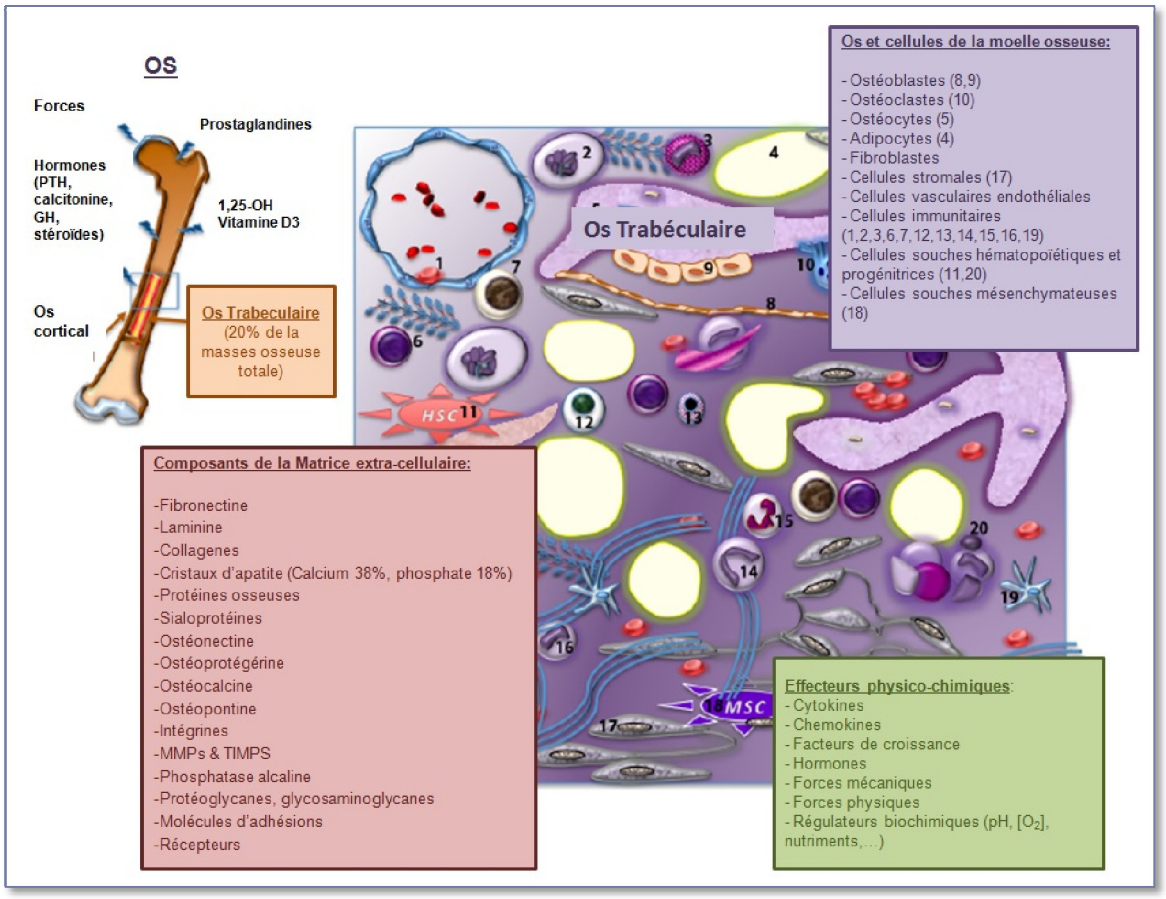


Figure 5: Microenvironnement osseux (modifié d'après Ferrarini et al. 2013).

D. Le gène de fusion TMPRSS2 :ETS

1. 2005, la révolution moléculaire dans le CaP

La mise en évidence, en 2005, de gènes de fusion impliquant d'une part un gène hormono-dépendant *TMPRSS2* (pour *trans-membrane protease serine 2*), codant une sérine protéase transmembranaire, et d'autre part un gène de la famille ETS, le gène *ERG* et dans une moindre mesure les membres du groupe PEA3/ETV, a représenté une révolution dans la connaissance biologique des tumeurs prostatiques (Petrovics et al. 2005; Tomlins et al. 2005; Demichelis et al. 2007; Perner et al. 2007; Rajput et al. 2007; Klezovitch et al. 2008). Le cancer de la prostate et les gènes de la famille ETS sont aujourd'hui étroitement associés (40 à 80% des CaP). Outre le gène *ERG*, les gènes *ETV1* (pour *ETS* variant *1*), *ETV4, ETV5, ELK4* (pour *ETS domain-containing protein ELK-4*), et dernièrement *FLI1* (pour *Friend leukemia virus integration 1*) ont été trouvés fusionnés à la partie 5'UTR du gène *TMPRSS2*. (Tomlins et al. 2008; Rickman et al. 2010; Hollenhorst et al. 2011; Paulo et al. 2012; Zhang et al. 2012). D'autres gènes de fusion n'impliquant pas les membres de la famille ETS ont également été décrits (Rubin et al. 2011).

Ces fusions entrainent la juxtaposition de la région promotrice et 5' non codante d'un gène hormono-dépendant (*TMPRSS2, SLC45A3, HERPUD1, NDRG1*) avec la séquence

codante d'un membre de la famille ETS par délétion intrachromosomale ou par translocation (Iljin et al. 2006; Attard et al. 2008; FitzGerald et al. 2008; Mehra et al. 2008). En conséquence, les produits des gènes de fusion *TMPRSS2-ETS* dans les cellules prostatiques sont des protéines ETS exprimées de façon massive et ectopique puisque leur expression est devenue hormono-dépendante, sensible à l'activité des récepteurs aux androgènes.

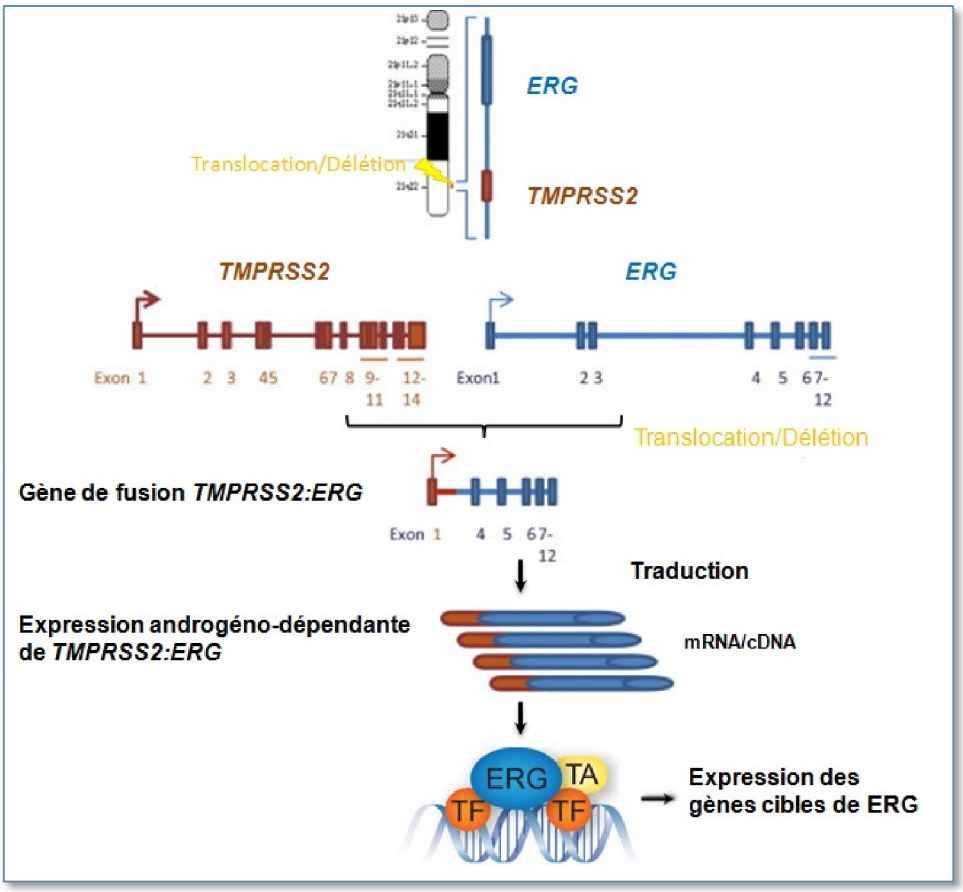


Figure 6: Formation du gène de fusion *TMPRSS2:ERG* par translocation/délétion dans le cancer de la prostate entrainant l'expression anormale et androgéno-dépendante du facteur ERG.

Une grande diversité de transcrits dérivant de ces gènes de fusion a été rapportée dans la littérature, résultant du site de fusion entre les gènes et de l'épissage alternatif (Hu et al. 2008). Parmi les gènes de fusion identifiés dans les CaP, la fusion *TMPRSS2 :ERG* est la plus fréquente (85% des cancers présentant la fusion) et en particulier la fusion entre l'exon 1 du gène *TMPRSS2* et l'exon 4 du gène *ERG* (exon 218) (figure 6) (Kumar-Sinha et al. 2008; Rubin et al. 2011). La protéine issue de cette fusion est une protéine ERG tronquée des 32 premiers acides aminés par rapport à la protéine ERG complète. Notre équipe s'est focalisée dans un premier temps sur l'étude fonctionnelle de la protéine ERG issue de cette fusion *TMPRSS2 :ERG,* même si l'étude fonctionnelle des fusions impliquant ETV1, ETV4, ETV5, ELK4 et FLI1 est envisagée dans un second temps

2. Rôle de TMPRSS2 :ERG dans le cancer de la prostate

Les protéines ERG, comme l'ensemble des membres de la famille ETS, sont des facteurs de transcription intervenant dans les voies de signalisation impliquées dans la croissance, la différenciation et la cancérogenèse (Beuzeboc et al. 2009). Après la découverte des gènes de fusion dans les CaP, des travaux ont rapidement montré une relation entre ces altérations moléculaires et des caractéristiques phénotypiques (Mosquera et al. 2009). L'expression anormale de ces translocations dans la prostate, joue un rôle évident dans la progression du cancer même si leurs rôles diagnostiques et pronostiques peuvent encore être discutés. La relevance fonctionnelle de ces facteurs de transcription issus des gènes de fusion constitue un nouvel enjeu dans la compréhension des mécanismes de développement et de progression du cancer de la prostate.

Plusieurs études ont cherché à caractériser le rôle de la fusion dans le CaP et ont confirmé que l'expression de ces fusions représente un événement moléculaire clé dans la progression du CaP. Les études cliniques tendent à associer la présence des gènes de fusion aux critères d'agressivité tumorale et à un mauvais pronostic : stade de haut grade, métastases (Mehra et al. 2008) même si paradoxalement aucune corrélation n'a été observée entre la présence de la fusion et le score de gleason. Des études fonctionnelles du gène de fusion dans des modèles murins ont montré que les souris transgéniques exprimant *TMPRSS2 :ERG* développent des lésions PIN (Klezovitch et al. 2008; Tomlins et al. 2008).

Des expériences de modulation *in cellulo* ont montré l'implication du gène de fusion dans la prolifération, la différenciation, la migration, l'invasion cellulaire (Klezovitch et al. 2008; Sun et al. 2008; Tomlins et al. 2008; Chng et al. 2012). Enfin des travaux ont cherché à identifier les gènes cibles de ERG, puisque la surexpression des protéines TMPRSS2 :ERG conduit à la dérégulation de ces derniers pouvant être impliqués dans des processus cellulaires et voies de signalisation clés dans la compréhension de l'histoire du CaP (figure 7) (Clark and Cooper 2009).

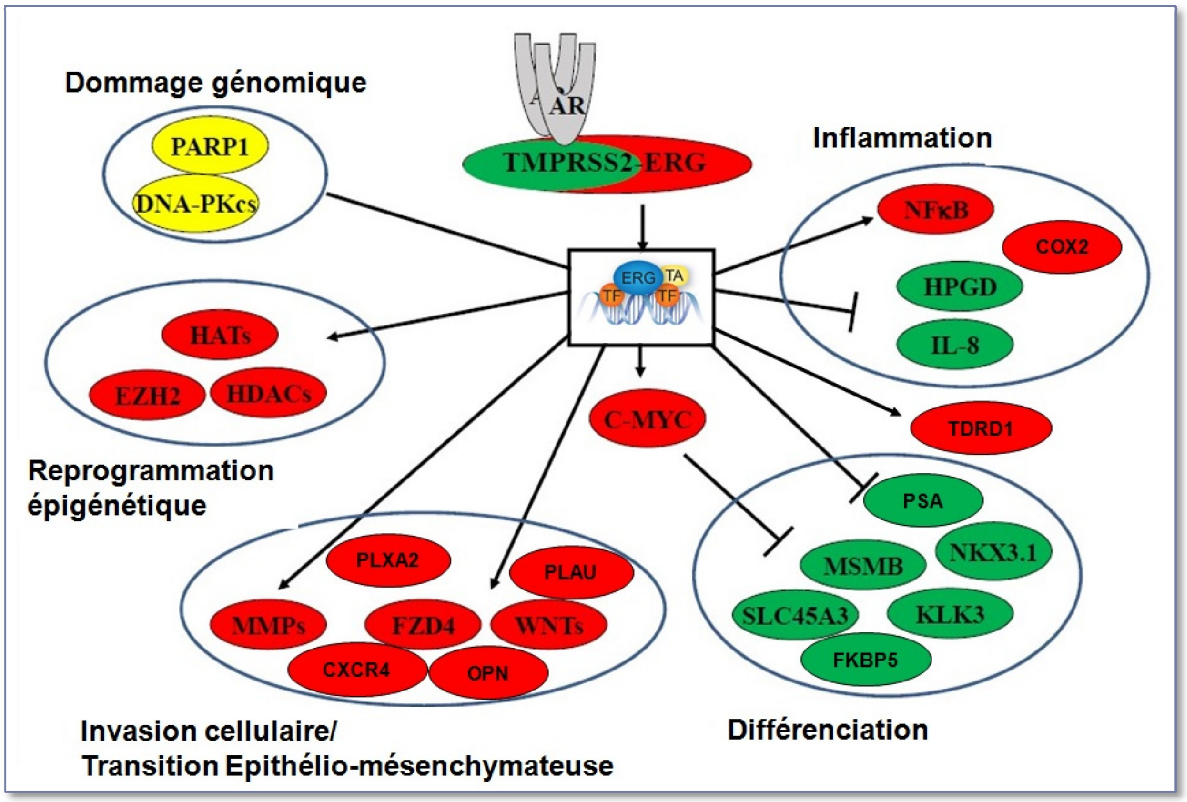


Figure 7: Gènes cibles de TMPRSS2:ERG dans le cancer de la prostate. En rouge: les gènes dont l'expression est augmentée par la fusion, en vert: les gènes dont l'expression est diminuée, en jaune : les protéines qui interagissent avec TMPRSS2 :ERG (modifié d'après Sreenath et al. 2011).

Le profil d'expression des gènes de fusion dans les tumeurs prostatiques est complexe. Au sein d'une même tumeur, il a en effet été observé une hétérogénéité de l'expression des fusions *TMPRSS2 :ERG* dans les différents foyers (Mehra et al. 2007). L'expression des gènes de fusion n'est pas le seul facteur impliqué dans l'initiation, la progression et l'invasion du CaP (Rubin et al. 2011). La synergie ou l'interférence de la fusion avec d'autres facteurs de transcription ou altérations génomiques peuvent contribuer à l'évolution de l'histoire du CaP.

3. Les interactions de TMPRSS2 :ERG

Dans le paragraphe IV.D., nous avions abordé la liste des partenaires de ERG. Puisque TMPRSS2 :ERG conserve les domaines ETS et PNT, importants dans les interactions de ERG avec ses partenaires, on peut supposer qu'il y ait conservation des interactants, (excepté les partenaires qui interagissent avec le domaine de ERG impliqué dans la liaison avec la protéine ESET).

La protéine issue de la fusion TMPRSS2 :ERG est capable d'inhiber la fixation du récepteur aux androgènes sur le promoteur de ses gènes cibles, et d'interagir physiquement avec ce récepteur, via son domaine ETS, en absence d'ADN (Yu et al. 2010; Chng et al. 2012). TMPRSS2 :ERG et AR peuvent également recruter des corépresseurs tels que les

HDACS1, HDAC2 et EZH2 pour former un complexe répressif. EZH2 est une enzyme responsable de la triméthylation de la lysine 27 de l'histone H3 qui permettrait le recrutement de PRC1 (pour *polycomb repressive complex 2*) et de HDAC (Ren et al. 2012). ERG participe à la dérégulation de l'acétylation des histones en favorisant l'activité HDAC et en inhibant l'activité de cofacteur tel que CBP/P300 (Fortson et al. 2011).

Une étude protéomique visant à identifier des protéines interagissant avec ERG a permis de montrer que ERG interagit avec poly (ADP-ribose) polymerase (PARP) et la sous unité catalytique de la DNA-PKcs (pour *DNA protein kinase*) de façon indépendante à l'ADN et avec les protéines Ku70 et Ku80 (hétérodimère essentiel du complexe DNA-PK/PARP1) de manière DNA dépendante (Brenner et al. 2011). La PARP-1 fait partie intégrante de complexes protéiques se fixant sur le promoteur de certains gènes pour activer leur transcription. Au niveau de la chromatine, elle peut destabiliser les nucléosomes par parylation des histones. Enfin la parylation de certains facteurs de transcription peut modifier leurs affinités pour l'ADN ou pour leur corégulateurs.

VII. Etudes fonctionnelles du gène de fusion TMPRSS2 :ERG dans les métastases osseuses du CaP

Notre équipe s'intéresse depuis longtemps au rôle physiologique et pathologique des facteurs de transcription appartenant à la famille ETS, et en particulier de ERG. Lorsque je suis arrivé dans l'équipe, le projet post-doctoral qui m'a été confié était d'étudier la fonction du facteur de transcription ERG dans la mise en place du cartilage à partir des souris transgéniques déjà établies et caractérisées pour leur phénotype. Afin d'expliquer le vieillissement précoce observé chez les souris transgéniques DN-Erg, nous avons réalisé une étude transcriptomique comparative. Une liste de gènes dont l'expression est perturbée, directement ou non, par l'expression du transgène *DN-Erg*, a été obtenue. Parmi ces gènes, figure le gène de l'*Opn* qui code une protéine matricielle, abondante dans le tissu osseux, et largement décrit dans la littérature pour son implication dans l'invasion tumorale et les métastases de nombreux cancers.

Avec la découverte des fusions ERG dans le CaP, et puisque ERG régule l'expression de gènes impliqués dans la mise en place du cartilage et du squelette, la thématique de l'équipe a évolué vers l'étude du gène de fusion *TMPRSS2 :ERG* dans la progression métastatique à l'os du CaP. C'est dans ce contexte que j'ai initié l'étude de la régulation du gène de l'*OPN* par le facteur ERG et la protéine issue de la fusion *TMPRSS2 :ERG*. Les résultats obtenus ont permis de renforcer notre hypothèse sur l'implication du gène de fusion *TMPRSS2 :ERG* dans la formation des métastases osseuses du CaP et de poursuivre ce projet.

A. L'expression anormale du facteur de transcription ERG dans les cellules cancéreuses de prostate active l'Ostéopontine.

Pour cette étude, nous avons établi des lignées de cellules cancéreuses prostatiques PC3 et PC3c (lignée cellulaire dérivée de la lignée PC3) qui expriment stablement la fusion *TMPRSS2 :ERG* (Fradet et al. 2013).

L'expression du gène *Opn* est augmentée dans les cellules cancéreuses de prostate PC3c transfectées stablement par le vecteur d'expression codant le facteur ERG ou la protéine issue de la fusion *TMPRSS2-ERG*. L'étude du promoteur de l'*OPN* par mutagénèse dirigée, retard sur gel et par ChIP nous a permis de mettre en évidence et de caractériser la fixation des facteurs ERG et TMPRSS2-ERG *in cellulo*. Parallèlement à l'étude cellulaire et moléculaire, nous avons montré une corrélation significative entre l'expression du gène *OPN* et celle du gène de fusion *TMPRSS2-ERG* à partir des ARN d'échantillons humains de CaP obtenus par la collaboration avec la tumorothèque de la Cancéropôle Nord-Ouest. D'autre part, des études d'immunomarquages dans les tissus cancéreux prostatiques révèlent une colocalisation de l'OPN et de TMPRSS2-ERG. Ces résultats suggèrent donc que l'*OPN* est

un gène cible direct de *TMPRSS2-ERG* dans le CaP, et pourrait jouer un rôle dans la formation des métastases osseuses (Flajollet et al. 2011).

Publication relative à ce projet :

1. **Abnormal Expression of the ERG Transcription Factor in Prostate Cancer Cells Activates Osteopontin.**
   Flajollet S., Tian T., Flourens A., Tomavo N., Villers A., Bonnelye E., Aubert S., Leroy X., Duterque-Coquillaud M., Mol. Cancer Research, 2011

B. TMPRSS2 :ERG module la voie des prostaglandines dans les cellules cancéreuses de prostate

Afin d'étudier d'autres gènes cibles de la fusion *TMPRSS2-ERG* impliqués dans les métastases osseuses du CaP, et de caractériser de façon plus globale le transcriptome des clones cellulaires PC3 et PC3c exprimant stablement *TMPRSS2-ERG*, nous avons réalisé l'étude transcriptomique de ces cellules sur puces Agilent. Cette approche à grande échelle pour chaque modèle cellulaire, nous a permis d'obtenir le transcriptome de ces cellules et de réaliser l'étude comparative entre l'expression de la fusion *TMPRSS2 :ERG* et la condition contrôle (PC3c T1E4 versus PC3c PCDNA3 et iPC3 T1E4 versus iPC3 PLPCX).

De manière surprenante, parmi les gènes dont l'expression est perturbée par la présence de TMPRSS2 :ERG dans les cellules PC3, nous retrouvons 7 gènes sur les 9, soit 80 % des gènes décrits pour être dérégulés par le contact physique des cellules PC3 wt avec des ostéoblastes (Shiirevnyamba et al. 2011). Ces gènes sont principalement liés à l'homéostasie osseuse et aux échanges cellulaires, suggérant que l'expression de la fusion dans les cellules PC3 mime le dialogue et donc l'activité des cellules osseuses sur les cellules tumorales.

Parmi les gènes identifiés par l'étude transcriptomique, je me suis focalisé sur un cluster de gènes impliqués dans la voie de signalisation des prostaglandines (PGE2). Les prostaglandines sont présentes à de fortes concentrations dans les tissus tumoraux, et sont des médiateurs des signaux de transduction qui modulent la croissance, l'adhésion cellulaires et le tropisme à l'os. Une étude récente (Mohamed et al. 2011) a mis en évidence l'interférence de TMPRSS2-ERG dans la voie de signalisation des prostaglandines, au niveau de l'inhibition de l'expression de *15-hydroxy-prostaglandine dehydrogenase* (*HPGD*). HPGD intervient dans le catabolisme des prostaglandines (PGE2) et est considéré comme un gène suppresseur de tumeur. L'étude transcriptomique que nous avons menée dans les cellules PC3 confirme la modulation de l'expression de *HPGD* par TMPRSS2 :ERG et met en évidence l'augmentation du niveau d'expression des gènes *Phospholipase A2* (*PLPA2*) et *Cyclooxygenase 2* (*COX2*). PLPA2 et COX2 sont impliqués dans la voie de synthèse de PGE2. En conséquence, le taux de PGE2 augmente sévèrement dans les cellules PC3

TMPRSS2 :ERG par rapport aux cellules wt, c'est ce que nous avons observé par le dosage de ces molécules dans le milieu de culture.

Afin de montrer la régulation directe des gènes de ce cluster par la fusion *TMPRSS2 :ERG*, j'ai réalisé l'analyse de données de ChIP-seq générées par d'autres laboratoires et référencées dans des banques (NCBI SRA, ENA SRA). L'analyse des résultats de ChIP-seq réalisé dans la lignée cellulaire VCAP (positive pour la fusion), montre la fixation de ERG sur le promoteur des gènes *HPGD* et *COX2*. Nous avons complété notre étude par des expériences de ChIP dans les clones stables PC3 TMPRSS2 :ERG.

L'étude de l'expression des transcrits COX2 et du gène de fusion TMPRSS2-ERG à partir des ARN d'échantillons humains de CaP de notre banque d'échantillons de tumeurs prostatiques (collaboration avec la tumorothèque de la Cancéropôle Nord-Ouest) a permis d'établir une corrélation significative entre ces 2 gènes.

En conclusion, dans les tumeurs prostatiques, TMPRSS2 :ERG module la voie de signalisation des prostaglandines qui peut contribuer à la progression tumorale (Flajollet et al. en préparation).

C. TMPRSS2 :ERG régule *MMP9* et *PLXNA2*

Suite aux études transcriptomiques réalisées dans les cellules PC3 et PC3c, d'autres gènes cibles de TMPRSS2-ERG sont étudiés par les membres de l'équipe dans le cadre de ce projet (Thèse de Tian V. Tian et master 2 recherche de Carine Delliaux). Nous avons démontré que la fusion *TMPRSS2 :ERG* est capable d'augmenter la capacité migratoire et invasive des cellules cancéreuses prostatiques. En particulier, parmi les gènes différentiellement exprimés en réponse à la fusion, nous avons montré que les gènes *Matrix metallopeptidase 9* (*MMP-9*) et *Plexin A2* (*PLXA2*) sont des gènes cibles directs (Tian et al. 2013). L'identification de ces nouveaux gènes cibles renforce notre hypothèse sur le rôle du gène de fusion dans les métastases du CaP.

Publication relative à ce projet :

1. **Identification of novel TMPRSS2:ERG mechanisms in prostate cancer progression: involvement of MMP9 and PLXNA2**
   Tian V.T., Tomavo N., Huot L., Flourens A., Bonnelye E., Flajollet S., Hot D., Leroy X., de Launoit Y., Duterque-Coquillaud M., Oncogene, 2013

D. Perspectives thérapeutiques visant à cibler l'activité fonctionnelle de la fusion TMPRSS2 :ERG

La régulation hormonale est cruciale pour la fonction des cellules prostatiques normales mais aussi pour la croissance des cellules cancéreuses prostatiques et l'évolution des

métastases osseuses. L'hormonothérapie, pouvant être associée à une radiothérapie, est le traitement de référence des CaP. Différents types de traitements peuvent être envisagés pour empêcher l'action de la testostérone sur les cellules cancéreuses : soit en inhibant la biosynthèse des androgènes (Attard et al. 2009) ; soit en bloquant l'activité des récepteurs hormonaux des cellules (de Bono et al. 2011). Ces traitements sont efficaces, mais cependant transitoires, puisque les tumeurs développent invariablement des mécanismes de résistance favorisant la progression vers la phase terminale de la maladie. Les facteurs de transcription oncogènes, à l'instar des produits des gènes de fusion TMPRSS2-ETS, représentent des cibles thérapeutiques d'intérêt permettant de jouer sur l'amélioration des processus physiologiques dérégulés et d'intervenir de manière appropriée pour le traitement du patient.

Différentes approches peuvent être envisagées pour cibler directement ou indirectement l'activité d'un facteur de transcription dans le traitement des cancers : perturbation de l'expression protéique du facteur de transcription, inhibition indirecte, perturbation physique du facteur de transcription par interférence dans l'interaction avec l'ADN ou par interférence des interactions avec les cofacteurs (Pandolfi 2001; Darnell 2002).

Dans le cadre d'un projet collaboratif avec l'équipe du docteur Marie-Hélène David (Lille, INSERM, JPARC) visant à développer une nouvelle stratégie d'inhibition de la fixation de ERG à l'ADN, nous avons testé *in cellulo* des petites molécules (Nhili et al. 2013). Ces petits composés DB (pour diamidines hétérocycliques) ont la capacité d'interagir au niveau du petit sillon de la double hélice d'ADN au niveau d'une séquence spécifique et de rentrer en compétition avec la protéine pouvant s'y fixer. L'une des molécules (DB1255) présente la propriété d'interférer sur la fixation de ERG à son élément de réponse. Les résultats obtenus *in cellulo* confirme ceux obtenus *in vitro*. En effet, l'activité transcriptionnelle induite par ERG sur des vecteurs rapporteurs contenant un site EBS (promoteur artificiel et promoteur du gène de l'*OPN*) diminue significativement dans les cellules traitées par ces composés.

La recherche de composés chimiques capables d'interférer spécifiquement dans la liaison du facteur de transcription à l'ADN, ouvre des perspectives thérapeutiques encourageantes.

Publication relative à ce projet :

1. **Targeting the DNA-binding activity of the human ERG transcription factor using new heterocyclic dithiophene diamidines.**
   Nhili R., Peixoto P., Depauw S., Flajollet S., Dezitter X., Munde M.M., Ismail M.A., Kumar A., Farahat A.A., Stephens C.E., Duterque-Coquillaud M., David Wilson W., Boykin D.W., David-Cordonnier M.H., Nucleic Acids Res., 2013

Projet de recherche : Identification et caractérisation des partenaires du facteur de transcription TMPRSS2 :ERG

Les facteurs de transcription ERG, comme les autres membres de la famille ETS, ne peuvent réguler seuls la transcription de leurs gènes cibles. Leur activité fonctionnelle dépend profondément de la composition et de l'organisation spatiale des complexes dont ils font partie. Les interactions protéine-protéine jouent un rôle crucial dans la formation de ces complexes en constituant un réseau dynamique et adaptable (Laudet et al. 2007). La possibilité d'interférer sur ces interactions offre une nouvelle source de cibles thérapeutiques prometteuses dans la recherche pharmacologique (Koehler 2010). Le développement de telles molécules capables de moduler ces interactions requiert au préalable une connaissance approfondie du réseau transcriptionnel impliqué.

L'objectif général de ce programme de recherche est de contribuer au développement de cette stratégie thérapeutique pour le traitement des CaP qui expriment la fusion *TMPRSS2 :ERG*. Malgré le grand nombre de travaux réalisés visant à évaluer l'intérêt diagnostique et à caractériser le rôle biologique des fusions ETS dans le cancer de la prostate, peu de travaux se sont aventurés dans l'identification des interactants des facteurs TMPRSS2 :ERG et dans la dissection de ce réseau moléculaire (St John et al. 2012). L'une des pistes de travail que j'envisage de mener sera de caractériser les partenaires protéiques de la fusion *TMPRSS2 :ERG*, ainsi que les mécanismes moléculaires mis en jeu dans ce réseau d'interaction. Puisque les récepteurs nucléaires (RN) sont des partenaires potentiels de ERG, un des axes de recherche consistera à identifier les récepteurs nucléaires (RN) susceptibles d'interférer avec le réseau transcriptionnel du facteur TMPRSS2 :ERG et à caractériser ses connexions moléculaires.

I. Identification et caractérisation de nouveaux partenaires de la fusion TMPRSS2 :ERG

Avant d'envisager une quelconque action sur les interactions protéine-protéine, il est nécessaire de pouvoir répondre à ces questions :

A. Quels sont les partenaires transcriptionnels des facteurs TMPRSS2 :ERG?
B. Quels sont les interfaces protéiques impliquées dans cette interaction ?
C. Quels sont les mécanismes moléculaires et les voies de signalisation mis en jeu dans ces interactions ?
D. Comment s'organise le réseau moléculaire de TMPRSS2 :ERG au niveau du promoteur de ses gènes cibles?
E. Existe-t-il des molécules spécifiques ciblant les partenaires ou les interactions mises en jeu ?

A. Identification de l'interactome de TMPRSS2 :ERG

La difficulté de ce type d'étude réside dans l'affinité entre les partenaires, la nature des interactions stables ou transitoires, binaires ou multiprotéiques, l'abondance de certaines protéines, la nécessité de certaines modifications post-traductionnelles, et la contamination par des interactions non spécifiques. Nous allons donc entreprendre des approches complémentaires, qui possèdent chacunes leurs avantages et leurs limites, afin de rechercher d'une manière « globale » des nouveaux partenaires de la protéine issue de la fusion *TMPRSS2-ERG*. Pour cela nous envisageons 3 approches complémentaires : 2 approches de purification par chromatographie d'affinité couplée à la spectrométrie de masse et une approche de double hybride.

1. La technique de purification de complexes protéiques par GST pull-down couplée à la spectrométrie de masse

Au cours de la recherche de nouveaux partenaires du récepteurs RAR impliquant le domaine AF1, j'avais développé pendant ma thèse avec mon tuteur Christophe Rachez, une stratégie qui nous avait permis d'obtenir rapidement des protéines candidates (Flajollet et al. 2013b). Dans le cadre de ce projet, nous envisageons une approche similaire.

Puisque la protéine ERG issue de la fusion est comparable à la protéine ERG^{p55} (la perte des 32 premiers acides aminés n'affecte pas l'intégrité des domaines structuraux (Leong et al. 2009)), nous allons utiliser les constructions de la protéine ERG^{p55} pleine longueur fusionnée à la GST, déjà établies dans l'équipe pour produire en bactéries le facteur de transcription. La protéine ERG fusionnée à la GST est alors fixée à la résine Glutathion avant d'être mise en présence d'extraits cellulaires de cellules cancéreuses prostatiques. Les protéines fixées à la résine sont éluées, séparées par SDS-PAGE puis identifiées par spectrométrie de masse MALDI-TOF (pour *Matrix Assisted Laser*

Desorption/ionisation Time of Flight). L'analyse par spectrométrie de masse est réalisée dans l'unité, en collaboration avec l'équipe d'Oleg Melnyk par Hervé Drobecq.

Nous avons initié des expériences préliminaires mettant en jeu la protéine GST-ERG et des extraits nucléaires de cellules PC3. Malgré la présence de bandes contaminantes ou correspondantes aux protéines GST-ERG, susceptibles de masquer les partenaires potentiels, l'analyse par spectrométrie de masse a permis d'identifier une dizaine de protéines candidates pour une interaction avec ERG (figure 8).

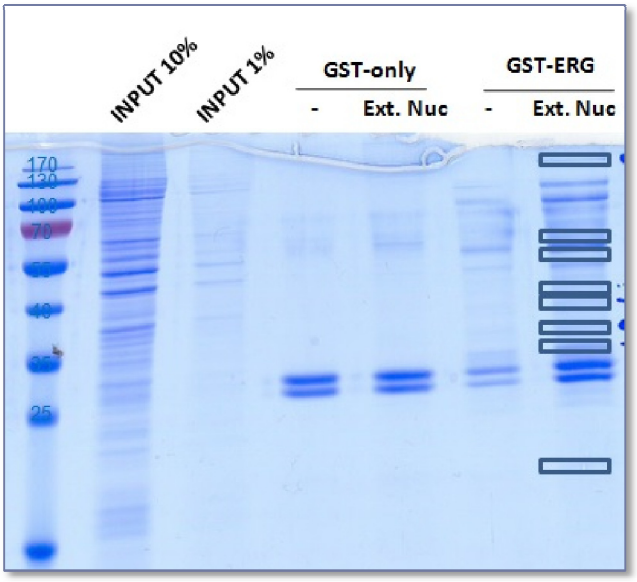


Figure 8: Isolation puis identification des protéines interagissant avec la protéine GST-ERG. Les protéines ERG fusionnées à la GST (GST-ERG) ou GST seule (GST) sont immobilisées sur une matrice glutathion et incubées en présence d'extraits nucléaires de cellules PC3 ou tampon seul (-). Les protéines présentes dans les complexes GST-ERG/interactants sont ensuite isolées sur gel SDS-PAGE (10%). Les rectangles correspondent aux bandes (protéines) qui ont été analysées par spectrométrie de masse.

Parmi les protéines isolées et identifiées par spectrométrie de masse, nous retrouvons plusieurs protéines dont l'interaction avec TMPRSS2 :ERG a déjà été décrite (Brenner et al. 2011). En particulier la PARP1 et les protéines KU70/XRCC6 et KU80/XCRR5 (voir paragraphe VI.D.3). Nous retrouvons également des protéines telles que la tubuline et HSP90 dont une interaction avec ERG a été mentionnée (voir paragraphe IV.D). D'autre part un certain nombre de facteurs d'initiation et d'élongation transcriptionnelle ont été identifiés parmi les protéines fixées à GST-ERG.

Afin de réaliser l'analyse d'un plus grand nombre de partenaires, nous envisageons d'améliorer:

- la coloration, afin d'augmenter la sensibilité du seuil de détection
- la séparation des protéines, par électrophorèse bidimensionnelle permettra de séparer les protéines en fonction de leur point isoélectrique et de leur masse, et de concentrer les protéines en un spot.

2. La technique de "purification en tandem par Tag"

Une autre approche pourra être envisagée : la méthode de purification de complexes protéiques dite de "purification en tandem par Tag" (Tandem Affinity Purification by Tag, ou TAP-Tag), qui permettra d'identifier les partenaires de la protéine d'intérêt tout en maintenant un niveau d'expression physiologique de la protéine et en conservant les conditions natives. Cette technique nécessite d'établir les constructions codant la protéine ERG doublement étiquetée, les clones cellulaires qui expriment stablement cette construction.

Nous utiliserons les modèles cellulaires de cancer de prostate PC3 et PC3c, qui n'expriment pas les fusions *TMPRSS2 :ETS*. Nous réaliserons des clones stables exprimant la protéine de fusion TMPRSS2 :ERG fusionnée au module de purification. Le complexe protéique bâti autour de la protéine appât TMPRSS2 :ERG-taguée sera extrait puis purifié par chromatographie d'affinité en deux étapes avant d'être analysé par spectrométrie de masse.

3. L'approche double-hybride

Nous n'excluons pas d'utiliser l'approche double-hybride pour identifier de nouveaux partenaires de TMPRSS2 :ERG dans le cadre de notre projet même si cette technique utilisée pour l'identification de partenaires de ERG chez le xénope n'avait permis de mettre en évidence qu'un seul interactant pour ERG : la protéine HSP90 (Deramaudt et al. 2001).

L'approche double-hybride sera réalisée chez la levure en criblant une banque d'expression d'ADNc obtenue à partir de cellules humaines de cancer de prostate (human prostate Matchmaker TmcDNA library commercialisée par la société Clontech) avec la protéine appât TMPRSS2-ERG.

4. Pertinence et validation des interactions

L'existence et la pertinence des interactions de TMPRSS2 :ERG avec les partenaires potentiels seront ensuite étudiées. L'analyse bibliographique permettra de vérifier si la protéine candidate est connue pour interagir avec le facteur ERG ou l'un des membres de la famille ETS. D'autre part sur la base de plusieurs critères, tels que la localisation nucléaire, les propriétés fonctionnelles, l'implication ou l'altération d'expression dans le CaP, nous sélectionnerons les protéines candidates avant de poursuivre l'étude de façon plus approfondie.

Nous réaliserons des expériences *in vitro* de GST pull-down et *in cellulo* de co-immunoprécipitation et immunocytochimie afin de confirmer l'interaction directe entre TMPRSS2 :ERG et les partenaires potentiels.

B. Caractérisation des interactions moléculaires

L'interaction protéique sera ensuite caractérisée par les techniques de co-rétention protéique et de co-immunoprécipitation. Nous déterminerons les domaines de ERG et du partenaire impliqué dans cette interaction. Pour cela, nous utiliserons les protéines de fusion GST-ERG ou ERG-Tagguée délétées des domaines fonctionnels afin de délimiter la zone minimale d'interaction nécessaire à la fixation du partenaire. L'interaction de ERG avec PARP1 nécessite le recrutement de DNA-PKcs. Cette interaction est dépendante du domaine ETS de ERG, et plus précisément des acides aminés YYDKN (Y373) (Brenner et al. 2011). C'est ce que nous avons vérifié par l'utilisation d'un mutant de délétion GST-ERG ΔETS qui entraine la perte d'interaction avec PARP1.

Il n'est pas exclu que des modifications post-traductionnelles ou la présence d'autres partenaires participent ou interfèrent dans ces interactions. Nous en tiendrons compte au cours de notre étude.

La caractérisation des interactions moléculaires permettra de définir une cartographie des domaines TMPRSS2 :ERG impliqués dans l'interaction.

C. Etude de l'activité fonctionnelle et des mécanismes moléculaires mis en jeu

La relevance fonctionnelle de l'interaction mise en évidence sera évaluée. L'expression du cofacteur sera modulée par surexpression ou par déplétion par la technique d'ARN interférence puis l'activité transcriptionnelle sera mésurée. Nous disposons de vecteurs rapporteurs luciférase activés par ERG (enhancer du virus du polyome, promoteurs des gènes cibles de ERG que nous avons caractérisés, comme par exemple celui de l'*OPN*).

Il est maintenant bien établi que le recrutement des facteurs de transcription et de leurs partenaires au niveau du promoteur est spécifique. Précédemment, j'ai montré le recrutement différentiel des cofacteurs AF9 et BRD4 par les récepteur des rétinoïdes RAR sur le promoteur des gènes induits en réponse aux rétinoïdes (Flajollet et al. 2013b).

Avec la recherche de nouveaux gènes cibles de la fusion *TMPRSS2 :ERG* impliqués dans les métastases osseuses du CaP, nous disposons de modèles cellulaires PC3 et PC3c et de listes de gènes, cibles potentiels de la fusion. En fonction des partenaires identifiés, on peut envisager de développer des clones cellulaires exprimant ou non la fusion dans lesquels nous aurons modulé l'expression des protéines étudiées (surexpression ou déplétion par RNA interférence). Lorsque ces modèles cellulaires seront caractérisés, on réalisera une étude comparative du transcriptome afin de déterminer les gènes dépendants du complexe ERG-Partenaire(s) étudiés. Cette étude sera réalisée dans le cadre d'une

collaboration avec la plateforme Biopuce - *Laboratoire d'Etudes Transcriptomiques et de Génomique Appliqués* avec qui nous collaborons.

D. Modélisation des réseaux de régulation transcriptionnelle des gènes cibles de TMPRSS2 :ERG

L'expression anormale de la fusion TMPRSS2 :ERG dans les cellules cancéreuses de prostate va entrainer la fixation du module ERG au niveau de ses éléments de réponse présents dans les promoteurs des gènes cibles et une modulation d'expression de ces derniers. Cependant la régulation de l'expression transcriptionnelle d'un gène est le résultat de l'intégration des activités de plusieurs facteurs de transcription et cofacteurs (modules) au niveau de son promoteur. Il est donc probable que TMPRSS2 :ERG vienne perturber directement ou non, la fixation des autres modules présents sur le promoteur contribuant à la réponse transcriptionnelle observée.

Afin de modéliser ces mécanismes moléculaires au niveau d'un promoteur spécifique, nous envisageons de réaliser des expériences utilisant la technique de promoteur-Pull down (ou DNA pull down) avec un fragment du promoteur d'un gène cible de ERG impliqué dans le CaP, centré sur le site de fixation de ERG. La séquence oligonucléotidique, fixée à une bille streptavidine, sera testée en présence d'extraits protéiques nucléaires de cellules exprimant ou non la fusion. Les protéines retenues seront séparées sur gel électrophorèse puis caractérisées par spectrométrie de masse. Ces protéines correspondent à l'ensemble des facteurs de transcription qui se fixent dans la région promotrice correspondante du gène étudié. L'analyse comparative des profils d'interaction obtenus avec les extraits protéiques des cellules exprimant ou non la fusion, permettra de définir les facteurs de transcription recrutés ou au contraire empêchés par la fixation de la fusion *TMPRSS2 :ERG* sur son élément de réponse. En outre, par cette technique, de nouvelles protéines interagissant avec la fusion pourront être mises en évidence.

Dans un premier temps, les régions promotrices centrées sur les sites fonctionnels EBS des gènes cibles de ERG que nous avons caractérisés, tels que l'*OPN et COX2,* pourront servir à la recherche de nouveaux partenaires de ERG. Nous pourrons proposer un modèle intégré de la régulation transcriptionnelle de l'expression de ces gènes dans nos modèles cellulaires de CaP.

A terme, on peut envisager une stratégie de modélisation à grande échelle qui intégrera les données de ChIP-seq et de protéomique. En effet, la recherche de motifs particuliers significativement retrouvés au voisinage des sites de fixation de ERG devrait permettre d'établir les réseaux de régulation transcriptionnelle du facteur TMPRSS2 :ERG *in vivo* pour un cluster de gènes donnés. Cet aspect du projet nécessite l'implication forte des

bioinformaticiens qui pourrait se matérialiser par exemple par l'encadrement en cotutelle d'un étudiant.

E. Recherche de molécules pharmacologiques

Avec la découverte des gènes de fusion TMPRSS2 :ETS dans les CaP, et vu le rôle crucial des facteurs de transcription ETS, ces protéines représentent des cibles pertinentes dans la recherche de nouvelles thérapies personnalisées des cancers de la prostate.

Une stratégie évidente consisterait à inhiber l'expression du gène codant cette fusion par des siRNA qui seraient libérés dans l'environnement tumoral. Des essais cliniques chez l'homme utilisant l'approche des siRNA pour traiter certaines pathologies ont présenté une bonne efficacité. Des travaux de recherche visant à cibler par des siRNA vectorisés, par couplage au squalène pour former des nanoparticules, spécifiques de la jonction TMPRSS2-ERG dans le CaP (Laboratoire de L. Massade à Villejuif) sont actuellement réalisés.

Lors de notre collaboration avec l'équipe de Marie-Hélène David (Centre Jean-Pierre Aubert à Lille), nous avons mis en évidence le pouvoir inhibiteur de petites molécules spécifiques sur la fixation des facteurs ERG au niveau de leur élément de réponse. Malgré leur efficacité, les composés utilisés sont très toxiques au niveau cellulaire. Ils peuvent néanmoins servir d'outils capables de perturber l'interaction de ERG à l'ADN.

Au lieu de cibler l'interaction ADN-protéine, nous avons considéré l'interaction protéine-protéine pour inhiber l'activité fonctionnelle de ERG et donc proposer des nouvelles stratégies thérapeutiques. En fonction des partenaires que nous aurons identifiés, nous chercherons l'existence de molécules inhibitrices déjà décrites pour ces protéines et nous les testerons. Par ailleurs, au cours de notre projet, nous envisageons de déterminer précisément les interfaces d'interaction de ERG. On pourra alors concevoir avec l'aide des chimistes (Equipe de Oleg *Melnyk* au sein de laboratoire) des petits peptides capables d'interférer spécifiquement sur ces interactions et les tester *in vitro* et *in cellulo*.

II. Inter-relation entre TMPRSS2 :ETS et les récepteurs nucléaires.

A. Contexte scientifique

Parmi les protéines déjà connues pour interagir avec certains facteurs de transcription, on trouve les RNs (Gauthier et al. 1993). La famille des RNs comporte une quarantaine de membres qui exercent un rôle primordial dans un grand nombre d'aspects du fonctionnement cellulaire. Leur impact dans la physiologie cellulaire est directement corrélé à leur activation par leurs ligands respectifs et à la composition moléculaire des complexes dans lesquels ils sont intégrés. Par ces propriétés, les RNs sont des cibles thérapeutiques privilégiées dans un certain nombre de pathologies comme les cancers ou les désordres métaboliques (diabète, hyperlipidémies et arthérosclérose). En démontrant que les RNs activés par leurs ligands peuvent moduler l'activité fonctionnelle des oncogènes ETS, et réciproquement (Gauthier et al. 1993; Schneikert et al. 1996; Darby et al. 1997), cela ouvre des perspectives attrayantes pour inhiber les processus cellulaires inappropriés pour le fonctionnement « normal » de la cellule, et dirigés par les protéines ETS.

Au cours de précédents travaux, le laboratoire a exploré l'interaction fonctionnelle du facteur ERG avec plusieurs membres de la famille des récepteurs nucléaires (Vlaeminck-Guillem 2000; Vlaeminck-Guillem et al. 2003a; Vlaeminck-Guillem et al. 2003b). En particulier, une répression mutuelle entre ERG et les récepteurs nucléaires ERα, ERβ, TRα1 et 2, TRβ1, RARα, RXRα et le récepteur orphelin RORα a été démontrée et étudiée. Récemment il a été établi que les protéines issues du gène de fusion *TMPRSS2 :ERG,* dont l'expression est régulée par les androgènes, interagissent physiquement avec les récepteurs aux androgènes (RA) et interfèrent sur l'activité fonctionnelle de ces derniers. (Yu et al. 2010; Chng et al. 2012). ERG pourrait avoir un effet inhibiteur sur l'activité transcriptionnelle de AR, et réciproquement (Sun et al. 2008; Yu et al. 2010). Par ailleurs, les analyses de ChIP-seq ont mis en évidence que ERG et AR partagent des gènes cibles communs, et qu'ils peuvent coopérer dans un réseau transcriptionnel mettant en jeu par exemple les corépresseurs HDACs et EZH2 (Chng et al. 2012). Récemment, une étude a montré que ERG pourrait « détourner » la régulation transcriptionnelle dirigée par AR ligandé et activer l'expression de gènes androgéno-indépendants comme *SOX9* (Cai et al. 2013). Les mécanismes moléculaires restent à découvrir.

Dans le cadre de la recherche des partenaires de la protéine issue du gène de fusion TMPRSS2 :ERG, j'envisage de poursuivre cette étude initiée dans l'équipe qui avait pour but de déterminer les interactions entre le facteur ERG et les récepteurs nucléaires, et de l'étendre aux récepteurs nucléaires qui ont une relevance fonctionnelle dans le CaP, en particulier des récepteurs nucléaires récemment « adoptés ». Bien que cruciaux dans le

cancer de la prostate, je n'aborderai pas directement les interactions entre TMPRSS2 :ERG et des récepteurs aux androgènes qui sont décrits dans la littérature et étudiés par d'autres équipes de recherche.

B. Mise en évidence d'une interaction directe entre ERG et les récepteurs nucléaires

1. ER, TR et RAR

Une étude menée dans l'équipe avait permis de mettre en évidence une interaction entre ERG et les RNs ER, RAR et TR (Vlaeminck-Guillem et al. 2003b). Cette interaction a pour conséquence une répression mutuelle de l'activité fonctionnelle de ces facteurs de transcription. Ces récepteurs nucléaires sont présents au sein de la prostate et ont été montrés pour avoir un rôle physio-pathologique dans le CaP. Les deux isotypes du récepteur aux oestrogènes ont un effet opposé dans les cellules cancéreuses prostatiques. Alors que ERα joue un rôle dans la prolifération et la survie de ces cellules, ERβ intervient dans l'apoptose et dans des mécanismes anti-prolifératifs (McPherson et al. 2007). Le récepteur à l'hormone thyroidienne activé augmente quant à lui la prolifération de certaines cellules tumorales (LNCaP) (Hsieh and Juang 2005). L'acide rétinoïque et ses récepteurs (RAR et RXR) sont impliqués dans le développement et la régulation de la croissance de la glande prostatique (pour revue : (Pasquali et al. 2006)).

Nous vérifierons dans un premier temps l'interaction des récepteurs ER, TR et RAR avec la protéine issue du gène TMPRSS2 :ERG dans les clones stables de cellules CaP par co-immunoprecipitation. Nous caractériserons ensuite les relations qui existent entre les partenaires. D'autre part, l'étude transcriptomique des cellules traitées par le ligand du récepteur permettra de compléter l'étude et de connaitre les gènes corégulés par ces réseaux transcriptionnels.

2. Les autres récepteurs nucléaires

La famille des RNs comporte une quarantaine de membres (48 à ce jour), or seuls quelques-uns ont été testés pour interagir avec ERG. Nous proposons d'étendre cette étude aux RNs qui sont relevant dans le CaP et dont le ligand est connu, notamment à: PPAR, VDR (pour *Vitamin D Receptor*), LXR (pour *liver X receptors* ou *récepteurs des oxystérols)*, FXR (pour *Farnesoid X receptor*), PXR/SXR (pour *Pregnane X Receptor/Xenobiotic sensing nuclear receptor*), et CAR (pour *Constitutive androstane receptor*). La plupart de ces RNs sont des récepteurs « orphelins récemment adoptés » et dont les ligands identifiés sont des dérivés de lipides alimentaires (Mouzat and Lobaccaro 2006). Pour chacun, une implication dans le CaP a été décrite. Pour certains récepteurs (PPAR et VDR), une association a déjà été établie avec l'un des membres de la famille ETS (Tolon et al. 2000), voire suggérée avec TMPRSS2 :ERG (Washington and Weigel 2010). En effet, l'activation de VDR semble

participer à l'augmentation de l'expression du facteur TMPRSS2-ERG dans des lignées de cellules prostatiques et dans le même temps inhiber l'expression de ses gènes cibles par un mécanisme qui reste à découvrir (Washington and Weigel 2010).

Récepteur Nucléaire	Implication dans CaP	Interaction avec ERG ou les autres membres de la famille ETS
AR	Crucial (Lambet al 2013)	Réseau intégré AR/ERG (Setlur et al 2010; Yu Manni et al 2010)
Era	Marika J. Linja et al, 2003	- TMPRSS2:ERG est régulé par Era (Setlur et al 2008) -Réprime l'activité transcriptionnelle de ERG (Vlaeminck-guillem et al 2003)
Erb	Marika J. Linja et al, 2003	- TMPRSS2:ERG est régulé négativement par Erb (Setlur et al 2008)
Tra		-Interférence mutuelle avec ERG (Vlaeminck-guillem et al 2003)
TRb	-Modulation de la prolifération(Tsui et al 2008; Moeller et al 2013)	-Interférence mutuelle avec ERG (Vlaeminck-guillem et al 2003)
RARa	-Inhibe la prolifération des cellules (Keedwell et al 2004)	-Interférence mutuelle avec ERG (Vlaeminck-guillem et al 2003) -interactaction de RAR et RXR avec TEL (Meester-Smoor et al 2011)
RXRa	- Partenaire d'hétérodimérisation d'un grand nombre de RNs	-Interférence mutuelle avec ERG (Vlaeminck-guillem et al 2003)
RORa	-Réduit les propriétés invasives et migratoires des cellules CaP	-Interférence mutuelle avec ERG (Vlaeminck-guillem et al 2003)
PPARg	- Rôle dans le métabolisme et la prolifération cellulaire (Annicotte et al 2006; Rogenhofer et al 2012) - Modulation de la réponse inflammatoire	- Expression modulée par la surexpression de la fusion -Interaction avec ETS-1
VDR	- Diminue prolifération - Augmente différenciation et apoptose (Hendrickson et al 2011; Holich et al 2006)	- Augmentation de l'expression de VDR avec TMPRSS2:ERG - Vitamin D3 inhibe la croissance promue par T1E4 (Washington et Weigel 2010)
LXR	- Régulation l'homéostasie du cholestérol - Diminue la croissance tumorale - Augmente apoptose (rafts lipidiques) - Inhibe l'action répressive de EZH2 sur les gènes suppresseurs de tumeur	
FXR	-Régule le métabolisme des androgènes (kaeding et al 2008) -Inhibe la prolifération des cellules LAPC-4 (Houssin et al, non publié)	
PXR	-Régule l'homéostasie des androgènes -Inhibe la prolifération des cellules LAPC-4 (Zhang Cheng et al 2010)	
CAR	-Rôle important dans le métabolisme des substrats xenobiotiques	

Tableau 5: Récepteurs nucléaires impliqués dans le CaP et susceptibles d'interagir avec TMPRSS2 :ERG. (Liste non exhaustive)

Les approches moléculaires pour l'étude *in vitro* des interactions protéine-protéine seront utilisées dans un premier temps pour mettre en évidence ou pas, les interactions des RNs avec TMPRSS2 :ERG. Puis nous caractériserons le réseau transcriptionnel et l'expression des gènes cibles qui en dépendent.

Certains ligands de synthèse permettent en effet une interaction protéine-protéine plus forte, voire discriminative, se traduisant par des activités transcriptionnelles différentes (Flajollet et al. 2006). Ceci s'explique par la nature de la molécule qui affecte ou modifie significativement la structure du récepteur lors de son positionnement dans son site de liaison (Mouchon et al. 1999). Dans le cadre de ce projet, on peut émettre l'hypothèse que l'utilisation de ligands de synthèse pourrait accroitre la spécificité d'action de ces molécules et interférer sur l'interaction entre ERG et les RNs que nous aurons mise en évidence. Des études utilisant les techniques de co-rétention protéique et résonance plasmonique de surface (biacore) permettront de mesurer qualitativement et quantitativement l'interférence sur cette interaction *in vitro* avant de mener des études plus approfondies. Ce travail pourra être réalisé avec la plateforme interactions moléculaires dirigées par Pierre-Marie Danze de l'université de Lille 2.

C. Recherche combinatoire ERG/Récepteurs Nucléaires

Dans le cadre de l'étude de l'implication du gène de fusion TMPRSS2 :ERG dans la formation des métastases osseuses du CaP, nous avons identifié des gènes cibles de ERG. Pour plusieurs d'entre eux, nous avons caractérisé les sites de fixation (EBS) par une analyse bioinformatique du promoteur que nous avons ensuite validés expérimentalement par ChIP, retard sur gel ou mutation. L'analyse bioinformatique du promoteur de certains gènes révèle l'existence de sites potentiels de liaison pour des récepteurs nucléaires, qui se trouvent très proche voire chevauchant le site EBS. C'est le cas par exemple pour le gène *Métalloproteinase 1* (*MMP1*) qui code pour une protéase intervenant dans la dégradation du collagene de type I, composant majeur de la matrice extracellulaire osseuse.

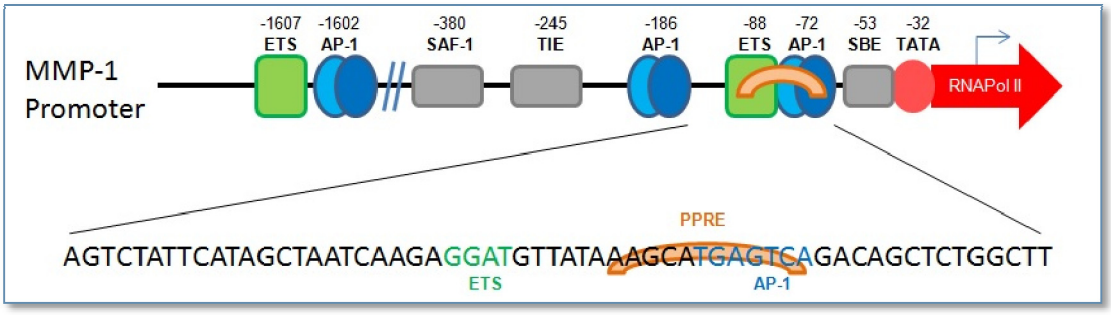


Figure 9: Analyse *in silico* du promoteur MMP1.

Parmi les protagonistes de la régulation de l'expression de *MMP1*, on trouve ERG, AP1 et PPARγ (Buttice et al. 1996; Francois et al. 2004). En effet, ERG, via une coopération fonctionnelle avec le dimère AP1, active l'expression de *MMP1* (Buttice et al. 1996). Par ailleurs, PPARγ activé par son ligand, la rosiglitazone entraine l'absence de la fixation de c-Fos/c-jun au niveau du promoteur de *MMP1* et une diminution de l'expression de ce gène (Francois et al. 2004). La combinatoire ERG/AP1/PPARγ que l'on peut rencontrer dans les CaP exprimant la fusion TMPRSS2 :ERG, n'a pourtant pas été étudiée. C'est ce que nous proposons de faire par des expériences de retard sur gel, d'immunoprécipitation de la chromatine, promoteur pull-down et de mesure de l'activité transcriptionnelle du gène rapporteur luciférase sous le contrôle du promoteur de *MMP1*.

Une analyse bioinformatique pour identifier les sites EBS associés aux sites de fixation des RNs sera réalisée afin d'identifier et regrouper les gènes dont l'expression est régulée par une combinatoire donnée.

III. Intégration du projet de recherche dans le cadre de l'UMR8161

L'objectif du projet que je propose, est de caractériser et d'étudier l'interactome du facteur de transcription TMPRSS2 :ERG dans les cellules cancéreuses de prostate. L'élaboration et la réalisation de ce projet s'appuient sur l'expertise que j'ai pu acquérir et est en parfaite adéquation avec la thématique de l'équipe que je souhaite intégrer à long terme.

Au cours de mon expérience doctorale et post-doctorale, j'ai acquis une expertise scientifique en biologie moléculaire et cellulaire. En particulier, j'ai étudié les mécanismes impliqués dans la régulation de la transcription et les voies de signalisation dirigées par les récepteurs nucléaires, le recrutement des facteurs de transcription sur le promoteur des gènes, celui de partenaires coactivateurs, leurs modifications structurales de l'organisation chromatinienne du promoteur. D'autre part, après avoir étudié l'implication du facteur de transcription ERG dans la mise en place du cartilage, je me suis intéressé à l'étude du rôle du gène de fusion TMPRSS2-ERG dans le cancer de la prostate.

Depuis 2008, j'ai rejoint l'équipe ″Protéines Ets, régulation transcriptionnelle et pathologies associées″ au sein de l'UMR8161 à l'Institut Biologie de Lille. Cette équipe a développé depuis longtemps une expertise dans la caractérisation fonctionnelle des protéines ETS. Depuis 2008, elle s'est focalisée sur l'étude de l'implication de la protéine issue des gènes de fusion *TMPRSS2 :ETS* dans les métastases osseuses du cancer de la prostate. Suite à une étude transcriptomique de cellules cancéreuses de prostate surexprimant le gène de fusion *TMPRSS2 :ERG*, nous avons mis en évidence des gènes cibles impliqués dans le tropisme des cellules cancéreuses à l'os.

Afin de mieux comprendre les réseaux transcriptionnels dirigés par ce facteur TMPRSS2 :ERG et de rechercher de nouvelles cibles potentielles pour mieux diriger l'action thérapeutique dans le cancer de la prostate, je propose de conduire cet axe de recherche qui est complémentaire à la thématique de l'équipe. La réalisation de ce projet bénéficiera de mon expertise scientifique et technique, ainsi que des outils nécessaires déjà établis tels que les vecteurs d'expression, les modèles cellulaires de CaP, les clones caractérisés surexprimant stablement la fusion. Je pourrais m'appuyer sur les collaborations étroites déjà établies avec des équipes de recherche, et les différentes plateformes (Biopuces-Transcriptomique et génomique appliquée de l'Institut Pasteur, Interactions moléculaires-Imagerie moléculaire et cellulaire de l'Université de Lille 2, Tumorothèque de la Cancéropôle Nord-ouest, Animalerie de l'Institut Pasteur), pour bénéficier des techniques et compétences complémentaires utiles. Enfin, j'espère obtenir un contrat qui me permette de poursuivre ce travail au sein de l'UMR8161, et de faire que cette thématique devienne un axe important dans l'équipe.

IV. Conclusion

Après avoir participé à l'audition des différents morceaux interprétés par le chef d'orchestre TMPRSS2 :ERG dans le registre de la métastase et du tropisme des cellules cancéreuses de la prostate vers l'os*, il faut s'intéresser à la composition de l'orchestre symphonique et du groupe instrumental* des récepteurs nucléaires*, ainsi qu'au décryptage des codes qui existe entre le chef d'orchestre et les instruments. Le défi étant, à terme, de pouvoir casser la baguette du chef d'orchestre ou à défaut d'utiliser diverses sourdines adaptées à chaque instrument pour atténuer ou modifier le son.*

Cette image de l'orchestre symphonique reflète assez bien le mécanisme très complexe de la régulation transcriptionnelle de l'expression des gènes. A l'évidence, une meilleure connaissance des réseaux transcriptionnels, et dans le cas du cancer de la prostate, de ceux impliquant le facteur de transcription ERG anormalement présent dans la majorité des CaP, pourrait avoir des conséquences importantes en thérapie. Ce projet devrait permettre l'identification de cibles moléculaires qui constituent autant de nouvelles cibles thérapeutiques des cancers prostatiques exprimant les gènes de fusion.

Il convient de garder en mémoire le fait que le cancer de la prostate est une maladie multifactorielle liée à plusieurs facteurs constitutifs de l'individu et liés à l'environnement, aux modes et conditions de vie. Toutes les molécules présentes dans l'environnement peuvent interagir avec certains récepteurs nucléaires et favoriser ou au contraire ralentir le développement du cancer. Etudier les relations entre TMPRSS2 :ERG et ces récepteurs nucléaires activés par ces molécules vont permettre de mieux prévenir, contrôler et agir.

« Quels sont les coactivateurs impliqués ? Quelle structure tridimensionnelle de ERG est responsable des interactions avec ces coactivateurs ? Quels domaines des coactivateurs permettent leur recrutement par le facteur ERG ? » sont les questions qui alimentent les conclusions et les perspectives des mémoires de thèse de l'équipe. Avec les avancées technologiques de haut-débit en transcriptomique et protéomique, les ressources et analyses bioinformatiques, et les enjeux d'une médecine personnalisée, je relève le défi pour répondre à ces questions.

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Annexes

Article 1

Distinct roles of the steroid receptor coactivator 1 and of MED1 in retinoid-induced transcription and cellular differentiation.
Flajollet S, Lefebvre B, Rachez C, Lefebvre P (2006)
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Article 2

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Article 3

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Article 4

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Article 5

Non-classical transcriptional regulation of HLA-G: an update.
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Article 6

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Article 7

Increased adipogenesis in cultured embryonic chondrocytes and in adult bone marrow of dominant negative Erg transgenic mice.
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Article 8

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Distinct Roles of the Steroid Receptor Coactivator 1 and of MED1 in Retinoid-induced Transcription and Cellular Differentiation*

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Retinoic acid receptors (RARs) are the molecular relays of retinoid action on transcription, cellular differentiation and apoptosis. Transcriptional activation of retinoid-regulated promoters requires the dismissal of corepressors and the recruitment of coactivators to promoter-bound RAR. RARs recruit *in vitro* a plethora of coactivators whose actual contribution to retinoid-induced transcription is poorly characterized *in vivo*. Embryonal carcinoma P19 cells, which are highly sensitive to retinoids, were depleted from archetypical coactivators by RNAi. SRC1-deficient P19 cells showed severely compromised retinoid-induced responses, in agreement with the supposed role of SRC1 as a RAR coactivator. Unexpectedly, Med1/TRAP220/DRIP205-depleted cells exhibited an exacerbated response to retinoids, both in terms transcriptional responses and of cellular differentiation. Med1 depletion affected TFIIH and cdk9 detection at the prototypical retinoid-regulated RARβ2 promoter, and favored a higher RNA polymerase II detection in transcribed regions of the *RARβ2* gene. Furthermore, the nature of the ligand impacted strongly on the ability of RARs to interact with a given coactivator and to activate transcription in intact cells. Thus RAR accomplishes transcriptional activation as a function of the ligand structure, by recruiting regulatory complexes which control distinct molecular events at retinoid-regulated promoters.

Coactivators (CoAs)[5] have multiple roles in transcriptional regulation: they are key structural components of multiprotein complexes, notably by interacting with transactivating domains of transcription factors, other coactivators or components of the basal transcription machinery. They possess enzymatic activities, catalyzing post-translational modifications of histones and of other transcriptional regulators. Their functional integrity is therefore required to recruit the basal transcriptional machinery to activate gene transcription. Transcriptional activation by liganded nuclear receptors is a paradigm to study promoter activation in response to small hydrophobic molecules, and this process is achieved by the sequential dismissal of corepressors and recruitment of distinct classes of coactivators, each serving one or several specific functions. Many of these functions are targeted to chromatin which, under its compacted form, precludes gene expression.

All-trans retinoic acid receptors (RARs) belong to the nuclear hormone receptors (NRs) superfamily and act as ligand-inducible transcription factors. The acquisition of a transcriptional activity by RARs results from structural transitions occurring in the ligand binding domain or LBD, leading to the formation of an hydrophobic coactivator binding pocket, with which an L*XX*LL motif from a coactivator molecule will interact (1, 2). A charge clamp stabilizes this interaction, allowing the docking of multiprotein coactivator complexes. It is thought that transcriptional activation by RARs requires the sequential recruitment of (reviewed in Ref. 3): (i) ATP-dependent chromatin remodeling complexes which affect the mobility of nucleosomes to alleviate, in most cases, chromatin-based repression. More specifically, tight binding of RXR/RAR heterodimers to DNA requires an ATP-dependent ISWI-mediated chromatin remodeling activity (4). (ii) Acetyl transferases such as the p160-related coactivator family (SRC1, 2, and 3), the integrator complex CBP/p300 and pCAF (5, 6). The recruitment of these coactivators favors histone acetylation at least for some retinoid-regulated promoters (7). (iii) The mediator complex (DRIP/TRAP/SMCC, Ref. 8), which allows the phosphorylation of RNA polymerase II (RNApol2) by TFIIH and its conversion into an elongation-competent form (8).

Whereas being relatively well established for specific model systems (7, 9), this mechanism is however not universal. We have established that the retinoid-controlled RARβ2 promoter is, in P19 embryonal carcinoma cells, highly responsive to retinoid stimulation and that histones associated to this promoter are constitutively acetylated (10, 11). Transcriptional activation of this promoter is correlated with histone H3 phosphorylation, a post-translational modification reminiscent of those occurring in immediate-early gene promoters (11). A much less

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[5] The abbreviations used are: CoA, Coactivator; RAR, retinoic acid receptors; DMEM, Dulbecco's modified Eagle's medium; Z, benzyloxycarbonyl; fmk, fluoromethylketone; GST, glutathione *S*-transferase; wt, wild type; ChIP, chromatin immunoprecipitation assay; shRNA, small hairpin RNA; wt, wild type; RNAi, interference RNA.

explored area is the actual contribution of each class of coactivator to RAR-mediated transactivation.

We investigated this latter question by assessing the respective role of p160 coactivators and of the mediator complex in retinoid-induced transcription and cellular differentiation. P19 cells are multipotent and differentiate into endoderm, mesoderm, or ectoderm depending on the chemical inducer and culture conditions (12). Retinoids promote P19 cells differentiation into neurons and glial cells, and the α-isotype of RAR is critical for this process to take place (13). P19 cells are thus an appropriate developmental system to study the role of retinoids, RARs and their coactivators in neuronal differentiation. Moreover, the expression of RARβ2 is critical for all-trans retinoic acid (atRA)-induced differentiation of P19 cells (14), thus providing a model in which transcriptional and differentiation processes can be studied simultaneously. We have manipulated p160-related factors and DRIP205/TRAP220/Med1 (hereafter termed Med1 according to the unified nomenclature, Ref. 15) expression levels in P19 cells using RNAi, and the consequences of mRNA knock-down on transcriptional and differentiation events have been characterized. In initial experiments, we discovered that a decreased level of expression of SRC2 or of SRC3 induced cell death, suggesting a contribution of these proteins to yet unknown critical biological processes. We therefore focused our study on SRC1 and Med1, which are two potentially important players in retinoid-induced transcription.

SRC1 has been shown to play a critical role, although partially filled by SRC2, in steroid-induced tissue development (16, 17) and transcriptional regulation by steroid and thyroid hormones (18, 19). Its contribution to retinoid-controlled transcription is much less documented, but SRC1 binds physically to RAR (6, 20) and its overexpression in P19 cells increases RXR/RAR heterodimers transcriptional activity (6, 10). The acetyl transferase activity of SRC1 (21) is dispensable for atRA-induced transcription (5).

Hypomorphic Med1 mice highlighted the role of this coactivator in hepatic and cardiovascular development (22), and mouse embryonic fibroblasts (MEFs) isolated from Med1$^{-/-}$ mice show strongly impaired thyroid hormone receptor-dependent transcription (23). One-hybrid assays revealed a moderate contribution of Med1 to the AF2 function of RARα, which was not detected at the level of p21 expression, an endogenous gene regulated by RARα (23). Thus, although Med1 interacts physically with RARα through L*XX*LL motifs (24), it remains to be established whether it actually serves as a coactivator for RARs.

MATERIALS AND METHODS

Plasmids—Oligonucleotides encoding for small hairpin RNAs were synthesized with appropriate loop and cohesive ends sequences according to the plasmid provider instructions. Oligonucleotides were cloned into the pSHAG plasmid (obtained from G. Hannon) or into pSilencer 2.1-U6 (Ambion Inc., Austin, TX). Oligonucleotide sequences were selected according to siRNA design guidelines (25). Two or three siRNAs were designed for each target mRNAs and tested for their efficiencies to selectively decrease target gene expression. Selected siRNAs were: si-Luciferase: cttacgctgagtacttcga; si-SRC1: tgaccgcaccatccatcct; si-Med1: cgtacccacagccagtgtc. All constructions were checked by sequencing. Detailed sequences are available upon request.

Cell Culture and Transfections—HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Biowhittaker), 1,000 units of penicillin and 10 μg of streptomycin per ml. One day prior to transfection, cells were plated in 6-well plates in DMEM supplemented with 10% fetal calf serum, 1,000 units/ml penicillin, and 10 μg/ml streptomycin. Each well was transfected using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen), with a DNA mixture including 1 μg of a RA-responsive firefly luciferase reporter gene containing three repeats of a composite GRE-RARE ($GRARE_3$-tk Luc), 0.25 μg of the expression vectors pSG5hRARα and pSG5RXGRα, the latter containing mutations in the P box to confer specific binding to a glucocorticoid response element (26), 50 ng of a control plasmid tk-luciferase (tk-*Renilla*, Promega), and 2.5 μg of pSilencer Control, pSilencerMed1 or pShagSRC1. The total amount of transfected DNA was adjusted to 4 μg per well. After a 5-h incubation with the DNA mix, cells were washed and cultured in fresh medium. 16 h later, cells were challenged with 1 μM atRA overnight. Cells were harvested and luciferase activities were assayed with the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase values were normalized to those of *Renilla* luciferase values.

Generation of P19 Cell Subclones—P19 cells with a significantly decreased coactivator expression were obtained as follows: the pSHAG-SRC1 or the pSilencerMed1 plasmid was cotransfected with a vector encoding the green fluorescent protein (pEGFP, Clontech) in a 1:10 ratio. Selection of resistant clones was carried out for 10–15 days in DMEM containing 300 μg/ml G418 (SRC1) or 350 μg/ml hygromycine (Med1). After selection based on antibiotics resistance and EGFP expression, resistant colonies were isolated, expanded, and characterized for Med1 or SRC1 expression by RT-PCR and Western blot analysis.

Cellular Extracts Preparation and Western Blot Analysis—Cellular extract preparation and Western blot analysis were carried out as previously described (10, 27). Immunodetections were performed using a polyclonal anti-SRC1 antibody (M-341, Santa Cruz Biotechnology), a polyclonal anti-Med1 antibody (C-19, Santa Cruz Biotechnology) and an anti-actin monoclonal antibody (ac-15, Sigma).

Cell Cycle Analysis—10^6 cells were trypsinized, washed once with culture medium, twice with PBS 1×, and fixed with 90% ethanol/phosphate-buffered saline overnight at −20 °C. Cells were rehydrated, washed twice in PBS 1×, and stained for 30 min in a 50 μg/ml propidium iodide solution containing 0.25 μg/ml RNase and 0.1% Triton X-100. The cell cycle repartition of fixed cells was analyzed by flow cytometry in a EPICS XL-MCL^{TM} cytometer (Beckman-Coulter), and quantified with the WinCycle software (Phoenix Flow Systems). When indicated, cells were treated with 40 μM of the caspase inhibitor z-VAD.fmk (Bachem) for 24 h.

Neuronal Differentiation—Neural differentiation was induced as follows: 10^7 P19 cells were cultured for 4 days in 60-mm bacterial grade Petri dishes in DMEM-10% fetal calf serum. Cell aggre-

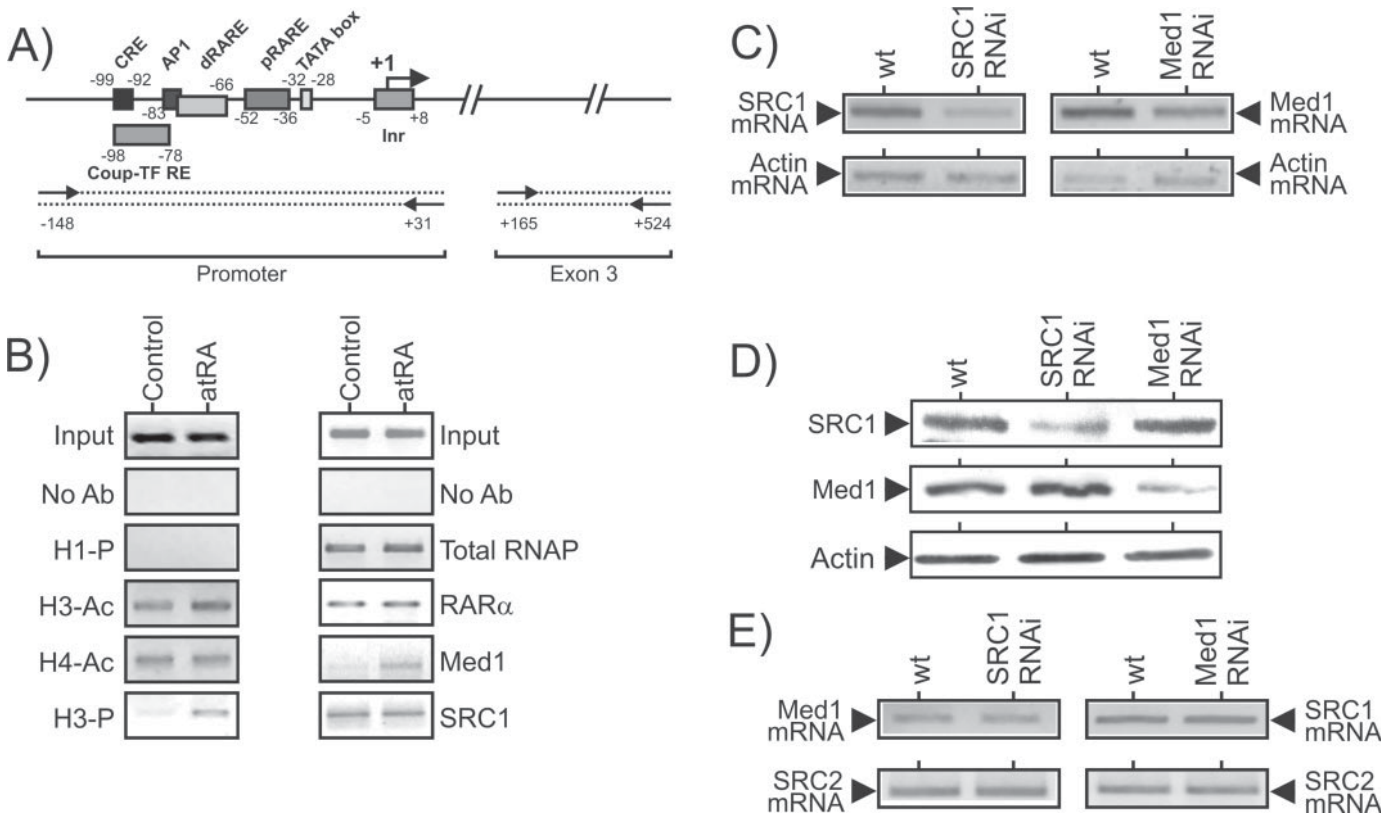


FIGURE 1. *A*, schematic organization of the RARβ2 promoter. *B*, RNA polymerase II, CoAs, RARα loading and histone modifications at the RARβ2 promoter. wtP19 cells were treated for 2 h with 1 μM atRA, ChIP assays were carried out using anti-RNApol2 Ab, anti-Med1, anti-RARα, as well as anti-phospho or acetylated histones antibodies. Immunoprecipitated RARβ2 promoter DNA was quantified by semi-quantitative PCR. *C–E*, characterization of SRC1RNAi and Med1RNAi P19 cell lines. *C*, SRC1 and Med1 mRNA content of wt, SRC1RNAi and Med1RNAi P19 subclones were carried out by RT-PCR. *D*, SRC1 and Med1 protein expression in P19 subclones were carried out by Western blot analysis of whole cell lysates. *E*, specificity of shRNAs. Med1, SRC1, and SRC2/TIF2 expression were characterized by RT-PCR in each cellular background.

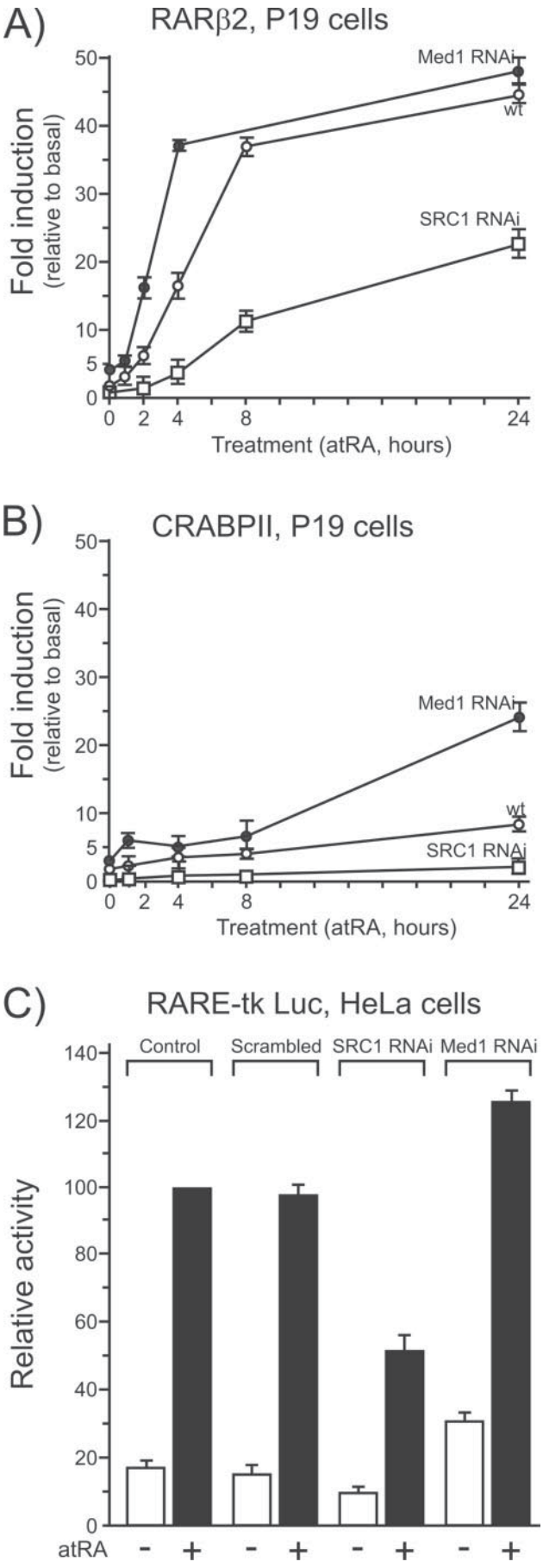
gates were dissociated by trypsin treatment and grown in tissue culture dishes in DMEM-10% fetal calf serum for 1 day. Adherent cells were then grown in the presence of atRA for 48 h at the indicated concentrations, followed by 48 h in the absence of atRA. Neuronal differentiation was assessed by Western blot analysis of whole cell extracts using a mouse anti-βIII tubulin antibody (clone 2G10, Upstate Biotech, Inc.).

RNA Preparation and RT-PCR—Total RNA was isolated using RNeasy Kit according to the manufacturer's protocol (Qiagen), and RT-PCR was carried out as described (28). Amplification conditions were adjusted to be within the linear range. Primers were designed to amplify cDNAs fragments ranging in size from 300 to 600 bp and were: actin primers: 5′-atcatgtttgagaccttcaa-3′ and 5′-catctcttgctcgaagtcca-3′; SRC1 primers, 5′-aatgtgttcagtcaagctgtccag-3′ and 5′-tggttattcagtcagtagctgctg-3′; Med1 primers, 5′-gcatgagcatcaaagatcgg-3′ and 5′-ggttctgtgagtcaacatcc-3′; SRC2 primers, 5′-cccgttttcccacagcagta-3′ and 5′-tgctgtttccacccatgctc-3′; SRC3 primers, 5′-aagcccctccacaacagttt-3′ and 5′-cagcagtatttctgatcggg-3′; RARβ2 primers, 5′- tggatgttctgtcagtgagtcccg-3′ and 5′-gctccgctgtcatctcatagctctc-3′; CRABPII primers, 5′- ccaggtggaaggatgtgttc-3′, 5′- attggtcagttctcggctcc-3′.

Quantitative PCR—mRARβ2 transcripts were detected as described in Ref. 28. CRABPII transcripts were assayed using an "Assay on Demand" kit from Applied Biosystems. The 18 S primers and Vic probe were purchased from Applied Biosystems. Reactions (40 cycles) and data analysis were carried out on an ABI Prism 7700 using the SDS software (PerkinElmer-Applied Biosystems). Expression levels of RARβ2 and CRABPII transcripts were normalized to 18 S RNA levels, and relative levels of expression of each transcript were calculated using the $2^{-\Delta\Delta Ct}$ method.

Chromatin Immunoprecipitation Assays—ChIP assays were performed as described in Refs. 10, 11, 27, and 29. Anti-acetylated H3 (06-599) and H4 (06-598), anti-phosphorylated Ser^{10} histone H3 (07-081) antibodies were from Upstate Biotech, Inc. Anti-RAR (C-20), anti-SRC1 (M-341), anti-Med1 (M-255), anti-RNApolII (C-21), anti-ERCC3/TFIIH (S-19), anti-cdk7 C-19), anti-cdk9 (H-169), and anti-Med17 (G-17) were from Santa Cruz Biotechnology. Anti-phospho-Ser^5 RNApolII (H14) was from Covance. ChIP analysis was performed at least in triplicate using distinct DNA preparations. When indicated (Fig. 3), DNA was quantified by Q-PCR using the ABI PRISM 7700 sequence detection system. The RARβ2 promoter sequence was in this case amplified from −78 to +38 with the following primers: Forward: TTGAAGGTTAGCAGCCCGG, Reverse: CTTCTGTCACACGGAATGAAAGAT, probe FAM/TAMRA: AAGGTTCACCGAAAGTTCACTCGCA. DNA was quantified and results expressed relative to input DNA, after subtracting nonspecifically bound DNA as assayed using nonspecific IgG in the ChIP assay.

GST Pull-down Assays—GST pull-down experiments were performed as described previously (20, 30).



RESULTS

The Transcriptional Activation Process in EC P19 Cells—P19 cells express all isotypes of RAR and RXR, and many of the established or putative corepressors (CoRs) and CoAs for RARs, including SRC1 and Med1 (see supplementary Fig. S1). The detection of transcriptional regulators, as well as of histone modifications occurring at the RARβ2 promoter (Fig. 1*A*) upon atRA challenge were carried out in this cellular background by ChIP assays (Fig. 1*B*). Histones H3 and H4 were constitutively acetylated, whereas ligand-dependent histone H3 phosphorylation was observed. No phosphorylated H1 could be detected on this promoter. SRC1 and RARα binding was constitutive and ligand-insensitive, whereas the basal level of Med1 detection increased in the presence of atRA. Mediator complex recruitment is facilitated, in a chromatinized environment, by histone acetylation (31), providing a molecular basis for the constitutive high detection of the mediator complex to the RARβ2 promoter, where both H3 and H4 are acetylated. Also consistent with this hypothesis is the finding that SRC1 is constitutively associated to the promoter, thus promoting the permanent recruitment of CBP/p300, and tethering of HAT activity to the RARβ2 promoter. Thus SRC1 and Med1 are likely to play a role in the transcriptional control of the RARβ2 promoter, which exists in a state poised for transcription.

Characterization of P19 Cells Deficient in SRC1 or Med1—P19 subclones overexpressing small hairpin RNA (shRNA) targeting either the luciferase gene (control), SRC1, or Med1 were selected. Efficiencies of SRC1 and Med1 mRNAs knock-downs were characterized in several subclones and compared with wild type (wt) cells. Wild-type cells behaved similarly to cells expressing an shRNA targeting the luciferase gene, which left unchanged SRC-1 and Med1 protein levels and did not affect RA-induced gene transcription (data not shown). Results obtained with representative subclones are shown (Fig. 1*C*) and were similar for several subclones: mRNA and, more importantly, protein levels were significantly decreased in both cases by more than 80% (Fig. 1*D*). In the case of Med1, we noted that the protein content decreased more strongly than mRNA levels, in agreement with observations showing that RNAi may also act on RNA translation. We monitored Med1 mRNA levels in SRC1RNAi cells and *vice versa*, as well as that of SRC2, which have been shown to be up-regulated in SRC1$^{-/-}$ mice (16). No nonspecific alteration of the expression of coactivators was observed (Fig. 1*E*), showing that selected shRNAs affected specifically the stability of targeted mRNAs. OAS1 expression was

FIGURE 2. **Transcriptional regulation of the RARβ2 and CRABPII promoters in wt, Med1-, and SRC1-deficient backgrounds.** *A*, kinetics study of RARβ2 promoter activation. mRNA transcripts levels coding for RARβ2 were assessed by real time PCR after 1, 4, 8, 16, or 24 h of treatment with 1 μM atRA. The steady-state level of each mRNA species was assayed as described under "Materials and Methods" and normalized to 18 S RNA level. Results are expressed relative to the basal level observed in wtP19, which was arbitrarily set to one. *B*, Q-PCR analysis of CRABPII transcripts. *C*, transcriptional activation of a retinoic acid-inducible reporter gene in HeLa cells. HeLa cells were co-transfected with expression vectors coding for RXRα, RARα, and the DR5-tk-Luc reporter gene. Vectors encoding for a scrambled, antiSRC1, or antiMed1 shRNA were transfected as indicated, and luciferase activity were assayed and normalized as indicated under "Materials and Methods." The luciferase activity detected in control cells upon atRA treatment was arbitrarily set to 100%.

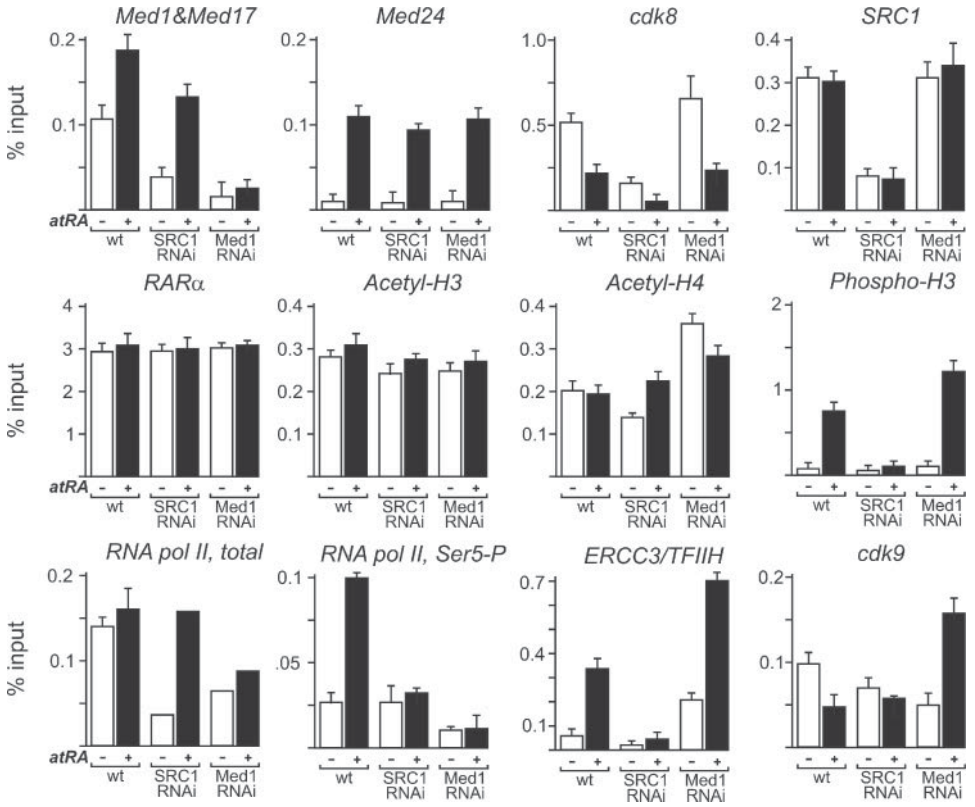


FIGURE 3. **Histone post-translational modifications and transcription factor loading at the RARβ2 promoter.** P19 cells were treated or not by 1 μM atRA for 1 h and the association of phosphorylated histone H3, acetylated histone H3 or H4, RNApol2, RARα, SRC1, and of subunits of the mediator complex to the RARβ2 promoter was analyzed by ChIP assays using antibodies as indicated in Fig. 1. The association of phosphorylated RNApol2, cdk8, cdk9, and TFIIH/ERCC3 to the RARβ2 promoter was similarly monitored by ChIP assays. Immunoprecipitated DNA was quantified by Q-PCR and expressed as a percentage of input DNA corrected from the background signal.

not up-regulated in P19 subclones (data not shown), indicating that siRNAs did not induce an interferon-like response (32). Thus by these criteria, shRNA overexpression triggered a specific degradation of targeted mRNAs.

SRC1 and Med1 Have Distinct Contributions to the Transcriptional Regulation of Retinoid-regulated Genes—Functional consequences of SRC1 or Med1 knock-down were assessed by monitoring the activity of two retinoid-responsive genes (Fig. 2). Retinoid responsiveness is conferred to the RARβ2 gene promoter by a retinoic acid response element (RARE) organized as a direct repeat of a hexanucleotide separated by a 5-bp spacer (DR5) (Refs. 33 and 34, see Fig. 1*A*). Quantitative PCR analysis (Fig. 2*A*) showed that RARβ2 transcripts accumulated in wtP19 cells according to a first order kinetics, reaching a plateau after an 8-h treatment with 1 μM atRA. Accumulation of RARβ2 mRNAs in SRC1RNAi cells followed similar kinetics, although the maximal level was decreased when compared with wtP19 (45-fold induction *versus* 20-fold). Most notably, Med1 knock-down promoted a much faster mRNA synthesis at earlier time points, without affecting significantly the maximal level which was reached, as in wtP19 cells, after an 8-h treatment. Of note, the basal expression of the *RARβ2* gene was strongly increased in Med1RNAi cells (4–5-fold), hinting at a repressive role for this protein in this cellular context. Overexpression of the appropriate coactivator was able to rescue transcriptional activity in depleted cells (data not shown).

To investigate whether both coregulators exerted a similar control on other retinoid-regulated genes, we monitored cytoplasmic retinoic acid-binding protein II (*CRABPII*) gene expression by Q-PCR, whose expression is regulated through a DR1 and a DR2 RAREs (35). In wtP19, CRABPII transcripts accumulated very slowly, on an almost linear, zero order kinetics (Fig. 2*B*). Knocking down SRC1 expression dramatically decreased atRA-induced CRABPII mRNA synthesis. Med1 depletion increased the basal level of expression of CRABPII but, in contrast to the *RARβ2* gene, allowed a more efficient ligand-dependent accumulation of CRABPII transcripts. Similar results were obtained with different P19 subclones. Med1 and SRC1 depletion impacted similarly on the response of a retinoid-inducible synthetic reporter gene in transiently transfected HeLa cells (Fig. 2*C*).

CRABPII participates in retinoid-mediated transcription by interacting directly with RXR/RAR heterodimers and increasing their transcriptional activity. This activity requires the binding of retinoids to CRABPII (36). In light of these data, the decreased expression of the *RARβ2* gene in SRC1RNAi cells could be interpreted as a result of a decreased expression of CRABPII. However, we obtained similar results using a panel of synthetic retinoids binding or not to CRABPII (data not shown), excluding a possible contribution of a decreased CRABPII expression to the observed phenotype.

Taken together, our data suggest that SRC1 acts as a coactivator in distinct cellular backgrounds, whereas Med1 exerts a repressive activity on various retinoid-regulated promoters, irrespective of the cell type.

SRC1 and Med1 Knock-downs Affect Transcriptional Events at the RARβ2 Promoter—Both *RARβ2* and *CRABPII* gene transcription are affected by SRC1 or Med1 knock-downs. To focus solely on early events leading to transcriptional activation, we selected to study those occurring at the RARβ2 gene promoter after a 2-h stimulation by atRA. Indeed, at this early time point, coactivator knock-down does not alter significantly CRABPII transcript accumulation, whereas RARβ2 gene transcription is clearly affected. Although knock-down effects are more pronounced at later time points for the *CRABPII* gene, it is likely that events unrelated to primary transcriptional regulation will be involved in this process. We thus investigated whether SRC1 and Med1 knock-downs altered H3 and H4 post-translational modifications and transcription factors detection at the RARβ2 promoter (Fig. 3).

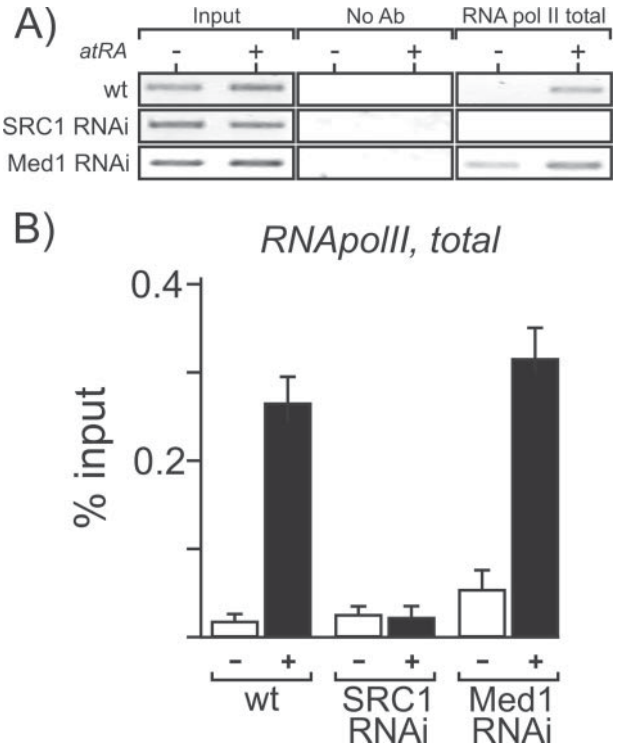


FIGURE 4. **Association of RNApol2 to the exon 3 of the *RARβ* gene.** *A*, RNApol2 loading was monitored by ChIP assay using the anti "total" RNApol2 antibody, and immunoprecipitated DNA was quantified by semi-quantitative PCR as described in the legend to Fig. 1. A representative result is shown. *B*, quantification of immunoprecipitated exon 3 DNA. Results from 2 or 3 independent experiments were quantified by gel scanning and averaged. Results are expressed as described in the legend to Fig. 3.

To monitor Med1 loading, ChIPs experiments were initially carried out using a mix of two antibodies directed against Med1 and Med17, two components of the core complex (also named PC2). As expected, knocking down Med1 expression prevented its association to the RARβ2 promoter, and therefore that of Med17 as well. Of note, SRC1 knock-down also affected Med1 detection, and most especially the basal level of association with the RARβ2 promoter.

Because the mediator complex is organized into submodules which can be isolated functionally and physically, we concluded that either the mediator complex is absent from the promoter in Med1RNAi cells, or that it undergoes significant conformational/structural changes preventing epitope recognition or that the mediator complex may exist under a Med1-free form. We thus used another antibody targeted at the tail of the core mediator complex (37). Several proteins are part of the tail of the core complex, including Med24/TRAP100. Med24 has a broad contribution to transcriptional activation (38), and despite the presence of 6 L*XX*LL motifs, does not interact *in vitro* with RARα or RXRα (39, 40). In wtP19, a clear ligand-dependent association of Med24 was detected in the presence of atRA, and a similar pattern was observed in SRC1RNAi and Med1RNAi cells, suggesting that Med1 depletion does not affect the association of other components of the mediator complex to the RARβ2 promoter. cdk8, a component of the repressive kinase complex of the mediator complex (41), is associated to the promoter under basal conditions and cannot be detected after agonist treatment (Fig. 3). Med1 knock-down did not alter cdk8 interaction with the promoter region, which was not detected on the promoter in all three cellular backgrounds. However, cdk8 detection at the RARβ2 promoter was sharply reduced in SRC1-depleted cells, suggesting that SRC1 affects, directly or indirectly, cdk8 association to the promoter, a phenomenon which could be related to the lower density of promoter-bound Med1&Med17 in SRC1 RNAi cells. The possibility that cdk8 is still present but not detectable in this configuration cannot however be excluded at this point.

The constitutive, ligand-insensitive SRC1 binding was severely compromised in the SRC1-deficient background but not affected in Med1 RNAi cells. As expected from its role as a primary transcriptional activator, RARα binding was not significantly altered in SRC1- or in Med1-deficient cells and remained constitutively high in each cellular background.

Histone post-translational modifications were then monitored. In wtP19 cells, a constitutive acetylation of H3 and H4 was detected, as previously shown (Fig. 1). In both SRC1RNAi and Med1RNAi cells, H3 acetylation was not significantly altered. In contrast, SRC1 knock-down caused a slight but consistent decrease in the basal acetylation level of H4, which became ligand-sensitive, increasing 2-fold upon atRA treatment. Med1 knock-down impacted moderately on H4 acetylation levels, with no detectable increase upon atRA challenge. H3 phosphorylation increased in wtP19 upon atRA treatment, as well as in Med1-deficient cells. This post-translational modification was abrogated in SRC1RNAi cells, confirming the relationship between transcriptional activation of the RARβ2 promoter and H3 phosphorylation.

RNApol2 detection in the promoter region suggested that RNApol2 binding could decrease in the Med1-deficient background, suggesting a faster promoter clearance. Conversion of RNApol2 to an elongation-competent form depends on the orchestrated activity of cyclin-dependent kinases. We therefore monitored the recruitment of two cyclin-dependent kinases involved in the regulation of RNApol2 activity, cdk7 and cdk8. cdk8 binding was monitored using an antibody directed against cdk8 itself, whereas cdk7 loading was assayed by immunoprecipitating the ERCC3 subunit of TFIIH, to which cdk7 is associated. In addition, phosphorylation of Ser5 of RNApol2 CTD was followed as an index of TFIIH activity. Upon atRA treatment, phosphorylation of Ser^5 increased concomitantly to TFIIH detection in wtP19. Very strikingly, this correlation was lost in Med1RNAi cells, in which TFIIH was constantly detected to the promoter, and where no Ser5P RNApol2 could be detected. In addition, cdk9 ChIP assays evidenced a stronger association of this kinase to the RARβ2 promoter in atRA-treated. These data suggested that the high density of TFIIH at the RARβ2 promoter in Med1-depleted cells could promote a faster dissociation of RNApol2 from promoter sequences, although we cannot not rule out at this stage that this reflects merely a RNApol2 epitope masking in these conditions.

To test further this hypothesis, we carried out ChIP assays to detect RNApol2 on exon 3 of the *RARβ2* gene (Fig. 4). RNApol2 detection increased in a ligand-dependent manner in transcriptionally active cells (wt and Med1 RNAi cells), thus establishing a strict correlation between RNApol2 detection at exon 3 and

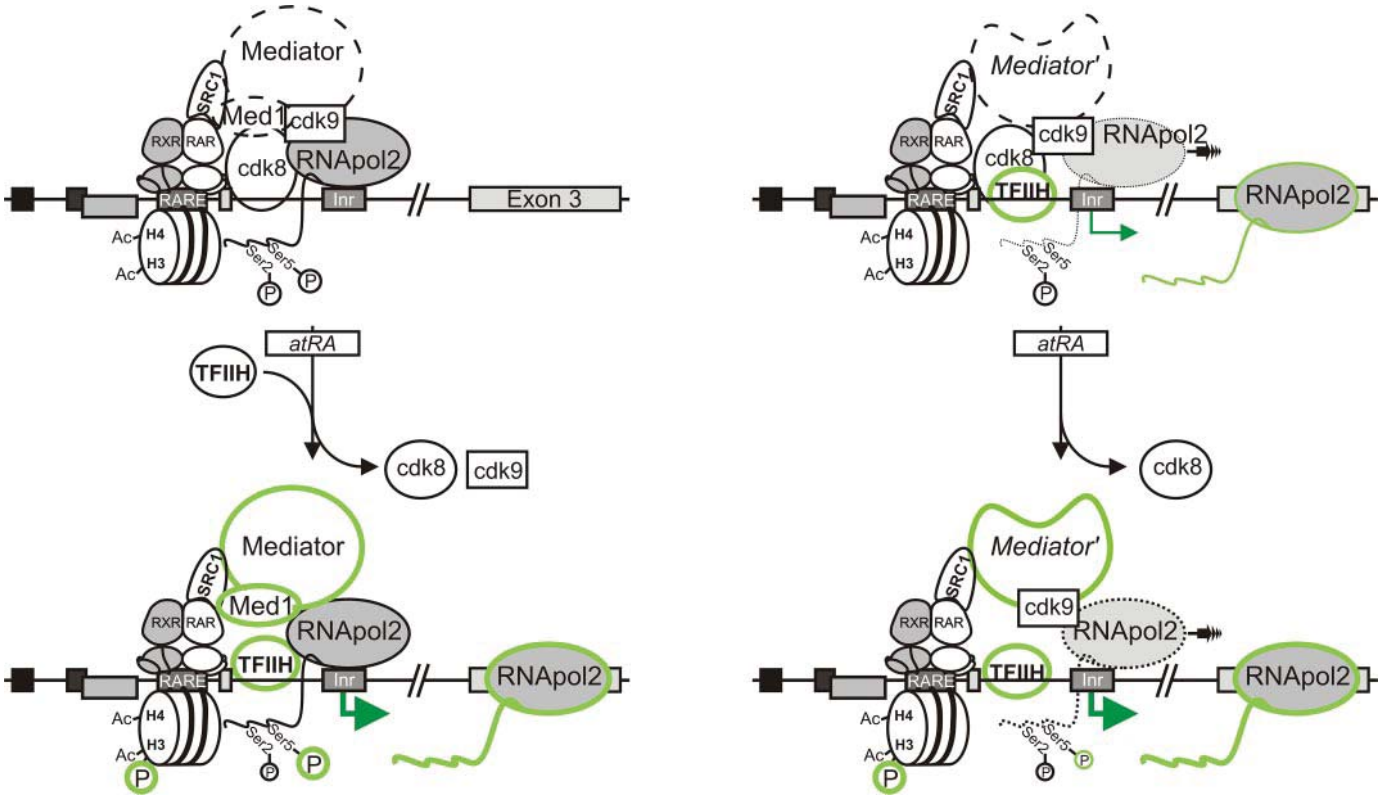


FIGURE 5. **Model summarizing molecular events occurring at the RARβ2 promoter and exon 3.** *Green lines* indicate major differences between transcriptional complexes in atRA-stimulated wtP19 or Med1siRNA cells. The most prominent features of Med1-depleted cells is the ligand-independent association of TFIIH and of cdk9 to the RARβ2 promoter, as well as their ligand-dependent recruitment leading to exacerbated tethering of this two kinases upon ligand stimulation.

transcriptional activity. Quite strikingly, RNApol2 detection was ligand-insensitive in Med1-deficient cells, providing a molecular basis for the increased basal transcriptional activity of the *RARβ2* gene in this background. A model summarizing these observations and interpreting ChIP assays as a change in association of the monitored factors, is presented in Fig. 5.

SRC1 and Med1 Regulate atRA-induced Cellular Differentiation—atRA-induced P19 differentiation is accompanied by cell growth arrest (42), apoptosis, and accumulation of neuronal markers such as βIII tubulin. wtP19 accumulated βIII tubulin as a function of atRA concentration (Fig. 6*A*). SRC1RNAi cells exhibited a lower βIII tubulin expression, underlining a lower propensity to differentiate upon atRA treatment. In contrast, Med1 knock-down increased the ability of P19 cells to express spontaneously βIII tubulin, and this accumulation remained ligand-dependent.

Differentiation is intimately linked to apoptosis in P19 cells (43). Apoptosis was quantified in wt, SRC1RNAi and Med1RNAi cells by flow cytometry (Fig. 6*B*). atRA-induced cell death was dose-dependent in wtP19 cells, to reach 7% of the cellular population. SRC1RNAi cells were highly resistant to apoptosis, which increased only marginally above control. In contrast, Med1RNAi cells underwent massive apoptosis, affecting 20% of the cells after a 48-h treatment with 1 μM atRA (44, 45). atRA-stimulated Med1RNAi cells were thus treated with the caspase pan-inhibitor zVAD-fmk. After a 24-h treatment, this inhibitor prevented atRA-induced apoptosis (Fig. 6*C*), but was barely active after a 48-h atRA treatment (data not shown), indicating that apoptosis becomes, at this stage, irreversible. Thus Med1 knock-down favors differentiation-induced apoptosis of P19 cells, showing that altering an upstream event, *i.e.* transcriptional activation by retinoids, impacts on long term processes such as cellular differentiation and apoptosis.

SRC1 Knock-down Impairs Selectively the Transcriptional Response to Synthetic Retinoids—We demonstrated that the structure of retinoids affect the ability of RXRα/RARα heterodimers to interact with coactivators (20). A prediction from these results is that the loss of expression of a given coactivator could selectively impair the transcriptional response to a specific retinoid. Using GST pull-downs experiments designed to monitor the association of coactivators to a RXRα/RARα heterodimer bound to a DR5 RARE (20), we assessed the ability of several RARα ligands to promote heterodimers interaction with SRC1 or Med1 *in vitro* (Fig. 7*A*), Med1 interacted with RARα in a ligand-dependent manner, irrespective of the nature of the retinoid. In contrast, the interaction with SRC1 was conditioned by the nature of the ligand, displaying the strongest interaction in the presence of TTNPB, a RAR synthetic panagonist (46). The ability of these ligands to activate the RARβ2 promoter in the wt, SRC1-, and Med1-deficient cellular background was quantified (Fig. 7*B*). In wtP19 cells, atRA, TTNPB, and Ch55, another synthetic RAR panagonist (47), were equally able to stimulate mRNA transcription from the RARβ2 promoter. In SRC1-deficient cells, the efficacy of these retinoids to promote transcription was strongly affected, as it could be predicted (see Fig. 2). However, the activity of TTNPB was, in this assay, much more dramatically affected than those of atRA and

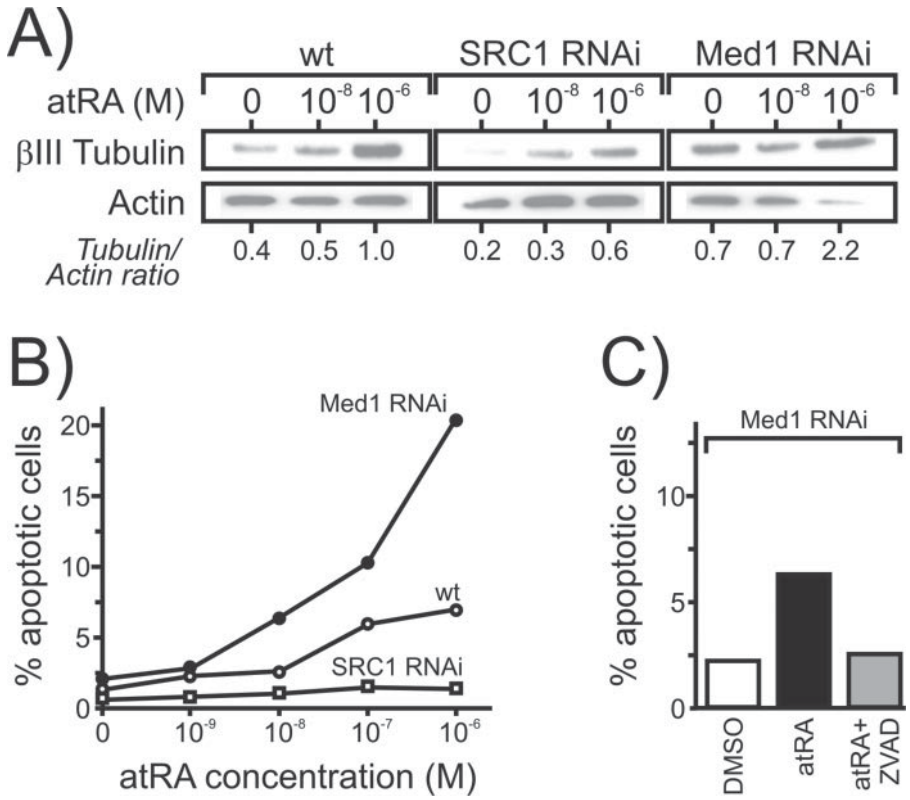


FIGURE 6. **Neuronal differentiation of P19 cells subclones.** *A*, altered expression of differentiation markers in SRC1RNAi and Med1RNAi cells. P19 cells were submitted to differentiation conditions in the presence of atRA, and whole cells lysates were analyzed for their content in βIII tubulin. *B*, apoptosis in P19 cells. Cells were allowed to differentiate as in *A*, and their cell cycle repartition was analyzed by flow cytometry. The repartition in the sub-G_1 phase was plotted as a function of atRA concentration. *C*, apoptosis of P19 cells is a caspase-dependent process. Med1RNAi cells were grown and analyzed as in *B* in the presence of 1 μM atRA and zVAD.fmk for 24 h. The cell repartition in the sub-G_1 phase was assayed by flow cytometry.

Ch55. Med1 deficiency affected positively RARβ2 basal expression, as shown in Fig. 2. Thus TTNPB-mediated activation of the RARβ2 promoter is highly sensitive to SRC1 levels, showing that synthetic retinoids may select among several coactivators to regulate transcription. Similar assays were carried out for the CRABPII gene. Whereas Med1-deficient cells exhibited a stronger response irrespective of the ligand used, as observed for the *RARβ2* gene, the differential effect of SRC1 depletion could not be precisely quantified, due to the very low residual activity of this gene in a SRC1-deficient background (see Fig. 2 and data not shown).

DISCUSSION

Transcriptional activation by NRs is dependent on the sequential recruitment and dismissal of corepressors and coactivators at the promoter of regulated genes. A plethora of proteins with RAR corepressive or coactivating activities have been identified but their relative role in retinoid-regulated transcription has not yet been clearly established (reviewed in Ref. 48). In a seminal study, Kawasaki *et al.* (49) demonstrated that p300 and CBP had distinct roles in atRA-induced differentiation and transcription in F9 cells. Using hammerhead ribozymes, they showed that p300 is required, but not CBP, for atRA-induced differentiation of F9 cells. p300 and CBP had opposite roles in the transcriptional regulation of inhibitors of cyclin-dependent kinases, $p21^{Cip1}$ and $p27^{Kip1}$. Korzus *et al.* (5) showed that pCAF is required for retinoid-induced transcription in Rat-1 cells. Finally, RAR-driven transcription is only mildly affected in $Med1^{-/-}$ MEFs (23), despite the broad implication of the Mediator complex and Med1 ligand-dependent interaction *in vitro* with multiple NRs, including RARs (24).

We aimed at defining the respective role of p160 coactivators and of Med1, the mediator complex subunit which mediates its physical interaction with RARα *in vitro*, in P19 cells, a relevant model of retinoid action at the transcriptional and cellular differentiation levels. Selective inhibition of the expression of either SRC1 or Med1 in these cells led to distinct transcriptional effects: SRC1 knock-down impaired the transcriptional activation of two retinoid-regulated genes, CRABPII and RARβ2, in agreement with its proposed role as a coactivator. More unexpectedly, Med1 knock-down greatly increased retinoid sensitivity of both genes. Notably, the basal level of transcription of these genes was enhanced by 3–5-fold, suggesting that Med1 exerts a repressive activity on both promoters. The mediator complex associates to the RARβ2 promoter in the absence of ligand (our results and Ref. 50). Cdk8, which is part of the repressive kinase module of the mediator complex, phosphorylates the cyclin H subunit of TFIIH, inhibiting TFIIH kinase activity. This prevents RNApol2 CTD phosphorylation, thereby exerting a repressive effect on transcription (51). The behavior of the cdk8 module was not altered in any case, excluding the possibility that an altered cdk8 structure or association could lead to transcriptional derepression. However, it should be noted that Med1 depletion induces gene-specific repression or induction in yeast, and that functional links between yMed1 and Srb10, the ortholog of mammalian cdk8, have been identified (52).

Retinoid-induced transcription occurs efficiently either with severely diminished (our data) or abolished Med1 expression (23). The constitutive detection of RNApol2 in wtP19 is also observed in the Med1-deficient background, showing that the conversion to the elongation-competent state may be Med1-independent. ChIP experiments with antiMed1 and antiMed17 antibodies failed to detect the association of these two subunits to the RARβ2 promoter. However, Med24 was detected on the promoter, showing that the mediator complex, which has probably a different

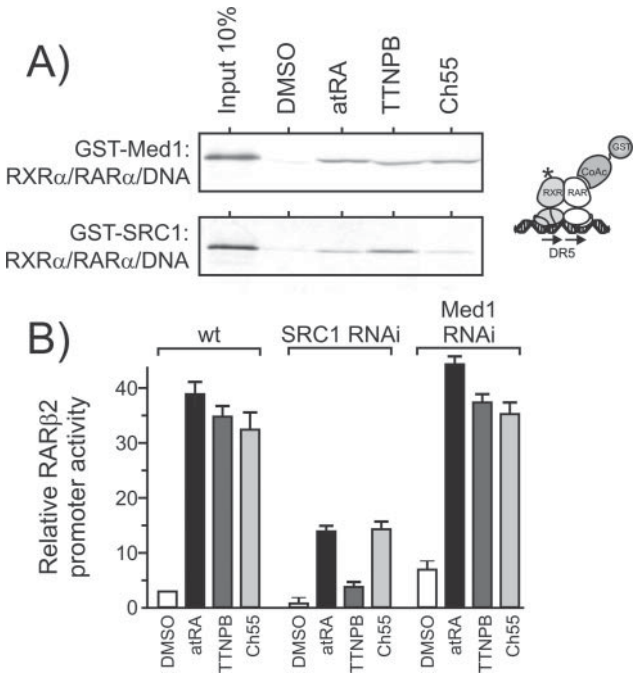


FIGURE 7. **SRC1-dependent activation of the RARβ2 promoter.** *A*, RARα interacts in a ligand-dependent manner with SRC1 and Med1. GST pull-down experiments were carried using a GST-SRC1-(382–842) or a GST-Med1-(527–970) fusion protein and radiolabeled RXRα. GST-bound complexes were analyzed by SDS-PAGE and RXRα was detected by autoradiography. *B*, transcriptional activation of the RARβ2 promoter in the SRC1 or Med1 background. Cells were challenged with 1 μM atRA and RNAs were analyzed for their content in RARβ2 transcripts by Q-PCR as in Fig. 2. Results are expressed relative to the level of expression of the *RARβ2* gene in SRC1RNAi cells, for which the level of expression in the absence of ligand was set to 1.

molecular composition in the absence of Med1 (52), is present. Thus a compromised recruitment of Med1 promotes a more efficient transcriptional activation by retinoids in P19 cells. This finding is consistent with the mild positive effect of Med1 knock-out on RAR-mediated transcription in MEFs (23), and with the hypothesis that the mediator complex is recruited indirectly to the promoter through interactions with CBP/p300 (31). In the same report, study of mediator complex recruitment to p300 in a chromatinized environment provided data suggesting that Med1 recruitment to p300 is facilitated by histone acetylation. This would explain why we and others (see Figs. 1, 3, and Ref. 50) could detect constitutively the mediator complex, since both H3 and H4 are constitutively acetylated at the RARβ2 promoter. Also consistent with this hypothesis is the finding that SRC1 is always detected at the RARβ2 promoter, which in turn would recruit CBP/p300 and thus tether HAT activity to the RARb2 promoter. Indeed, acetyl-H4 levels are decreased in SRC1-depleted cells, and we noted that Med1/Med17 loading was affected in this setting (Fig. 3).

RNApol2 detection is however not indicative of promoter activation: in the SRC1-deficient background, RNApol2 association is ligand-sensitive, but transcriptional activation is severely blunted. In this background, H4 acetylation also becomes ligand-sensitive and is reproducibly decreased compared with wt and Med1RNAi levels. HAT activity has been associated to SRC1 (21), but is not selective for either H3 or H4. Similarly, CBP and p300 displays HAT activities targeted at H3 and H4, and pCAF acetylates preferentially H3 (53). It is therefore unclear whether the observed decrease in H4 acetylation is due solely to the loss of SRC1 association to the promoter, or to the loss of an associated HAT such as CBP/p300. In this regard, it is worth noting that binding of transcriptional activators to HAT dramatically alters HAT activity (54), thus making a formal identification of the H4 acetylase associated to the RARβ2 promoter still elusive. Histone H3 phosphorylation is detected in the wt and Med1-deficient background, in which efficient transcription is observed, and H3 phosphorylation is lost in SRC1RNAi cells, in which a weak transcriptional activity occurs. Interestingly, phosphorylation of H3 increases H3 K14 acetylation by yeast GCN5 (55), and we observed that H3 acetylation is decreased, but not abolished, in the SRC1-deficient background. Whether a similar process occurs at the RARβ2 promoter will require kinetics studies and the identification of the histone H3 kinase, which is distinct from Rsk2 and Msk1 kinases (11).

RNApol2 detection was decreased at the RARβ2 promoter in the Med1-deficient background, suggesting a faster promoter clearance. Conversion of RNApol2 to an elongation-competent form depends on the orchestrated activity of cyclin-dependent kinases. Cdk8 exerts a repressive activity by phosphorylating RNApol2 CTD on Serine 2 and 5, prior to preinitiation complex assembly, whereas cdk7, a component of the TFIIH complex, promotes the conversion toward an elongation mode by phosphorylating Ser^5. cdk9, a component of the positive transcription elongation factor b (P-TEFb), phosphorylates Ser^2 of the CTD and favors transcript elongation, but associates to a number of RNApol2-regulated promoters and phosphorylates RNApol2 CTD when RNA pol2 is in the promoter clearance mode (56). Upon atRA treatment, phosphorylation of Ser^5 increased concomitantly to TFIIH detection in wtP19. This correlation was lost in Med1RNAi cells, in which TFIIH was always detected on the promoter and where no Ser5P RNApol2 could be detected. In addition, association of cdk9, which decreased upon atRA treatment in wtP19, bound more strongly to the RARβ2 promoter in Med1RNAi cells challenged with atRA. Taken together, these data suggest that Med1 depletion may favor both the conversion of RNApol2 into an elongation-competent form, and relieves the elongation blockade shortly after the initiation of transcription, thus facilitating its access to transcribed regions.

P19 cells differentiate into neurons and glials cells upon exposure to atRA. Most neurons exhibit a GABAergic or a cholinergic phenotype (57, 58), and express neuronal markers including βIII tubulin. atRA also reduces the proliferation rate due to an increased duration of the S phase (42). P19 differentiation is linked to atRA-induced apoptosis and is an RAR- and RXR-dependent process (43). Flow cytometry studies revealed that Med1RNAi cells, which expressed a differentiation marker in an atRA-independent fashion, were prone to caspase-regulated apoptosis. This suggests that altering an upstream event, *i.e.* transcriptional activation by retinoids, impacts on a long-term process, *i.e.* cellular differentiation. This relationship is further demonstrated by the fact that SRC1-deficient cells, which display a weak transcriptional response to atRA, exhibit a strongly diminished expression of neuronal markers.

Ligand docking into the ligand binding pocket induces structural changes which are a function of retinoid structure. Indeed, atRA or 9-cis RA-bound RARα exhibits distinct abilities to release SMRT (59), and synthetic agonists confer to RARα a varying selectivity for coactivators *in vitro*, which is also conditioned by DNA binding (20). Our data suggest that liganded RARα interacts with SRC1 with different avidities *in vivo*: SRC1 knock-down affected more severely TTNPB-mediated gene activation, in agreement with the higher affinity of TTNPB·RARα complex for SRC1 *in vitro*. Finally, it suggests that alteration of the expression level of a given coactivator, for example as a consequence of a pathological condition, will lead to a dramatic change in ligand sensitivity of target cells.

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The core component of the mammalian SWI/SNF complex SMARCD3/BAF60c is a coactivator for the nuclear retinoic acid receptor

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Abstract

Retinoic acid receptors (RARs) activate transcription by recruiting coactivator complexes such as histone acetyltransferases (HAT) and the mediator complex, to increase chromatin accessibility by general transcription factors and to promote transcription initiation. Indirect evidences have suggested a role for the ATP-dependent chromatin remodeling complex SWI/SNF in RAR-mediated transcription. Here we demonstrate that two highly related subunits of the core SWI/SNF complex, BAF60c1 and BAF60c2, interact physically with retinoid receptors and are coactivators for RARs. This coactivating property is dependent on SRC1 expression, showing that HATs and SWI/SNF cooperate in this retinoid-controlled transcriptional process.

Keywords: Transcription; Chromatin; Nuclear receptors; Coactivator

1. Introduction

A fundamental role of eukaryotic transcription factors is to overcome the physical barrier that chromatin organization imposes on the access of these transcriptional regulators to DNA. Evolution has designed two main strategies to modulate chromatin structure and to favor transcription factors binding to DNA. The first one relies on the covalent modification of amino-acids located in histone tails, such as acetylation, phosphorylation, methylation. These post-translational modifications, which target in most cases, but not exclusively, histone H3 and histone H4, modify the role played by nucleosomal DNA sequences. These covalent modifications impact on the affinity of histone tails for DNA, thereby increasing the physical accessibility of cis-acting sequences. Moreover, these post-translational modifications create novel docking sites on nucleosomes, allowing the tight binding of proteins to modified histones, thereby controlling the structure, function and dynamics of chromatin. Introducing non-covalent modifications of chromatin structure is the second strategy used, which is based on the recruitment, to regulated promoters, of evolutionary conserved ATPase-containing complexes. Several ATPase-dependent remodeling complexes have been identified, such as the mating type switch/sucrose non-fermenting (SWI/SNF), the imitation of switch (ISWI), the nucleosome remodeling and deacetylating (NuRD) and the chromatin accessibility (ChRAC) complexes. Their action leads to several structural modifications, ranging from the loss of nucleosome(s) to extraction of histone H2A and H2B (Eberharter and Becker, 2004).

The human SWI/SNF complexes organize around two ATPases: Brahma (hBRM) and brahma-related gene 1 (BRG1) (Kingston and Narlikar, 1999; Peterson, 2002). These ATPases possess a bromodomain, allowing their binding to acetylated histones. Several hBRM- or BRG1-containing complexes have been isolated (Mohrmann and Verrijzer, 2005), and a minimal complex made of hBRM or BRG1 [which are mutually

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exclusive and functionally distinct subunits (Bultman et al., 2000)], SNF5/INI1, the Brahma associated factors BAF155 and BAF170, is essential for efficient chromatin remodeling in vitro (Phelan et al., 1999).

Several SWI/SNF complexes (BRM, BRG1, BAF or PBAF), characterized by a distinct subunit composition, have been isolated from mammalian cells (Wang et al., 1996). The core component of the BRM, BRG1, BAF or PBAF complexes are an ATPase subunit, SNF5/INI1, BAF155, BAF170, BAF60, BAF53 and β-actin. Specific components of the PBAF complex are BAF180 and BAF200 (Yan et al., 2005), whereas BAF250 is found exclusively in the BAF complex (Roberts and Orkin, 2004). In addition, BAF60c is expressed in a cell-specific manner during development (Lickert et al., 2004), adding a layer of complexity to the structure, and very likely to the function of these multimeric complexes. While their roles in transcriptional regulation have been extensively characterized, other studies have pointed to their involvement in viral integration and expression, DNA synthesis and cell cycle control (Olave et al., 2002; Wang, 2003; De La Sema et al., 2006). Importantly, signaling pathways regulate BAF complexes by post-translational modifications (Bourachot et al., 2003; Simone et al., 2004).

Retinoic acid receptors (RARs and RXRs) are ligand-regulated transcription factors belonging to the nuclear receptor (NR) superfamily, which comprises 48 members in the human genome. NRs can be regulated by endocrine signals such as steroids or dietary cues such as fatty acids, which behave as ligands for these NRs, while a subset of this family (orphan receptors) is regulated in a ligand-independent manner. RARs activate transcription upon agonist binding, which induces the sequential dismissal of corepressor complexes and the recruitment of coactivator complexes (Lefebvre et al., 2005). Several types of coactivator complexes are known to be recruited to RARs upon agonist binding, and we recently demonstrated that SRC1, a member of the p160 family of coactivator, is critical for the regulation of two retinoid-regulated genes, RARβ2 and CRABPII. In contrast, Med1/DRIP205/TRAP220, a component of the mediator complex interacting directly with RAR, appeared to have a repressive role in this model (Flajollet et al., 2006).

The use of a RAR-dependent, in vitro transcription system suggested that hSWI/SNF complexes are involved in late steps of the transcriptional activation of a retinoid-inducible, chromatinized template (Dilworth et al., 2000). Gene ablation of the polybromo protein BAF180 gene led to a partial decrease of the expression of RARβ2 gene, whereas CRABPII expression was totally abrogated in this BAF180-deficient background (Wang et al., 2004). Therefore transcriptional regulation by RARs seems to involve distinct coactivator complexes, including the hSWI/SNF and ISWI complexes, the mediator complex, histone acetyl transferases (HATs such as CBP/p300, pCAF) and p160 proteins. The role of BAF180 in retinoid-mediated transcription suggested that PBAF is an important coactivator for RARs. However, the mild phenotype of BAF180$^{-/-}$ mice suggests that not all retinoid-regulated genes require PBAF for optimal expression. PBAF regulates selectively vitamin D receptor (VDR)- and peroxisome proliferator activated receptor gamma (PPARγ)-mediated transcription in vitro, but not SP1 or SREBP1c-mediated transcription (Lemon et al., 2001).

The interaction between nuclear receptors and SWI/SNF complexes may occur through distinct subunits of the complex. The glucocorticoid receptor, whose ability to activate several target genes is dependent on BRG-1 expression, interacts physically with BRG-1, BAF57 and BAF60c (Fryer and Archer, 1998; Hsiao et al., 2003; Trotter and Archer, 2004). The estrogen receptor is able to engage interactions with BRG-1, BAF57 and BAF250 (Ichinose et al., 1997; Belandia et al., 2002), whereas BAF60c is a promiscuous partner for several nuclear receptors, including PPARγ, RXRα, FXRα, SF1 and RORα1 (Debril et al., 2004). Other interactions have also been demonstrated, including Brm/SHP, BAF57/androgen receptor, BAF60a/progesterone receptor, BAF60a/FXR (Hsiao et al., 2003; Kemper et al., 2004; Link et al., 2005). SWI/SNF complexes act in most instance as coactivators for nuclear receptors, including PPARγ (Salma et al., 2004), although they may exert a repressive activity toward various transcription factors (Murphy et al., 1999) through their association with corepressors (Underhill et al., 2000; Sif et al., 2001; Deckert and Struhl, 2002; Battaglioli et al., 2002), including nuclear receptors (Kemper et al., 2004).

As part of an effort to characterize more precisely the nature and the functions of coactivators and corepressors of RARs (Martin et al., 2003, 2005; Lefebvre et al., 2006; Flajollet et al., 2006), we undertook a study aiming at characterizing the role of SWI/SNF complexes in RAR-mediated transcription. We demonstrate here that BAF60c proteins exert RAR-coactivating properties essentially through a direct interaction with the RAR coactivator binding interface. In addition, BAF60c coactivating property is dependent on SRC1 expression, although neither BAF60c1 or BAF60c2 are able to engage direct interaction with this p160 protein.

2. Materials and methods

2.1. Reagents

atRA was obtained from Sigma. DNA restriction and modification enzymes were purchased from Promega.

2.2. Plasmids

The plasmids encoding for BAF60c1 and BAF60c2 (pSG5- and pGST-) were a gift from J. Auwerx (IGBMC, Illkirch, France), The pGST-BAF57 expression vector was a gift from B. Belandia (I.C.L., U.K.) whereas pGST-BAF250 and pCIneo-BAF250 were from Z. Nie (N.I.H., U.S.A.). GST fusion proteins encoded for the full length version of BAF57, BAF60c1 and BAF60c2, whereas the 382–842 fragment of SRC1 and the 1670–2171 fragment of BAF250 were fused to GST. p(GRARE)3tkLuc, pSG5-RXGR, pSG5-hRARα, pSG5-RARαΔAB, pSG5-RARαΔE/F, pSG5-RARα-K244, pSG5-RARα-K244,262A and pSHAG-SRC1 were described in Lefebvre et al. (1998a), Mouchon et al. (1999), and Flajollet et al. (2006). The ERE_3-tk Luc reporter gene, the pCMV-ERα expression vector have been described in Geffroy et al. (2005). The GRE_3-tk Luc and the pRSV-rGR constructs are described in Prima et al. (2000). The pSilencerBAF60c encoding for a anti-BAF60c1&2 small hairpin RNA was synthetized and cloned following a strategy described elsewhere (Flajollet et al., 2006). The selected anti-BAF60c siRNA was: cagatgggttccaagtgaag.

2.3. GST-pulldown assays

Glutathione-*S*-transferase (GST)-fusion proteins expressed in BL21/DE3-pLysS bacteria were adsorbed on glutathione-Sepharose 4B beads as previously described (Lefebvre et al., 1998b). ^{35}S-labeled proteins ([^{35}S]methionine was from GE Healthcare) were synthesized using the in vitro coupled transcription/translation kit from Promega (T7 TNT Quick Rabbit Reticulocyte kit). Three microlitres of each reaction were diluted in 150 μL of GST binding buffer (20 mM Tris–HCl, pH 7.8, 100 mM KCl, 0.05% NP40, 1 mM DTT, 20% glycerol) and pre-incubated in the absence or the presence of ligand for 1 h at room temperature with gentle rotation. Extracts were then incubated for 1 h at room temperature with an equal amount of fusion protein bound to GST-Sepharose, as verified by Coomassie Blue staining. Unbound material was removed by three successive washes (20 mM Tris–HCl, pH 7.4, 100 mM KCl, 0.1% NP40, 1 mM DTT, 20% glycerol). Resin-bound proteins were eluted in PAGE-SDS loading buffer and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Labeled proteins were visualized with a Molecular Dynamics Storm 860 phosphorimager (Molecular Dynamics). At least three independent experiments were carried out with different bacterial extracts for each experiment.

2.4. HeLa and P19 culture and transfection

P19 EC and HeLa Tet-On cells were maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Biowhittaker), 1000 U penicillin and 10 μg streptomycin per mL. One day prior to transfection, cells were plated in six-wells plates. Each well was transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions, with a DNA mixture including 1 μg of the DR5-driven, RA-responsive firefly luciferase reporter gene [GRARE3-tk luc (Depoix et al., 2001) referred to as DR5-tk Luc in the manuscript], 50 ng of a control plasmid tk-renilla luciferase (Promega) and various amounts of other plasmids as indicated in each legend. The total amount of transfected DNA was adjusted to 4 μg per well. When indicated, the RAR expression vector was substituted for a ER expression vector (Geffroy et al., 2005) or a GR expression vector (Prima et al., 2000) and the adequate reporter gene. Twenty-four hours after transfection, cells were challenged overnight with 1 μM atRA. Cells were harvested and luciferase activities were assayed with the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase values were normalized to those of renilla.

2.5. Cellular extracts preparation, coimmunoprecipitation assays and Western blot analysis

Cellular extract preparation and Western blot analysis were carried out as previously described (Lefebvre et al., 2002a). For coimmunoprecipitation experiments, cells were treated for 2 h with 1 μM atRA or left untreated and lysed as described previously (Martin et al., 2005). Immunodetections were performed using the following antibodies: monoclonal anti-actin antibody (ac-15, Sigma), monoclonal anti-SMARCD3 antibody (M01, Abnova Corp.), polyclonal anti-BRG1, anti-BAF250, anti-SRC1 and anti-RARα (sc-17796, sc-32761, M-341 and sc-551, respectively, Santa Cruz Biotech.).

2.6. Immunofluorescence and GFP detection

The experimental procedure is as in Prima et al. (2000).

2.7. RNA preparation, reverse transcription-PCR, and quantitative PCR oligonucleotides

Total RNA purification and RT-PCRs were carried out as described (Brand et al., 2002). Amplification conditions were adjusted to be within linear range. Sequences of primers used for PCR were: β-actin: 5′-atcatgtttgagaccttcaa-3′ and 5′-catctcttgctcgaagtcca-3′; mSMARCD3 (209-866): 5′-gcgcgcaaagccacgaaaa-3′ and 5′-tccggtgccactcaacaagg-3′; BAF60c2: 5′-gcgcgcaaagccacgaaaa-3′ and 5′-tccggtgccactcaacaagg-3′; SRC1: 5′-aatgtgttcagtcaagctgtccag-3′ and 5′-tggttattcagtcagtagctgctg-3′.

2.8. Quantitative PCR

Mouse RARβ2 transcripts were detected and analyzed as described in Flajollet et al. (2006).

2.9. Chromatin immunoprecipitation assays

ChIP assays were performed as described in Lefebvre et al. (2002b) and Flajollet et al. (2006) using anti-RAR (C-20) and anti-SRC1 (M-341) antibodies. Briefly, formaldehyde-crosslinked chromatin was sonicated and immuno-precipitated with either a non-specific IgG or the indicated antibody. ChIP analysis were performed at least in triplicate using distinct DNA preparations. Immuno-precipitated DNA was purified and quantified, after a first characterization by regular PCR (32 cycles), by Q-PCR using an ABI PRISM 7700 sequence detection system. The RARβ2 promoter sequence was in this case amplified from −78 to +38 with the following primers: forward: ttgaaggttagcagcccgg, reverse: cttctgtcacacggaatgaaagat; probe FAM/TAMRA: aaggttcaccgaaagttcactcgca. The exon 3 sequence was amplified using the following primers: 5′-tcagtggattcacccaggc-3′ and 5′-tcgggacgagctcctcag-3′ with the FAM/TAMRA probe 5′-cagcaccggcatactgctcaa-3′. DNA was quantified and results expressed relative to input DNA, after subtracting non-specifically bound DNA as assayed using a non-specific IgG in the ChIP assay. Assays were run in triplicate.

3. Results

3.1. BAF60c1 and BAF60c2 exert coactivating properties on RAR-mediated transcription

Several components of SWI/SNF complexes have been shown to contain the LXXLL nuclear receptor interaction motif (Mohrmann and Verrijzer, 2005), such as BAF250, BAF60c and BAF170. The ability of BAF57, BAF250 and BAF60c proteins to potentiate retinoid-induced transcription was first assessed in a transient transfection assay in HeLa cells, and compared to that of p160 proteins (SRC1, 2 and 3, Fig. 1A). In our system, transactivation is mediated solely through RAR, since RXR is non-permissive in this setting. SRC1 and SRC2 potentiated strongly the ligand-dependent activation of a DR5 RARE-driven reporter gene in this cellular background (four-fold), whereas SRC3 (pCIP/AIB1) was not active in our system. Overexpressing BAF57 and BAF250 did not modify significantly basal and retinoid-induced activities of the reporter gene. In contrast, both BAF60c1 and BAF60c2 displayed clearly detectable RAR-coactivating properties, and increased the retinoic acid (RA)-induced activity by two-fold. As control experiments, we also assessed the coactivating properties of SRC1, BAF57, BAF250 and BAF60c2 on ERα and GR transcriptional activity in strictly similar conditions (Fig. 1B and C). In contrast to RARα, both ERα and GR transcriptional activities were sensitive to BAF57 and BAF250 overexpression, but BAF60c2 was inactive in these assays, as previously published (Belandia et al., 2002; Nie et al., 2000).

We thus tested BAF60c1&2 function in P19 cells, a RA-sensitive embryonal carcinoma cell line which is a cellular model suitable to study both transcriptional and differentiation processes initiated by retinoids (Lefebvre et al., 2002a; Flajollet et al., 2006). BAF60c1&2 expression was therefore either knocked down using a small interfering RNA or increased upon transfection of a BAF60c2 expression vector in this cell line (Fig. 1D,

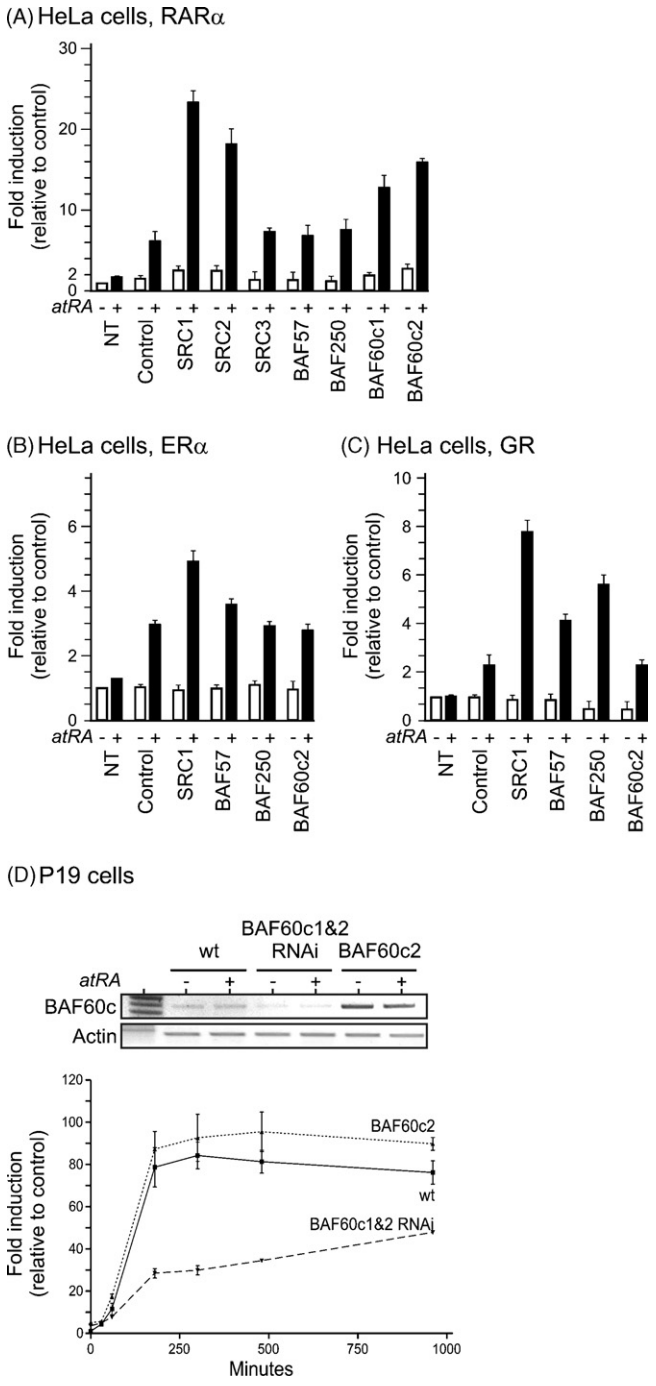

Fig. 1. BAF60c proteins possess RAR-coactivating properties in HeLa and P19 cells. (A) BAF60c1 and BAF60c2 overexpression potentiate RA-induced transcription. HeLa cells were transfected with the DR5-tk Luc reporter gene (NT), with this reporter gene and expression vectors coding for RARα and RXRα (control), or with the DR5-tk Luc reporter, RARα and RXRα expression vectors and an expression vector coding for the indicated protein. Twenty-four hours after transfection, cells were challenged or not with 100 nM atRA for 16 h. Renilla and firefly luciferase activities were assayed and expressed as a fold induction over the basal level of control cells, which was arbitrarily set to 1. (B) Coactivating properties of SRC1, BAF57, BAF250 and BAF60c2 on ERα transcriptional activities. HeLa cells were transfected as in (A) using an ERE_3-tk Luc reporter gene and a pCMV-ERα expression vector. (C) Coactivating properties of SRC1, BAF57, BAF250 and BAF60c2 on GR transcriptional activities. HeLa cells were transfected as in (A) using an GRE_3-tk Luc reporter gene and a pRSV-GR expression vector. (D) Knockdown of the expression of BAF60c1&2 affects the transcriptional response of the RARβ2 promoter to atRA. P19 cells were transfected either with a plasmid encoding for an antiBAF60c1&2 shRNA (BAF60c1&2 RNAi) or an expression vector coding for BAF60c2. The level of expression of the BAF60c1&2 mRNA was assayed by RT-PCR (upper panel). The level of expression of the RARβ2 gene was assessed by RT-QPCR at different times and expressed as a fold induction over the basal level of untreated cells, set arbitrarily to 1.

upper panel). Monitoring the expression by quantitative PCR of the endogenous RARβ2 gene, which is also regulated through a DR5 RARE (de Thé et al., 1990), showed that overexpression of BAF60c2 exerted a mild potentiation on RA-induced RARβ2 mRNA accumulation, suggesting that the level of expression of endogenous BAF60c2 in P19 cells is not as rate-limiting as it is in HeLa cells. However, knocking down BAF60c1&2 expression had a strong impact on the activity of the RARβ2 gene, which became noticeable after 1 h of RA treatment (Fig. 1D, lower panel). After a 3 h treatment, the observed inhibition reached more than 60% compared to wild type P19 cells, and was still of about 40% after a 16 h challenge. Taken together, our data suggest that BAF60c proteins are potential coactivators for RARα in two distinct cellular backgrounds.

3.2. The BAF complex interacts with RARα in intact cells

Our results showed a functional interaction in intact cells between BAF60c proteins and RXR/RAR heterodimers, bound either to plasmid DNA (transiently transfected reporter gene) or to a fully chromatinized DNA template (RARβ2 promoter). Coimmunoprecipitations were thus carried out using P19 cellular extracts to investigate the physical interaction between endogenous RARα and these SWI/SNF complex subunits (Fig. 2A). Immunoprecipitating endogenous RARα allowed the detection of BAF60c1&2, which were interacting constitutively and in a ligand-insensitive manner with RARα. BAF250 displayed a similar pattern of interaction to that of BAF60c. Immunofluorescence studies showed that BAF60c was localized in both the cytoplasmic and nuclear compartment, whereas an overexpressed EGFP-tagged hRARα was exclusively detected in the nucleus (Fig. 2B).

To further investigate the pattern of interaction of BAF60c subunits with the promoter-associated RARα, we carried out chromatin immunoprecipitation assays on the RARβ2 promoter (Fig. 2C). In agreement with our previous results (Lefebvre et al., 2002b; Flajollet et al., 2006), both RARα and SRC1 were constitutively bound to the promoter in a ligand-insensitive manner. As expected from coimmunoprecipitation assays, BAF60c proteins were tethered to the promoter in a ligand-insensitive manner. In contrast, BAF60c proteins could not be detected at exon 3 of the RARβ2 gene (Fig. 2D), whereas RNA polymerase 2 density increased at this locus in a ligand-dependent manner, as previously reported (Flajollet et al., 2006). Thus immunoprecipitation assays suggest that, despite having both LXXLL motifs, BAF250 and BAF60c proteins interact with RARα in a ligand-independent manner, much like SRC1. Whether this applies to both BAF60c1 and BAF60c2 cannot be determined at

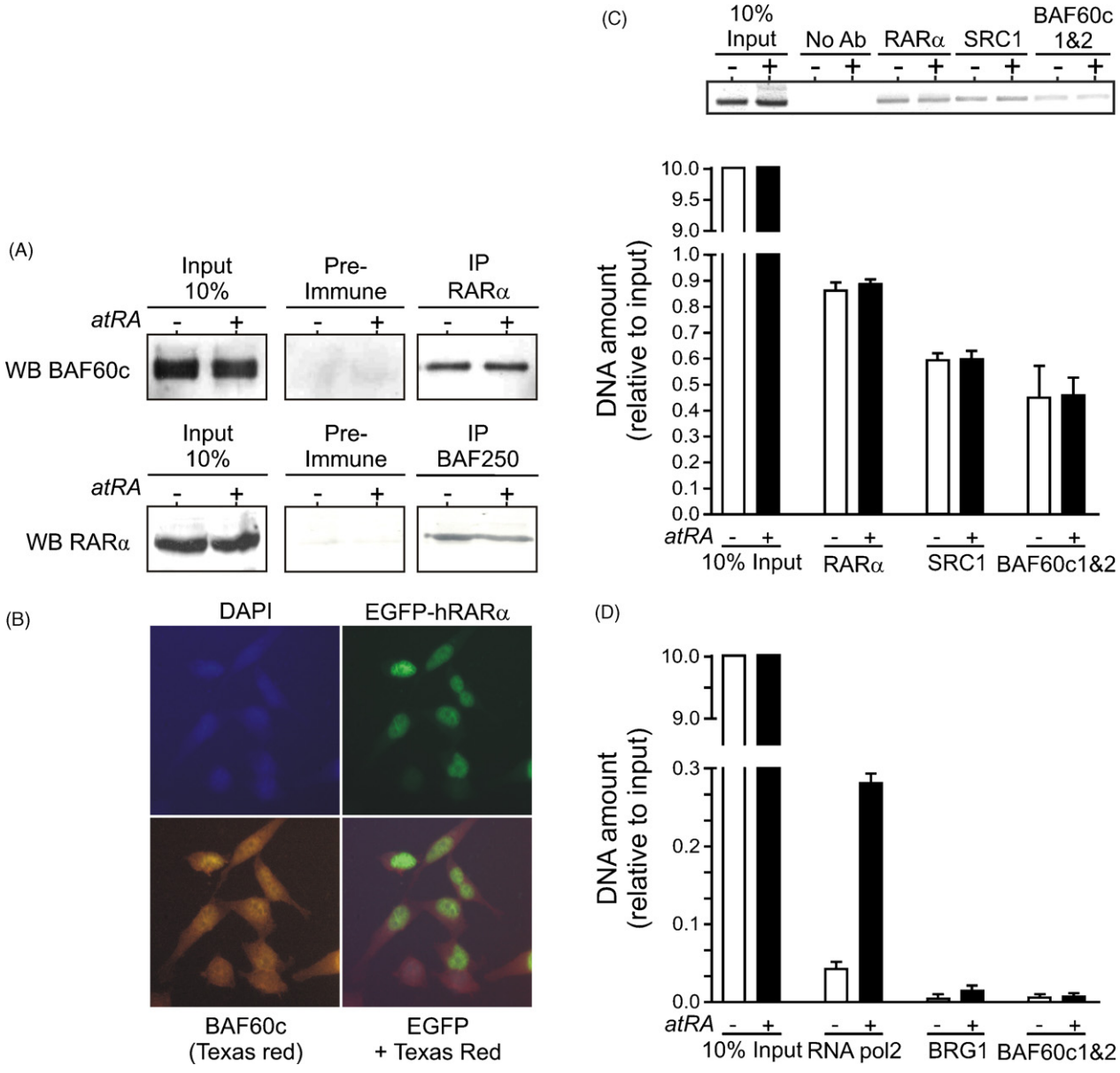

Fig. 2. BAF60c1&2 interact with RARα in intact cells. (A) BAF60c proteins interact with RARα in a ligand-independent manner. RARα or BAF250 were immunoprecipitated from P19 whole cell extracts, and the association of BAF60c or RARα with the RARα or BAF250 precipitate was detected by Western blot analysis. (B) Colocalization of RARα and BAF60c proteins. HeLa cells were transfected with an EGFP-hRARα expression vector and fixed with paraformaldehyde 24 h after transfection. Endogenous BAF60c was detected by indirect immunofluorescence. (C) BAF60c proteins bind to the RARβ2 promoter. The RARβ2 promoter immunoprecipitated from formaldehyde-crosslinked chromatin by antibodies against RARα, SRC1 or BAF60c1&2 was subjected to regular PCR (upper panel) or to Q-PCR (lower diagram) using RARβ2 promoter-specific primers. Results are expressed relative to the PCR signal detected with 10% input DNA after subtraction of the signal generated in control (no antibody, No Ab) immunoprecipitates ($n = 3$). (D) RNA polymerase 2, but not BAF60c1&2, associates to exon 3 of the RARβ2 gene. Immunoprecipitated DNA was amplified with primers specific for the RARβ2 exon 3 region (+165 to +524) and quantified as described in Flajollet et al. (2006) and above.

this point, since immunoprecipitations were carried out with an antibody recognizing both isoforms.

3.3. p160 coactivators act in synergy with BAF60c proteins on a retinoid-regulated promoter

Results described above suggest that distinct components of the SWI/SNF complex may engage interactions with promoter-bound RAR/RXR heterodimers. Since SRC1 and BAF60c1&2 bound to the RARβ2 promoter with a similar pattern, we tested whether p160 and BAF60c proteins could interact functionally (Fig. 3). Hela cells were transfected with the DR5-tk Luc reporter gene, and RARα and RXRα overexpression conferred RA sensibility to this system (Fig. 3A). Overexpression of either SRC1 or SRC2 increased both the basal and the RA-induced luciferase activity, whereas SRC3 and p300 were inactive (Fig. 3A). As shown above, BAF60c2 displayed coactivating properties in this system, and interestingly, co-expression of either SRC1 or SRC2 with BAF60c2 yielded an additive response. A identical result was obtained upon overexpression of BAF60c1 (data not

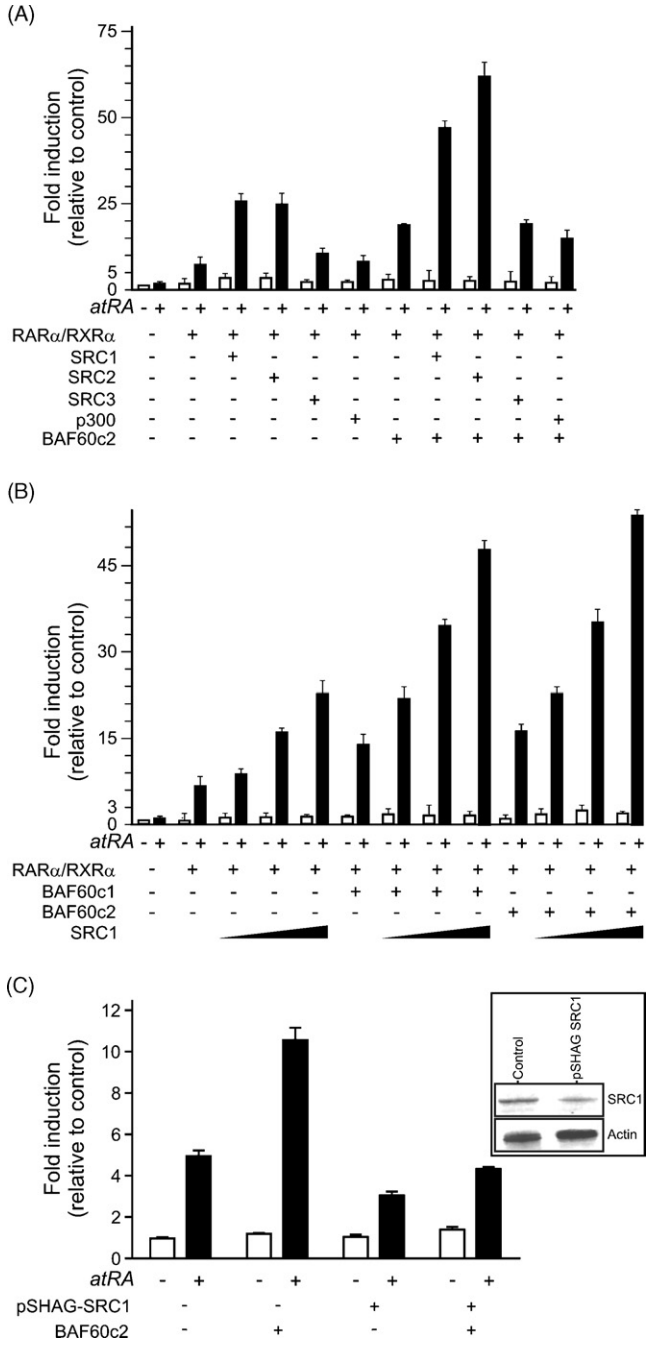

Fig. 3. BAF60c1 and BAF60c2 coactivate RARα in a SRC1-dependent manner. (A) SRC1 synergizes with BAF60c1&2. HeLa cells were transfected with the DR5-tk Luc reporter gene and the indicated combination of expression vectors. The luciferase activity was assayed after challenging cells with vehicle (DMSO, − lanes) or 100 nM atRA (+ lanes). Results are expressed as in Fig. 1. (B) Dose-dependency of SRC1 synergy with BAF60c1 or BAF60c2. HeLa cells were transfected with the indicated combination of expression vectors and the DR5-tk Luc reporter gene, with increasing amount of SRC1 expression vector (50, 250 and 500 ng). Luciferase assays were assayed and are displayed as described in Fig. 1. (C) Down-regulation of SRC1 expression affects BAF60c2 coactivating capacity. HeLa cells were transfected with the DR5-tk Luc.

shown). The additive effect between BAF60c1 or BAF60c2 with SRC1 was clearly dose-dependent, as shown in Fig. 3B.

HeLa cells were then transiently transfected with a plasmid encoding for a shRNA directed against SRC1 mRNA (pSHAG-SRC1, Fig. 3C). In agreement with a ca. 60% transfection efficiency, SRC1 expression was decreased by 40–50% when assayed by Western blot analysis of whole cell extracts (inset, Fig. 3C). In SRC1-depleted cells, overexpression of BAF60c2, together with RARα and RXRα did not enhance significantly the transcriptional response of the reporter gene to atRA, in contrast to control cells (Fig. 3C). From this, we conclude that the BAF60c coactivating property is exerted through a SRC1-dependent mechanism.

3.4. BAF57 and BAF250, but not BAF60c1&2, interact directly with SRC1

The functional potentiation and the detected physical interactions suggested that BAF60c proteins could interact physically with SRC1. We therefore tested this hypothesis using a GST pulldown approach, in which BAF proteins were fused to GST and used as a bait for labeled SRC1 (Fig. 4). BAF250 and BAF57 interacted with SRC1 (Fig. 4A). In sharp contrast, BAF60c proteins were unable to interact with SRC1, suggesting that BAF60c interaction with the RARα-containing complex might take place through RARα itself, or at least in a SRC1-independent manner. This hypothesis was further tested in the P19 cell background, in which a permanent depletion in SRC1 was induced upon stable transfection of a shRNA-encoding vector targeting SRC1 mRNA [Fig. 4B and Flajollet et al. (2006)]. ChIP assays in control cells confirmed the ligand-insensitive and constitutive interaction of SRC1 and BAF60c1&2 with the RARβ2 promoter (Fig. 4C). Knockdown of SRC1 expression resulted in a decreased association of this protein to the promoter. However, this depletion had no significant impact on BAF60c1&2 binding to the promoter, confirming that SRC1 and BAF60c1&2 do not interact physically with each other. These observations led us to speculate whether BAF60c polypeptides could interact directly with RARα.

3.5. Only BAF60c2 interacts with RARs in a AF2-dependent manner

GST pulldown experiments were thus performed to assess the RAR-binding properties of BAF subunits (Fig. 5). ER was first used as a prototypical interactant with SRC1, BAF60c1&2, BAF250 and BAF57. ERα turned out to interact in a strictly ligand-dependent manner with SRC1, BAF250 and BAF57. The interaction of ERα with BAF60c proteins was also ligand-dependent, but a significant basal interaction was detected (Fig. 5A). RARα, RARβ, and RARγ interacted with SRC1 in a ligand-dependent manner, and BAF250 exhibited a ligand-independent interaction with all three isotypes. Thus BAF250 may interact with RAR itself, in addition to SRC1 (Fig. 4). We noted however that RARγ interacted quite weakly with BAF proteins when compared to the α and β isotypes. Similarly, the interaction of BAF60c1 with RARs was constitutive and not influenced by RA, whereas BAF60c2 interacted constitutively with the three isotypes and atRA increased moderately this interaction with RARα and RARγ. Finally, BAF57 displayed a significant affinity only for RARβ, opening the possibility that

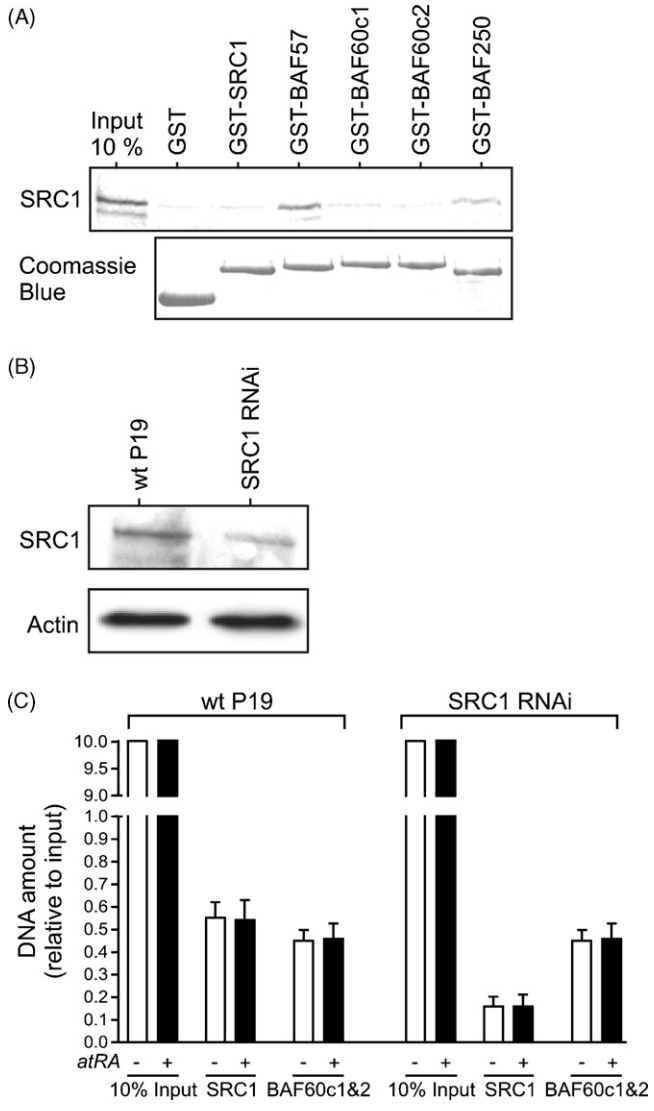

Fig. 4. BAF60c1&2 do not interact physically with SRC1. (A) In vitro interaction of BAF proteins with SRC1. Radiolabeled SRC1 was incubated with GST or GST fusion proteins as indicated. The GST pulldown procedure is described in Section 2. The lower panel shows a portion of the gel previously stained with Coomassie Blue to assess the quantity of GST fusion proteins used in the assay. (B) Knockdown of SRC1 expression in P19 cells. P19 cells stably expressing an anti-SRC1 shRNA were isolated and characterized (Flajollet et al., 2006). The level of expression of SRC1 in wt P19 and shRNA-expressing cells (SRC1 RNAi) was assessed by Western blot analysis of P19 whole cell extract (50 μg proteins/lane). (C) Knockdown of SRC1 expression does not prevent BAF60c1&2 tethering to the RARβ2 promoter. Chromatin immunoprecipitation with antiSRC1 or antiBAF60c antibodies were carried out as described in Fig. 2. Results are expressed relative to the PCR signal detected with 10% input DNA.

BAF57 could play a specific role in RARβ-mediated transcription. We also investigated whether BAF proteins interact with the RAR dimerization partner RXRα in a similar assay. Quite strikingly, only BAF60c proteins could engage detectable interaction with RXR, and again, only BAF60c2 exhibited both a basal and ligand-induced interaction with RXRα (Fig. 5A).

We thus sought to identify the molecular structure(s) allowing the interaction between BAF60c1 or BAF60c2 and RARα, using mutants of RARα in a GST pulldown assay (Fig. 5B and C). The isolated RARα LBD was sufficient to mediate the basal and ligand-dependent RARα-BAF60c2 interaction (Fig. 5B), and both the A/B and C domains were dispensable for this interaction, although we noted that deletion of the A and B domains altered moderately the affinity of BAF60c1&2 for RARα (Fig. 5B and C). Point mutants in the LBD at K244 and K262 in the RARα LBD prevent agonist-induced helix 12 folding, and thus inactivate the coactivator-RARα binding interface and RARα transcriptional activity [Fig. 5C and Mouchon et al. (1999)]. In GST-pulldown assays, mutations of K244 or of K244 and K262 were able to prevent the ligand-dependent recruitment of SRC1 to RARα, BAF60c1&2. In addition, the progressive alteration of the CoA binding interface (from hRARαK244A to hRARαK244,262A) decreased the basal interaction of both BAF60c1 and BAF60c2 with RARα. Similarly, deletion of a significant portion of the AF2 AD region (hRARαΔ376-408) prevented the ligand-dependent interaction of RARα with BAF60c2 and lowered the basal interaction with both isoforms. This indicates that the classical CoA binding interface is a very important structural determinant of BAF60c1&2 interaction with RARα. However, an yet undetermined domain in the RARα LBD is likely to contribute also to this ligand-independent interaction.

4. Discussion

Many studies have highlighted the function of the SWI/SNF complex in nuclear receptor-mediated transcriptional activation. Transcriptional coactivation of retinoic acid receptor by hSNF2 suggested a role of this complex in RAR-targeted chromatin remodeling (Chiba et al., 1994), and in vitro transcription assays revealed a facilitating role of the PBAF complex in transcriptional activation by several NRs (Lemon et al., 2001). Gene ablation of the PBAF-specific subunit BAF180 impacted differentially the response of two RA-target genes, RARβ2 and CRABPII (Wang et al., 2004), underlining the role of the PBAF complex in RAR-mediated transcription. However, the association of BAF250 to RARα-containing complex (Fig. 2A) suggest that BAF complexes may also play a functional role, at least in P19 cells. Finally, order of addition experiments in an in vitro transcription assay showed that the SWI/SNF complex participates to the transcriptional activation of a DR5-regulated chromatin template at late stages of the transcriptional process (Dilworth et al., 2000). However, how the SWI/SNF complex exerts its coactivating function, from a molecular point of view, on RAR-mediated transcription is not clear at this time.

Here we show that the effect of the SWI–SNF complex on RA-induced transcription may involve its targeting to RA-regulated promoters, through a direct interaction of RXR/RAR heterodimers with BAF60c proteins. We could show that both BAF60c1 and BAF60c2 are coactivators of RAR in two distinct cellular backgrounds. BAF60c coactivation was dependent on SRC1 expression, however, no physical interaction could be detected between these two components. Since both proteins interact with RAR through its coactivator binding interface, and thus probably involving the LXXLL motif borne by BAF60c subunits, one may speculate that this functional synergy reflects

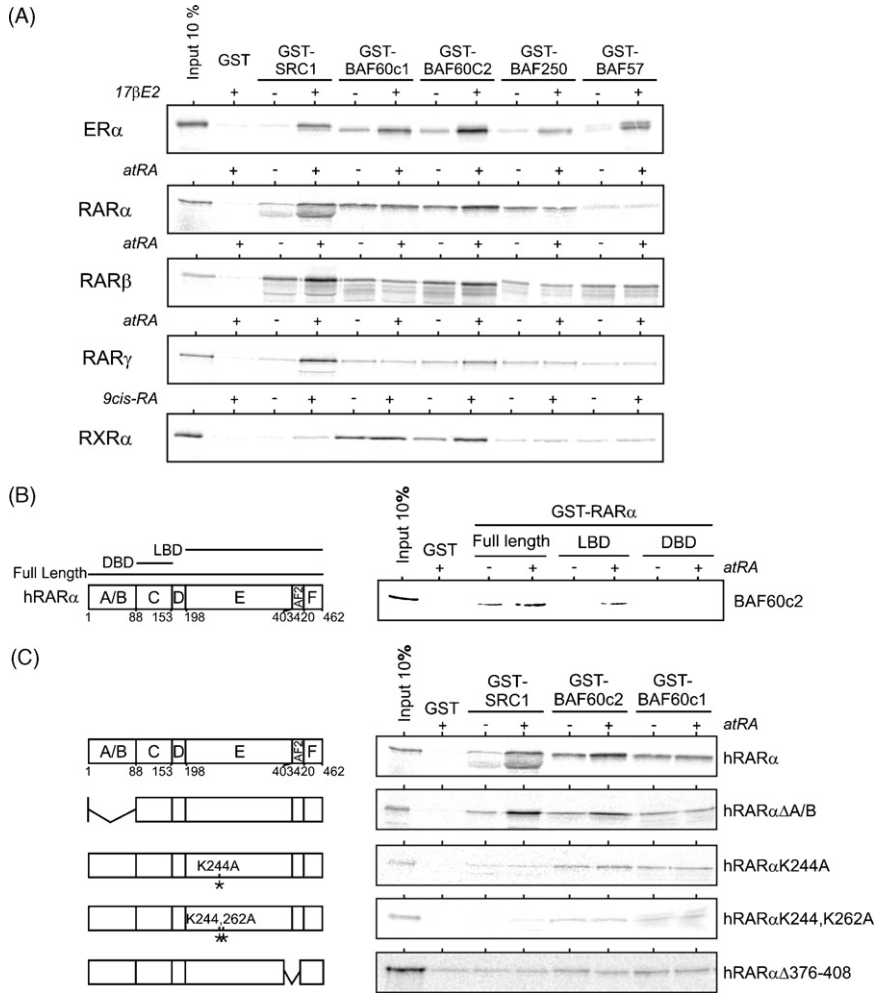

Fig. 5. Molecular interactions of SWI/SNF subunits. (A) Interaction of ERα, RARα, RARβ, RARγ and RXRα with BAF proteins. ^{35}S-labeled nuclear receptors were synthesized in vitro, and GST pulldowns were performed as described in Section 2. Ligand concentrations were 100 nM for each condition. (B and C) Mapping RARα domains interacting with BAF60c1 and BAF60c2. RARα or its derivatives were ^{35}S-labeled and tested for their interaction with BAF60c2 (B) or BAF60c1 and BAF60c2 (C). Left panels show a diagram of RARα and of the mutants used in GST pulldown experiments.

a sequential recruitment of SRC1 then of the SWI/SNF complex to RA-regulated promoters, as suggested previously (Dilworth et al., 2000). This scenario would be in agreement with the observed delayed recruitment of the SWI/SNF complex to the PPARγ2 promoter during preadipocyte differentiation (Salma et al., 2004). Interestingly, TFIIH recruitment to the PPARγ2 promoter is SWI/SNF-dependent, and TFIIH recruitment to the RARβ2 promoter is ligand-dependent, in opposition to SRC1 and the Med complex which are associated permanently to this promoter, irrespective of its activation state [Figs. 2 and 4 and Flajollet et al. (2006)]. We observed, by coimmunoprecipitation and ChIP assays, that RAR interacts with Brg1 in a ligand-dependent manner (data not shown). Taken together, these data therefore suggest that TFIIH recruitment to the RARβ2 promoter may be dependent on the association of a fully functional SWI/SNF complex.

Alternatively, the lack of detectable binding of SRC1 to RXR [data not shown and Mouchon et al. (1999)], the obligate RAR dimerization partner, and the ability of BAF60c1&2 to bind RXR, leaves open the possibility of the formation of a BAF60c/RXR/RAR/SRC1 complex. RXR-bound BAF60c could in this conformation synergize with RAR, by stabilizing the interaction of RAR with other components of the SWI/SNF complex such as Brg1. This would be reminiscent of the transcriptional potentiation of GATA4-regulated genes by BAF60c, which occurs through the stabilization of the interaction between transcriptional regulators and Brg1, allowing further cooperation between the SWI/SNF complex and HATs (Lickert et al., 2004). A in-depth investigation of the kinetics of the recruitment of the SWI/SNF complex to the promoter of endogenous RA-regulated genes is clearly needed to distinguish between these two possibilities. These "receptor-centered" hypothesis do not exclude, however, a model by which the SWI/SNF complex would interact directly with hyperacetylated histones or DNA through bromodomains or DNA-binding motifs (Mohrmann and Verrijzer, 2005). During the course of these study and of others (Lefebvre et al., 2002b; Martin et al., 2005; Flajollet et al., 2006), we noted that several coactivators, which interact in a ligand-dependent manner with RAR in vitro, such as SRC1, Med1 and to a lesser extent BAF60c2, are constitutively bound

to the RARβ2 promoter in intact cells. While this observation has been confirmed by others (Pavri et al., 2005), the molecular basis for this constitutive interaction is still unknown but may involve a receptor-independent recruitment.

Our results thus reveal an important role of BAF60c1 and BAF60c2 polypeptides in RA-dependent transcriptional regulation. Because BAF60c1 and BAF60c2 are expressed in a tissue-specific manner (Debril et al., 2004), this may impact on the cell-specific activity of RARs. Interestingly, this property is known to be dependent on the AF1 region, whose function can be modulated through a TFIIH-dependent phosphorylation (RochetteEgly et al., 1997; Keriel et al., 2002). Taken together, our data as well as those from others establish an important role for SWI/SNF complexes in RAR-regulated transcription. It will be important to determine whether these complexes act by facilitating the assembly of the preinitiation complex, and/or by favoring the very first steps of transcriptional elongation.

Acknowledgements

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The Elongation Complex Components BRD4 and MLLT3/AF9 Are Transcriptional Coactivators of Nuclear Retinoid Receptors

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Abstract

Nuclear all-trans retinoic acid receptors (RARs) initiate early transcriptional events which engage pluripotent cells to differentiate into specific lineages. RAR-controlled transactivation depends mostly on agonist-induced structural transitions in RAR C-terminus (AF-2), thus bridging coactivators or corepressors to chromatin, hence controlling preinitiation complex assembly. However, the contribution of other domains of RAR to its overall transcriptional activity remains poorly defined. A proteomic characterization of nuclear proteins interacting with RAR regions distinct from the AF-2 revealed unsuspected functional properties of the RAR N-terminus. Indeed, mass spectrometry fingerprinting identified the Bromodomain-containing protein 4 (BRD4) and ALL1-fused gene from chromosome 9 (AF9/MLLT3), known to associate with and regulates the activity of Positive Transcription Elongation Factor b (P-TEFb), as novel RAR coactivators. In addition to promoter sequences, RAR binds to genomic, transcribed regions of retinoid-regulated genes, in association with RNA polymerase II and as a function of P-TEFb activity. Knockdown of either AF9 or BRD4 expression affected differentially the neural differentiation of stem cell-like P19 cells. Clusters of retinoid-regulated genes were selectively dependent on BRD4 and/or AF9 expression, which correlated with RAR association to transcribed regions. Thus RAR establishes physical and functional links with components of the elongation complex, enabling the rapid retinoid-induced induction of genes required for neuronal differentiation. Our data thereby extends the previously known RAR interactome from classical transcriptional modulators to components of the elongation machinery, and unravel a functional role of RAR in transcriptional elongation.

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Introduction

Transcriptional activation by nuclear all-trans retinoic acid (atRA) receptors (RARs) stems from the concerted action of transcriptional coregulators whose role is to convert a repressive chromatin environment into an opened state, allowing the assembly of the transcription preinitiation complex (PIC). Chromatin opening and PIC assembly are the end result of ligand-induced conformational changes in the highly structured C-terminal activating function (AF)-2 domain of DNA-bound RARs, creating a protein-protein interaction interface that recognizes LXXLL-containing transcriptional coregulators. Distinct families of transcriptional coregulators are recruited to the RAR AF-2 in response to agonists, including the p160 family (SRC1, TIF2/GRIP1, AIB1/ACTR/pCIP), CBP/p300, which recruit or carry histone acetyltransferase activity, and the DRIP/TRAP/Mediator complex which controls the basal transcription initiation machinery [1].

The *Rarβ2* promoter is a paradigm for NR-mediated transactivation, and has provided considerable insights into RAR-controlled transcription. Detailed mechanistic studies using this promoter showed that RARα-driven transcription requires, in addition to the above mentioned transcriptional coregulators, protein complexes involved in DNA breakage and repair such as topoisomerase II, PARP-1 and PCNA [2–4] and appropriate post-translational modifications of corepressors [5]. Furthermore, histone H3 Serine10 (S10 H3) phosphorylation is concomitant to retinoid-induced *Rarβ2* activation [6]. This histone mark is known to favor the loading of the positive transcription elongation factor b (P-TEFb) on regulated promoters, which is further facilitated by BRD4/HUNK1, a bromodomain-containing transcription factor with high affinity for acetylated histones H3 and H4 and Mediator subunits [7–9]. Intriguingly, constitutively acetylated histones H3 and H4 reside at the *Rarβ2* promoter, favoring the permanent loading of RXR-RARα heterodimers onto the retinoic acid response element (RARE) located in this promoter [10]. In line

with the possible involvement of P-TEFb in *Rarβ2* promoter activation process, the kinase subunit of P-TEFb CDK9 associates to this promoter in a ligand-controlled manner [11]. Thus a functional role of P-TEFb in retinoid-induced activation of the *Rarβ2* promoter can be hypothesized on the basis of this physical colocalization.

Beside the ligand-regulated AF-2 region that encompasses the ligand binding domain (LBD), RARs harbor other functional domains such as the DNA binding domain (DBD) and the poorly characterized, unstructured, ligand-independent N-terminal AF-1 domain. Little is known about the exact roles of RARα domains outside of the LBD in transcriptional regulatory processes. In addition to its recognized role in direct protein-DNA interaction, the DBD interacts with transcription factors such as RXRs, c-jun, BLZF1, NF-IL6, myb and TEL [12]. Similarly, RARα AF-1 engages into intra-molecular interactions with RARα AF-2 to activate transcription, according to a mechanism involving the recruitment of TFIIH subunits cyclin H to AF-2, and of the kinase cdk7 to AF-1 [1]. We have therefore further investigated this question by purifying putative RARα coregulators able to interact with RARα domains distinct from the AF-2 domain. Mass spectrometry fingerprinting confirmed that RARα AF-1 interacts with the p62 subunit of TFIIH. More strikingly, this approach revealed that the two mutually exclusive P-TEFb interactants AF9/MLLT3 and BRD4/HUNK1 [13,14] bind to RARα in a ligand-independent manner, evidencing a physical connection between RAR and transcription elongation factors. AF9 and BRD4 played distinct roles in retinoid-induced transcription and neuronal differentiation as shown by microarray analysis of mRNAs from the mouse pluripotent cell line P19. We further show that RAR associates to transcribed regions of retinoid-regulated genes in an AF9 and BRD4-dependent manner, and as a function of P-TEFb activity.

Materials and Methods

Plasmids

Expression vectors for RAR and RXR, and reporter constructs have been described [15]. pSG5-RARα-ΔAF-1, and pcDNA3-AF-1 and pGFP-NLS-AF-1 were constructed by PCR amplification of the *Rarα* cDNA, followed by cloning in pSG5 (Stratagene, Santa Clara, CA), in pcDNA3 (Invitrogen, Carlsbad, CA) and in pEGFP-C1 (Clontech, Mountain View, CA) containing a nuclear translocation signal (NLS), respectively. cDNAs corresponding to the proteins identified by mass spectrometry were obtained as full length open reading frame clones from the Mammalian Gene Collection (N.I.H., Bethesda, MD) or from Origene (Rockville, MD). They were amplified by PCR and inserted in the pCRII vector by T/A cloning (Invitrogen). Cloned cDNAs were then inserted as EcoRI/XbaI or XhoI/XbaI fragments in pCMV-3×FLAG (Sigma, St Louis, MN) for expression in mammalian cells as a fusion with three FLAG epitope tags. The dominant-negative mutant of CDK9 (cdk9 D167N, pCMV cdk9-HA dn, [16] was a kind gift from A. Giordano (U.Penn., Philadelphia, USA). The *BRD4* cDNA fragment coding from residue 1 to 722 was cloned into pCMV-3xFLAG (Sigma). shRNA targeting *Af9* or *Brd4* mRNAs were cloned in pSIREN (Clontech) using the following oligonucleotides: *Brd4*: gatccgcctggagatgacatcgtcttattcaagagataagacgatgtcatctccaggtttttttctcgagg, *Af9*: gatccgtgagtgtgcaaagacccacctttcaagagaaaggtgggtctttgcacactctttttttagatctg and their complementary counterparts to generate ecotrophic retroviruses according to the manufacturer instructions. Complete sequences are available on request. All constructs were verified by automatic sequencing (MWG Gmbh, Ebersberg, Germany). Other expression vectors were purchased from GeneCopoeia (Rockville, Md, USA).

P19 Stable Clones Generation

Subconfluent P19 cells were transduced at MOI = 5 with lentiviral particles and further selected with puromycin. Individual clones were selected by the limited dilution method and characterized for the expression of either *Af9* or *Brd4*. Cell lines displaying a mRNA decreased expression above 80% were further characterized by western blotting. Initial experiments were carried out on two individual, independent subclones.

Transient Transfections

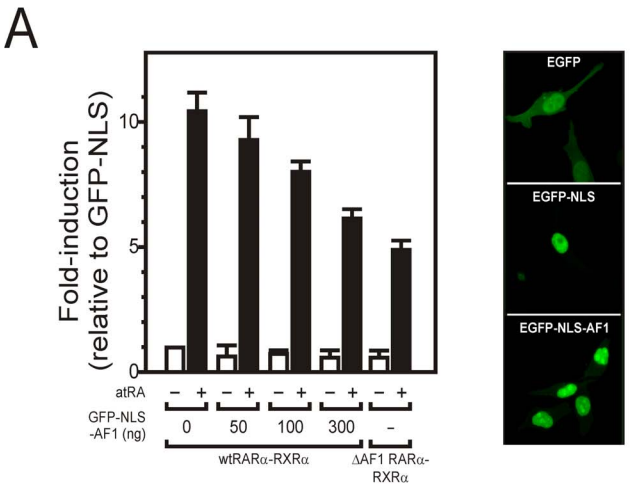
HeLa or P19 cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma). Transfections were carried out by the polyethyleneimine coprecipitation method with Exgen500 (Euromedex, Souffelweyersheim, FR) as described [11]. Twenty four hours after transfection, cells were treated overnight with 1 μM atRA and luciferase activity was quantified. Basal expression levels were arbitrarily set to 1 and data are expressed as the mean±SEM (n = 3–6). Luciferase assays were performed with the BrightGlo system (Promega, Madison, WI) and luciferase activity (as relative luciferase units, RLU) was measured with a Victor Light 1420 Luminescence counter (Perkin-Elmer, Waltham, MA).

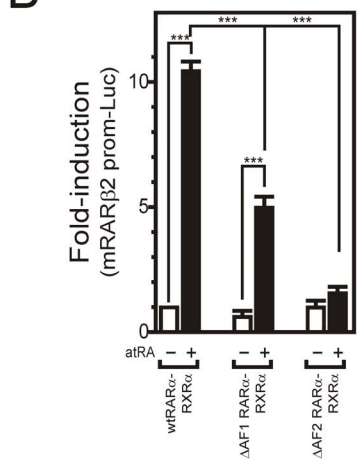
GST Pulldown Assays

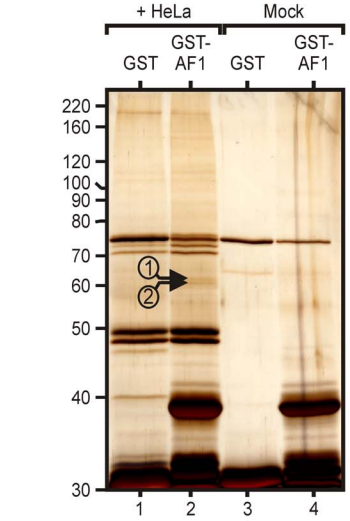
The following GST-RARα fusion proteins were used in these assays (Fig. 1E and Fig. S1A), with the numbers indicating the first and last amino acid of the RARα sequence: GST-hRARα (1–462); GST-AF-1 (1–92), GST-AF-1-DBD (1–158), GST-DBD (92–173); GST-DBD-LBD (92–462), GST-LBD (186–462). Glutathione S-transferase (GST) fusion protein expression and purification, and GST pulldown assays were performed according to Rachez et al. [17] with the following modifications. Immobilized GST fusion proteins were incubated in GST-binding buffer consisting of 20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 0.1% Nonidet P (NP)-40, 0.5 mM PMSF, 1 mM DTT, protease inhibitors (Sigma), containing 1 mg/mL BSA and 100 mM KCl. Immobilized proteins on beads (20 μg) were incubated at 4°C for 6–10 hours with 2–6 mg of HeLa nuclear extract; or 2 μg proteins on beads were incubated at 4°C for 4 hours in the presence or absence of atRA (Sigma) with proteins synthesized by *in vitro* translation (TNT-coupled reticulocyte lysate, Promega) with ^{35}S-methionine (GE-Healthcare, Waukesha, WI). After three washes in GST-binding buffer supplemented with 150 mM KCl and 0.3% NP-40, samples were resolved by SDS-PAGE, and detected either by silver nitrate staining or autoradiography followed by quantification with a Storm 860 phosphorimager (GE-Molecular Dynamics).

Immunoprecipitation and Immunoblotting

Whole cell extracts from 10^5 transfected HeLa cells were prepared in 0.25 mL lysis buffer [10 mM Tris·HCl, pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, protease inhibitors (Sigma) and 0.1 U DNaseI (Sigma)]. After centrifugation, cleared extracts were diluted 5 times in lysis buffer without NP-40 and incubated at 4°C for 3 hours with 80 μL anti-FLAG M2 affinity resin (Sigma), or with the anti-RARα monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) immobilized onto protein A sepharose (GE-Amersham) pre-equilibrated in lysis buffer. After four washes with lysis buffer adjusted to 0.15% NP-40, samples were resolved by SDS-PAGE and detected by western blot with appropriate primary antibodies, followed by incubation







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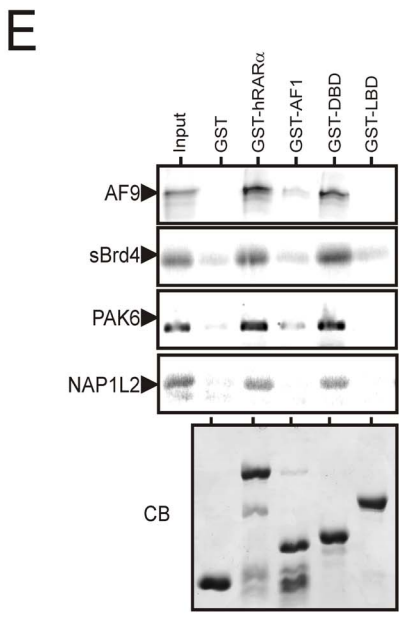
Protein names	Abbreviation	Protein ID	Band (% coverage)	Predicted molecular mass (kDa)
Serine/threonine kinase PAK6; p21-activated kinase	PAK6	Q9NQU5	1,2 (30%, 28%)	75
ALL1-fused gene from chromosome 9	AF9/MLLT3	P42568	1,2 (32%, 53%)	63
Nucleosome assembly protein 1-like 2	NAP1L2	Q9ULW6	2 (35%)	53
TFIIH complex p62 subunit	BTF2/TFIIH p62	P32780	1 (40%)	62
Bromodomain-containing protein 4	Hunk1/Brd4	NP_055114.1	1 (28%)	81
Retinoic acid receptor gamma	RARγ	P13631/ P22932	1 (55%)	50



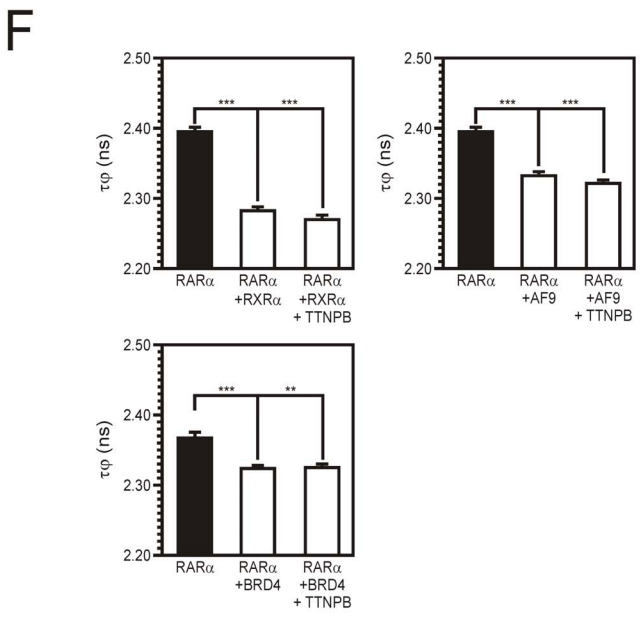


Figure 1. The N-terminus of RARα interacts with nuclear proteins. (A) A nucleus-targeted RARα AF-1 domain acts as a dominant negative receptor. HeLa cells were cotransfected with expression vectors coding for wild type (wt) RXRα, wtRARα, GFP-NLS and GFP-NLS-AF-1 at the indicated ratio together with a m*Rarβ2* promoter-driven reporter gene (mRARβ2-Luc). Cells were treated overnight with 1 μM atRA and luciferase activity was quantified. Basal expression levels were arbitrarily set to 1 and data are expressed as the mean±SEM (n = 3). *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$. (Right panel) Confocal laser microscopy of transfected HeLa cells. (B) The RARα AF-1 domain is transcriptionally active. HeLa cells were transfected with mRARβ2-Luc and expression vectors coding for wtRXRα, wtRARα, N–terminally ΔAF-1-RARα) or C-terminally truncated ΔAF-2-RARα) RARα. Cell treatment, luciferase assays and calculations are as in (A). (C, D) Isolation and identification of proteins interacting with the AF-1 transactivation motif of RARα. AF-1 fused to GST (GST-AF-1) or GST alone (GST) were immobilized on a matrix and incubated with HeLa nuclear extracts (+HeLa) or buffer alone (Mock). Numbers indicate bands that were subjected to mass spectrometry analysis. (D) The table indicates the name, protein abbreviation, the UniProtKB/TrEMBL entry, percentage of peptide coverage in two representative purifications, and the predicted molecular mass. (E) Target validation by GST pulldown assays. Various domains of RARα were expressed as fusion proteins to GST (left panel) and used as baits for ^{35}S-labeled shortBRD4 (sBRD4), AF9, PAK6 and NAP1L2. CB: Coomassie Blue staining of RAR derivatives adsorbed on glutathione-Sepharose. (F) Interaction of RARα with BRD4 or AF9. FLIM-based FRET fluorescence assays were performed to determine the lifetime of the donor (GFP) in the indicated conditions.
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with HRP-coupled secondary antibodies and detection with ECL+ (GE-Amersham).

Mass Spectrometric Analyses

Protein samples resolved by SDS-PAGE were detected by colloidal blue staining [17]. Bands were digested with trypsin and analyzed by Matrix-assisted laser desorption/ionization time-of-flight (Maldi-TOF) mass spectrometry. The SwissProt database was interrogated through the MS-Digest Search program [18].

Spot excision and in gel digestion. Protein spots were excised semi-automatically from 1D gels using the "Click and pick" mode of the Ettan Spot Picker (GE-Amersham Biosciences). Approximately ten plugs were excised from each band and put in the same well. In-gel digestion was performed using an automated protein digestion system (Ettan Digester; GE-Amersham Biosciences). Briefly, plugs were washed three times with 50 mM ammonium bicarbonate containing 50% methanol, then 50% acetonitrile containing 0.1% TFA. After two washing steps in 70% acetonitrile, plugs were dried and rehydrated with 10 μL of 20 mM ammonium bicarbonate containing 4 μg/mL trypsin (Promega) for 4 hours. Finally, a 20 mM ammonium bicarbonate solution was added overnight. The peptide mixture were then dried, and resuspended in 10 μL 0.1% TFA before MALDI-MS analysis.

Acquisition of mass spectrometric peptide maps, MALDI-TOF MS and database search and analysis. 1 μL of peptide mixture was mixed with 1 μL of DHB matrix solution (10 mg dihydrobenzoic acid in 50% methanol) on the MALDI target. MALDI-TOF MS was performed using a Voyager DE STR mass spectrometer (PerSeptive Biosystems) equipped with a 337.1 nm nitrogen laser and the delayed extraction facility. All spectra were acquired in a positive ion reflector mode. Typically, 200 laser shots were recorded per sample, and spectra were internally calibrated (using the DataExplorerTM software, PerSeptive Biosystems) using three peptides arising from trypsin autoproteolysis ([M+H]+842,5100; [M+H]+1045.5642; [M+H]+2211.1046). Tryptic monoisotopic peptide masses were searched for in the NCBI, using Protein Prospector (http://prospector.ucsf.edu/) with a mass tolerance setting of 50 ppm, with three missed cleavage sites as fixed parameters, and with carbamidomethylation and methionine oxidation as variable modifications. The database search identified multiple proteins in each band. However, proteins to be further tested were chosen among the possible candidates by comparing the relative abundance of the different peptides, the percentage of recovery of each protein and taking into account the full size of the proteins. MS analysis was carried out 4 times on independent samples and selected peptides were detected at least twice out of 4 analysis, but in most cases they were detected 3 or 4 times out of 4 analysis. Proteins of interest were selected from this type of analysis if peptide coverage was above 20%. Specific samples were re-analyzed and provided enhanced spectra of protein digests which resulted in significantly increased sequence coverage (above 35%) and confidence in protein identification.

RT-QPCR

RNAs were extracted as described in [11]. RT-QPCR analysis was carried out as described in [19]. When indicated, Taqman assayson demand (Life Technologies, Foster City, CA) where used: RARβ2 Mm01319674_m1 and Mm01319678_m15′; cdx1, Mm00438172_m1*and m00438173_gH; Hoxa1 Mm04208064_g1* and Mm00439359_m1; Stra8 Mm01165138_m1 and Mm00486473_m1*; HoxB4 Mm00657964_m1 and Mm01307004_mH.

Microarray Analysis of mRNAs

Microrray hybridization and scanning were carried out following the manufacturer instruction (Agilent, One-color Microrray Gene Expression Analysis) using mouse SurePrint 8*60K arrays. Data processing and analysis were performed as described in [20] using the Genespring 12.0 software (Agilent). Briefly, array integrity was visually inspected, and quality controls were performed based on PCA analysis. Data were filtered to exclude signals in the low 20% and averaged. A gene-level analysis was performed and data filtered and graphed as detailed in the legend to figures. Data were deposited on the ArrayExpress web site with the accession number E-MEXP-3669.

Chromatin Immunoprecipitation Assays

ChIP assays were carried out in duplicate as described in [11] using antibodies from Abcam (Cambridge, UK; AFF4, ab57077), Santa Cruz [TFIIH, sc293; cdk9, sc8339; cdk8, sc1521; RARα, sc551; RXRs, sc774; RNApol II, sc899; AF9(D17), sc32369; AF9(L15); sc32371; Brd4(H250), sc48772], Bethyl Laboratories (Montgomery, TX; AF9, A300-595A, A300-596A, A300-597A), Active Motif (Carlsbad, CA; Brd4, #39909). Bethyl antibodies were used as a 1:1:1 (vol:vol) mix in ChIP reactions; Brd4 was immunoprecipitated with a v:v mix of Santa Cruz and Active Motif antibodies. Results were acquired and quantified as described in [21] and [22].

FRET Assays

Human RARα was cloned into pEGFP-C1 (Clontech, Palo Alto, CA, USA) to be expressed as a C-terminal EGFP-fusion protein. hRXRα, AF9 and Brd4 were cloned into the pReceiver-M55 backbone (Genecopoeia, Rockville, Md, USA) to generate mCherry-fusion proteins. Initial experiments showed that N-terminally fused mCherry proteins, but not C-terminally fused proteins, were suitable for FRET experiments together with the EGFP-RAR. HeLa cells were plated on 32 mm diameter glass

coverslips 12 h before transfection with the FuGENE HD reagent (Roche Diagnostics, Rotkreuz, SW) according to the manufacturer's recommendations. For FLIM-FRET imaging, the glass coverslips were deposited into a POC (Perfusion, Open and Close) chamber and the culture medium was replaced by Leibovitz's 15 medium (L-15, Invitrogen). The frequency-domain FLIM measurements have been previously described in Leray et al [23]. The FLIM microscope is composed of a LIFA system (Lambert Instruments, Roden, The Netherlands) implemented on a spinning-disk confocal system (Yokogawa CSU-X1, Tokyo, Japan) adapted on a Leica (Lognes, FR) DMI6000B inverted microscope and equipped with a diode laser source emitting at a wavelength of 488 nm and whose intensity is modulated at 40 Mhz. Cells were imaged using a 63× oil-immersion objective (Leica HCX Plan Apo NA 1.4). Fluorescence emitted was then successively routed by a dichroic mirror (Semrock Di01-T405/488/568/647), spectrally filtered (Chroma, HQ545/30×) and detected with an intensified CCD camera (Li^2CAM, Lambert Instruments) modulated at the same frequency (40 MHz) and coupled to an optical zoom (×2). The phase fluorescence lifetime for each sample was calculated from the acquisition of 36 phase-shift images using the LI-FLIM software (Lambert Instruments), after calibrating the system with a reference fluorescein solution at 1 μM of known lifetime (4 ns). For each set of acquisition, means and standard error of the means (S.E.M.) were determined from at least 8 samples per condition using the GraphPad Prism v5.0 software (San Diego, CA).

Statistical Analysis

Values are reported as the mean±SEM, with 3–6 biological replicates (gene expression data). The statistical significance of differences amongst groups were determined using either a Student t-test (2 group) or ANOVA followed by a post-hoc test (Tukey) using GraphPad Prism v5.0. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

Results

RARα Interacts with Components of the SEC Complex

The AF-1 domain of RARα functions as an autonomous transactivation domain, as shown by squelching experiments in Hela cells using overexpressed, nuclear-targeted GFP fused to RARα AF-1 (Fig. 1A) and the defective transcriptional activity of RARα deleted from its N-terminal AF-1 domain (ΔAF-1-RARα, Fig. 1B), as previously reported in COS cells [24]. We thus identified novel RARα transcriptional coregulators using mass (MALDI-TOF) fingerprinting of HeLa nuclear proteins binding to the isolated RARα AF-1 domain (Fig. 1C and 1D). The p62 subunit of TFIIH (BTF2), known to interact physically and functionally with RAR AF-1 [25,26] was isolated, thus validating our experimental strategy. Isolated proteins interacted with RARα in a ligand-independent manner, and included the novel potential coregulators PAK6 (p21-activated kinase-6), AF9/MLLT3 (ALL1 fused gene from chromosome 9), NAP1L2 (nucleosome associated protein 1-like 2) and the short isoform of BRD4 (70–80 kDa, sBRD4). Although full-length Brd4 has been described in HeLa cells as occurring as a 180–200 kDa polypeptide [27], all of the identified peptide sequences mapped within the sequence of sBRD4, in agreement with a number of reports describing BRD4 as a lower molecular mass polypeptide of ca. 70–80 kDa [28,29].

Unexpectedly, we also identified RARγ as an AF-1 interactant. This suggested that RAR can homodimerize through this region and the LBD, a hypothesis which was confirmed by GST pulldown assays using labeled RARα deletion mutants and GST-hRARα AF-1. This assay indeed revealed that the RAR AF-1 interacts mostly with the LBD, hinting at inter-domain interactions (Fig. S1A). Therefore, our GST affinity matrix was constituted not only of the isolated AF-1, but also included full-length RARγ, and thus might have adsorbed proteins interacting, in a ligand-independent manner, not only with the isolated AF-1, but also with full length RARγ. Detected interactions were therefore validated using either the full length RARα or various RARα deletion mutants fused to GST as baits (Fig. 1E). All proteins interacted with full length RARα and showed a strong propensity to associate to the isolated RAR DBD. Preliminary experiments ruled out a possible contribution of the hinge region to the observed interactions (CR and PL, unpublished observations). PAK6, already described as an androgen and estrogen receptor corepressor [30,31], and TFIIH were not considered further. Thus only AF9 interacted detectably with the isolated AF-1 domain, as shown by GST pulldowns and coimmunoprecipitation assays (Fig. 1E and Fig. S1B). AF9 and BRD4 were thus chosen for further study, as both potentiated RARα transcriptional activity without interacting with the RARα LBD (Fig. 1E), in contrast to NAP1L2, which, in addition, was inactive in the transcription assay (Fig. S1C). Molecular interactions were further assessed by fluorescence resonance energy transfer (FRET) in living Hela cells. The use of N-terminally tagged proteins (eGFP for RARα, mCherry for RXRα, AF9 and Brd4) showed a significant decrease of the donor lifetime (eGFP) coupled to RARα in the presence of the known RAR dimerization partner acceptor RXRα. Similarly, both AF9 and Brd4 induced a decrease of the donor lifetime, showing clearly that these proteins interact closely with RARα. Interactions were not modified upon treatment with a synthetic pan-RAR agonist (TTNPB), confirming that these interactions are ligand-independent (Fig. 1F).

RARα-mediated Transcription is P-TEFB-dependent

P19 EC cells (noted $P19^{wt}$ thereafter) are stem cell-like, pluripotent cells differentiating into neurons and glial cells upon atRA treatment [32]. In this cell line, as well as in other mouse EC cell lines, the *Rarβ2* promoter is the archetypical atRA-regulated promoter, harboring a consensus DR5 retinoic acid response element (RARE) and potential cis-acting motifs for COUP-TFI/NR2F1 and CREB (Fig. 2A). atRA, but not overexpressed COUP-TFI or cAMP, induced *Rarβ2* expression in P19 cells (Fig. 2B), showing that the DR5 RARE is a major functional cis-acting element. As expected, the mRNA expression of the pluripotency marker *Pou5f1/Oct4* was downregulated in response to atRA, whereas that of control genes *Top2β*, *Tcf19* and *Rplp0* was unaffected (Fig. 2B). In these conditions, a ligand-dependent binding of RARα to the negative RARE from the *Pou5f1* promoter [33] was detected by chromatin immunoprecipitation (ChIP) assays, performed with a specific anti-RARα antibody [11,34] (Fig. 2C). RARα binding was concomitant to decreased RNA polymerase II (RNApol II) C-terminal domain (CTD) phosphorylation (S5P-RNApol II), a post-translational modification associated with promoter clearance and increased transcription. In similar conditions, S5P-RNApol II, but not RARα, associated to the retinoid-insensitive *Rplp0* ribosomal gene promoter (Fig. 2C). Thus the atRA-induced activity of the *Rarβ2* promoter depends mostly, if not exclusively, on RAR-driven events.

ChIP assays showed that RARα was constantly bound to the *Rarβ2* promoter, which harbored high levels of acetylated H3 histone irrespective of the presence of ligand (Fig. 2D and [6]), in agreement with the facilitating effect of histone acetylation on the binding of RAR-RXR heterodimers to nucleosomal DNA [10]. In contrast, a decreased density in the P-TEFb kinase subunit CDK9

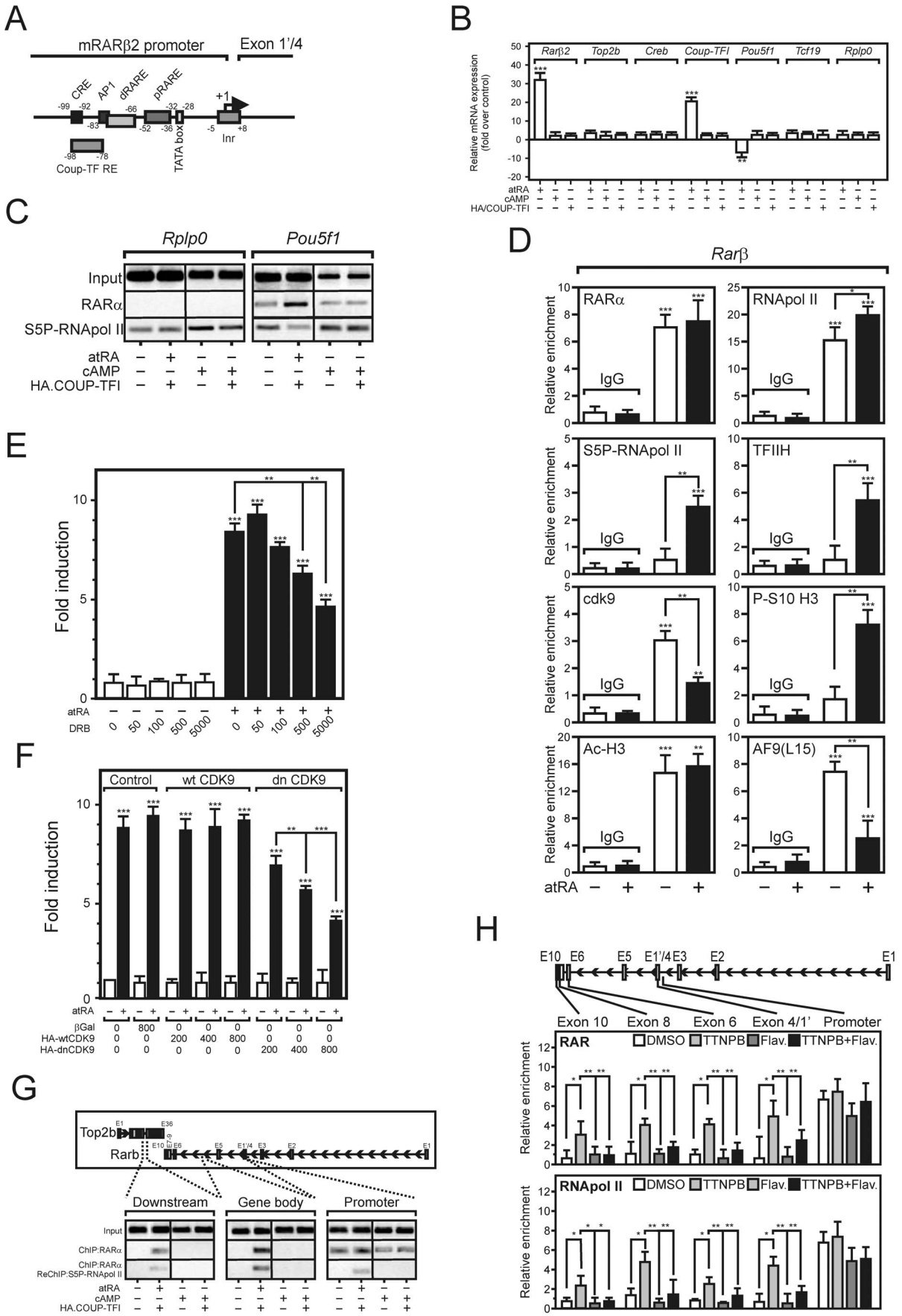

Figure 2. RAR localizes to transcribed regions of the Rarβ2 gene in a P-TEFB-dependent manner. (A) Structure of the mouse *Rarβ2* promoter. pRARE: proximal RARE; dRARE: distal RARE. (B) Gene expression in P19 cells. P19 cells were treated for 48 hours with DMSO, 1 μM all trans RA (atRA), 250 μM cAMP or transfected with a HA-tag COUP-TFI expression vector. Expression of *Rarβ2, Top2β*, *Creb*, *Coup-TFI*, *Tcf19* and *Rplp0* were quantified by RT-QPCR. Basal expression levels were arbitrarily set to 1 and data are expressed as the mean±SEM (n = 3). *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$. (C) RARα and phosphorylated RNApol II loading at the *Rplp0* and *Pou5f1/Oct4* promoters. P19 cells were treated as in (B) and ChIP assays were performed with indicated antibodies. (D) AF9 colocalizes to the *Rarβ2* promoter. P19 cells were treated for 4 hours with 1 μM atRA, and ChIP assays were carried out as described. The specific enrichment in *Rarβ2* promoter sequence is expressed after normalization to background values (*Myoglobin* gene). Data are expressed as the mean±SEM (n = 2). *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$. (E) DRB inhibition of the *Rarβ2* gene transcription. P19 cells were treated with the indicated combination of atRA (1 μM) and varying doses of DRB (50 to 5000 nM) for 4 hours. *Rarβ2* mRNA was quantified by RT-QPCR. (F) A CDK9 dominant negative mutant inhibits *Rarβ2* gene expression. P19 were cotransfected with increasing amount of pCMV-lacZ (control), pCMV-HA-wtCDK9, or pCMV-HA-dnCDK9 expression vectors at the indicated ratio, then treated 24 hours with 1 μM atRA. Gene expression was quantified as above and data expressed as the mean±SEM (n = 4). *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$. (G) RAR and phosphorylated RNApol II are detected at transcribed regions of the *Rarβ2* gene. P19 cells were treated as in (D) and ChIP/reChIP assays were performed. (H) P-TEFb inhibition prevents RAR association to *Rarβ2* elongated regions. P19 cells were treated for 4 hours with the indicated combination of TTNPB (1 μM) or flavopiridol (250 nM). ChIP assays (n = 2) were performed as in (D).
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and AF9 (Fig. 2D), known to associate to P-TEFb [35], was observed. Increased densities of phosphorylated histone H3 serine10 (P-S10 H3), of total (RNApol II) and of S5P-RNApol II were concomitant to *Rarβ2* activation, in agreement with the increased loading of the RNApol II kinase TFIIH (Fig. 2D and [11]).

P-TEFb catalyzes the phosphorylation of RNApol II on Ser2 and of the negative elongation factors NELF and DSIF [DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity-inducing factor] to promote the release of paused RNApol II [36]. DRB inhibited atRA-induced accumulation of *Rarβ2* mRNA in $P19^{wt}$ with an $EC_{50}\approx5$ μM, suggesting that CDK9 activity is necessary for *Rarβ2* promoter activation (Fig. 2E). Similarly, increasing amount of a dominant-negative (dn) mutant of CDK9 (CDK9 D167N) blunted *Rarβ2* promoter activation (Fig. 2F). Thus the sensitivity of the *Rarβ2* promoter to CDK9 inhibition reflects the association of this chromatinized cis-acting sequence to the P-TEFb kinase CDK9 (Fig. 2D).

Involvement of P-TEFB in Retinoid-mediated Transcription

The interaction of RARα with P-TEFb interactants raised the possibility that it could associate with elongating complexes. RARα association with various intronic or exonic regions of the *Rarβ2* locus was monitored by ChIP assays in P19 cells (Fig. 2G and 2H). RARα association with an intronic region occurred only after atRA treatment, and could also be observed, albeit to a much lower extent, downstream of the transcription termination site which mapped to the retinoid-insensitive 5′-flanking *Top2β* gene (Fig. 2G). ChIP-ReChIP assays demonstrated a colocalized, ligand-dependent accumulation of RAR and S5P-RNApol II on these transcribed regions (Fig. 2G). Thus RAR colocalizes with elongating RNApol II. To evaluate whether P-TEFb activity affects RAR association to transcribed regions of the *Rarβ2* gene, $P19^{wt}$ were treated with TTNPB with or without the CDK9 inhibitor flavopiridol (Fig. 2H). While flavopiridol did not perturb RAR recruitment to the *Rarβ2* promoter and tended to decrease RNApol II density at this location, it clearly decreased the atRA-induced accumulation of RNApol II and of RAR at several exonic regions. Thus P-TEFb activity is required for RAR association to *Rarβ2* transcribed regions. This observation was unexpected in the light of a previous report showing that RAR is absent from exon 6 of the *Rarβ2* gene in atRA-treated HeLa cells [37]. Although BRD4 or AF9 are readily expressed in these fibroblastic cells, the high level of sequestration of P-TEFb in an inactive from in this rapidly proliferative cell line may explain this discrepancy [38]. Moreover, RAR displays a distinct binding kinetics with the RARβ2 RARE, which, in non-differentiating HeLa cells, cycles over time with a period of 4 hours [37] but decreases over time in EC F9 cells [39], thus pointing to distinct cell-specific transcriptional activation mechanisms.

Functional Relevance of AF9 and BRD4/RARα Interaction in Retinoid-regulated Transcription

AF9 overexpression strongly potentiated the basal expression level of the *Rarβ2* gene without affecting its ligand responsiveness in $P19^{wt}$ (Fig. 3A), in agreement with its ligand-independent interaction with RARα (Fig. 1). The short isoform of BRD4 (sBrd4) exerted a significant effect on retinoid-induced RAR activity, whereas the full length BRD4 (lBrd4) was mostly active on the basal expression of the *Rarβ2* gene (Fig. 3B). Conversely, shRNA-mediated knockdown of *Af9* or of *Brd4* expression strongly blunted *Rarβ2* expression in P19 subclones [noted $P19^{Af9(-)}$ and $P19^{Brd4(-)}$, (Fig. 3C)]. To assess whether BRD4 and/or AF9 could play a role in transcription elongation, we assayed the abundance of *Rarβ2* mRNA using exon-specific PCR primer sets (Fig. 3D). After a 4 hour-induction, $P19^{wt}$ accumulated about half the amount of full length transcript when compared to 5′ transcripts, suggesting a fast initiation process but a poor processivity. Amplification of exon sequences showed that the loss of processivity occurred between exon 6 and exon 7 of the *Rarβ* gene, which may relate to yet undefined particular chromatin structures. We noted that the loss of processivity was not significant in Brd4-depleted cells. In contrast, $P19^{Af9(-)}$ and $P19^{Brd4(-)}$ accumulated less 3′-truncated transcripts (or produced less abortive 5′ transcripts), suggesting that these AF9 and BRD4 act mainly on initiation or early elongation events.

Transcription Elongation Factors and RAR Associate to the *Rarβ2* Gene Body

P-TEFb has been shown to bind to SEC, which contains the core component AFF4 and the coactivating AF9, or to BRD4 [38,40]. TFIIH also participates in transcriptional elongation [41]. The recruitment of these factors to the *Rarβ2* locus, as well as that of RAR and RXR, was thus monitored after retinoid treatment (Fig. 3E). In $P19^{wt}$, RNApol II was found within transcribed regions and at the promoter like the TFIIH subunit cdk7, whereas AFF4 density increased within the gene body. AF9 associated preferentially to the promoter region but was detected at significant levels at exonic regions, and BRD4 accumulated at the 3′ end of the gene. RAR was detected both at the promoter and exonic regions, whereas its heterodimeric partner RXR was surprisingly detected only at the promoter. $P19^{Brd4(-)}$displayed a decreased RNApol II density throughout

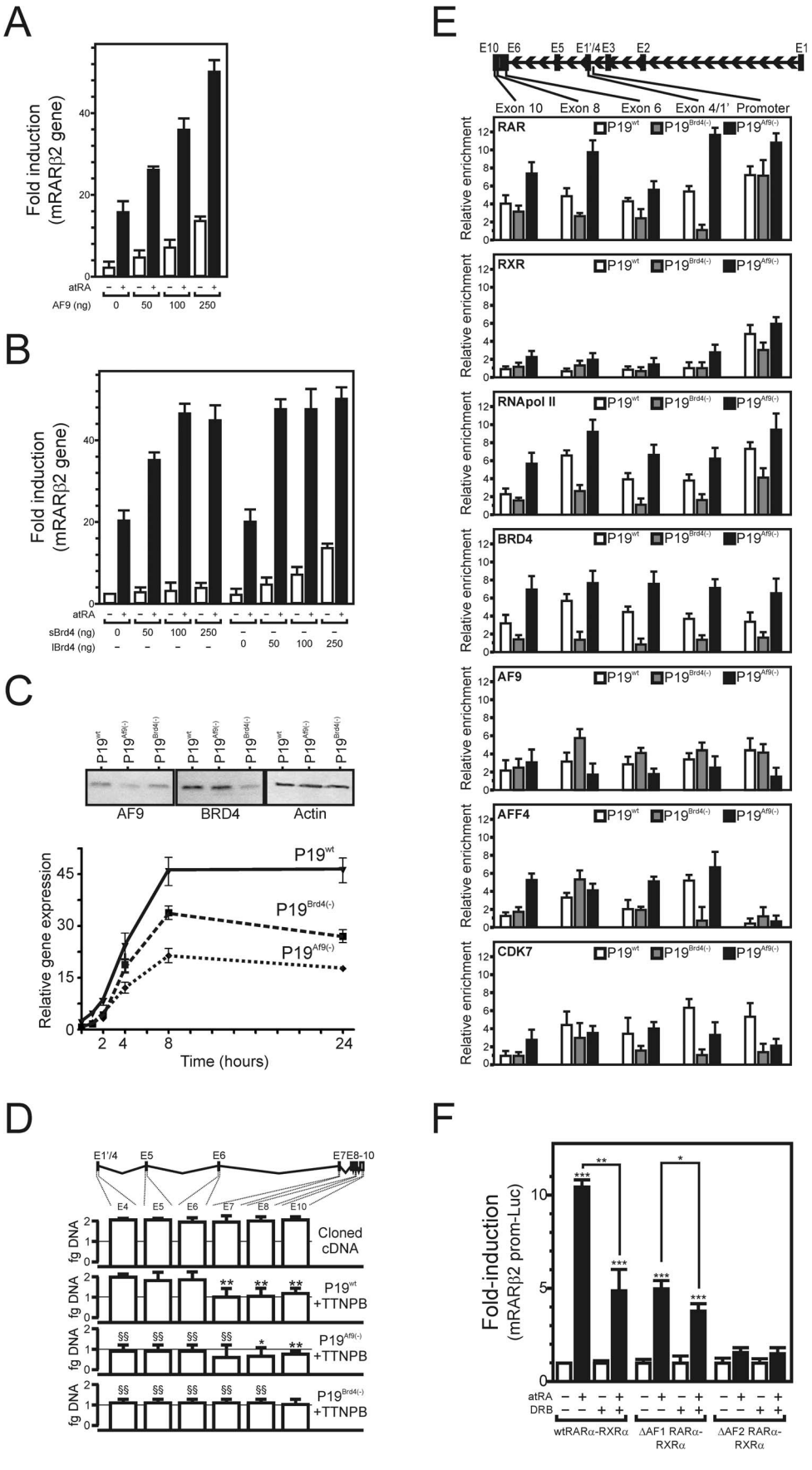

Figure 3. AF9 and BRD4 coactivate RARα in a ligand-independent manner. (A, B) P19 cells were transfected with the indicated amounts of AF9, sBRD4 or lBRD4 expression vectors for 24 hours with or without 1 μM atRA and *Rarβ2* gene expression was assayed by RT-QPCR. The basal expression level in non transfected, untreated cells was arbitrarily set to 1 and data were expressed as the mean±SEM (n = 5). *, p<0.05; **, p<0.01; ***, p<0.005. (C) *Af9* or *Brd4* knockdowns. (C, upper panel) AF9 or BRD4 expression was assayed by western blot analysis in P19wt, $P19^{Af9(-)}$ and $P19^{Brd4(-)}$. (C, lower panel) *Rarβ2* gene expression in AF9- or BRD4-depleted P19 cells. The time-dependent expression of *Rarβ2* upon stimulation with 1 μM TTNPB was quantified by RT-QPCR. (D) Exon-specific RT-QPCR assay of the *Rarβ2* mRNA. Cloned *mRarβ2* cDNA was used as a standard in PCR reaction, and used to select PCR primer sets displaying a similar efficiency (''Cloned cDNA''). *Rarβ2* mRNA from either $P19^{wt}$, $P19^{Af9(-)}$ or $P19^{Brd4(-)}$ was then formally quantified by Q-PCR. **, p<0.01, intra-sample comparison; §§, p<0.01, inter-sample comparison. (E) RAR associates to *Rarβ2* transcribed regions as a function of AF9 and BRD4 levels. $P19^{wt}$, $P19^{Af9(-)}$ or $P19^{Brd4(-)}$ were treated with 1 μM TTNPB for 1 hour and ChIP assays were performed. The specific enrichment in the different *Rarβ2* amplicons was assayed by Q-PCR and expressed normalized to background values (myoglobin gene). Data are expressed as the mean±SEM (n = 2). *, p<0.05; **, p<0.01; ***, p<0.005. (F) The AF-1 region of RAR confers DRB sensitivity to RA-induced transcription of the *Rarβ2* promoter. P19 cells were cotransfected as indicated with expression vectors coding for wtRXRα, wtRARα or ΔAF-1-RARα or ΔAF-2-RARα together with the mRARβ2-Luc reporter gene. Cells were treated 24 hours with 1 μM atRA and/or DRB and luciferase activity was quantified. Basal expression levels were arbitrarily set to 1 and data are expressed as the mean±SEM (n = 6). *, p<0.05; **, p<0.01; ***, p<0.005.
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the *Rarβ2* locus, in agreement with the observed diminished *Rarβ2* mRNA synthesis. AFF4 and AF9 densities were barely affected, however cdk7 association was decreased. *Brd4* knockdown also impacted negatively on RAR loading in elongated regions. In contrast, *Af9* silencing globally increased RNApol II, BRD4, RAR and, to a lower extent, AFF4 loading. This suggested that AF9 exerts a negative effect on BRD4 recruitment, and that the observed decreased transcriptional activity of *Rarβ2* upon AF9 depletion may correlate with decreased RNApol II processivity. Importantly, the cdk9 inhibitor DRB significantly blunted the transcriptional activity of full length RARα in the presence of atRA, but was much less efficient on the transcriptional activity of the N-terminally truncated RARα (ΔAF-1-RARα, Fig. 3F), suggesting that AF-1 integrity is required to confer P-TEFb dependency to RAR-mediated transcription.

AF9 and BRD4 Regulate Distinct Retinoid-regulated Gene Clusters

Retinoids trigger neuronal differentiation of P19 cells by regulating a network of RAR-driven genes. Indeed, 188 and 66 genes were up- or down-regulated respectively by more than 2-fold after a 4 hour-treatment of P19 embryoid bodies with atRA (Fig. S2A and Table S2). Functional annotation of these genes identified clusters of genes involved in embryo development and patterning (Fig. S2B), showing that P19 cells recapitulate initial transcriptional events leading to neuronal differentiation. For example, genes from the *Hox* cluster were significantly upregulated, as well as *Cyp26a1*, involved in the catabolism of RA.

We first asked whether BRD4 or AF9 played a role in controlling gene basal expression levels. While 31,948 genes were significantly expressed in $P19^{wt}$, only 397 or 459 genes were up- or down-regulated by more than 5-fold in untreated $P19^{Brd4(-)}$ or $P19^{Af9(-)}$, respectively (Fig. S3). Among these 2 sets of genes, *Hoxa4* was the only known retinoid-target gene to be down-regulated in both cellular backgrounds (Table S2), showing that these two elongation factors do not contribute to basal transcription of retinoid-regulated genes.

Gene expression levels were determined after a 4-hour treatment of $P19^{wt}$ with the RAR-specific agonist TTNPB. The gene program induced by this synthetic ligand was very similar to that induced by atRA (91% overlap, Fig. S4 and Table S3). Gene expression levels in stimulated conditions (TTNPB, 4 hours) were calculated for each cellular background and expressed as fold-change over basal levels in $P19^{wt}$ set to 1 for each gene (Fig. 4A and 4B). While examination of the gene expression pattern (Fig. S5A) showed a limited perturbation of the $P19^{wt}$ transcriptome, a gene-by-gene analysis revealed that genes involved in cellular differentiation displayed a differential sensitivity to BRD4 or AF9 levels (Fig. 4A). Importantly, many known atRA-target genes were selectively affected by AF9 or BRD4 silencing (Fig. 4B), pointing at distinct functions of these two elongation factors in atRA-induced transcription. This conclusion was validated by the fact that AF9 depletion did not affect P19 cell differentiation, as assayed by the increased expression of the 160kDa neurofilament, while *Brd4* knockdown blocked this process (Fig. S5B).

BRD4 has been recently described as having a limited contribution to the rapid induction of a subset of retinoid-regulated genes, in contrast to SEC which had a broad impact on this process in mouse ES cells [35]. We thus investigated whether BRD4 or/and AF9 could contribute directly to the rapid induction of RAR-controlled genes in P19 cells. Genes displaying a maximal expression (FC>2) after a 60 min.(cluster I, Fig. 5), 120 min. (cluster II, Fig. 5) or 240 min. (cluster III, Fig. 5) treatment with TTNPB were identified in $P19^{wt}$ cells. The expression of these clusters was monitored in $P19^{Brd4(-)}$ or $P19^{Af9(-)}$ to show that early-induced genes (clusters I and II) were more sensitive to *Brd4* or *Af9* knockdown than genes from cluster III. Thus both BRD4 and AF9 affect differentially atRA-regulated gene expression in our system, and favor the rapid induction of a limited set of genes.

AF9- and BRD4-independent Genes Do Not Recruit RAR to Promoters and Transcribed Regions

A close examination of the gene expression pattern by fold expression over time (Fig. 4D) also showed that some retinoid-regulated genes were insensitive to BRD4 or AF9 depletion. Gene expression data were therefore organized in 4 clusters of genes maintaining, or not, a significant induction rate (more than 50% of the gene activity in the wild type background) upon TTNPB treatment (4 hours, Fig. 6, top panel) in $P19^{Brd4(-)}$ or $P19^{Af9(-)}$ backgrounds. Four clusters were defined on the basis of gene induction sensitivity to BRD4 or AF9 depletion (AF9 and BRD4-independent, cluster A; BRD4-dependent and AF9-independent, cluster B; AF9-dependent and BRD4-independent, cluster C; AF9- and BRD4-dependent, cluster D). Three genes within each cluster harboring a RAR binding site within 30 kb of the gene boundaries as documented in mouse ES cells (Table S1, [42]) were selected for further characterization. The recruitment of RAR and of RNApol II was monitored by ChIP-PCR assays, due to the length of selected sequences, in TTNPB-treated $P19^{wt}$ cells at either a control upstream region devoid of potential RARE (UR), at the identified RAR DNA binding site (RAR BS), at the transcription start site (TSS) or within an exon without potential RARE (Exon, Fig. 6). Control UR associated neither to RAR nor to RNApol II in these conditions, while RAR was consistently

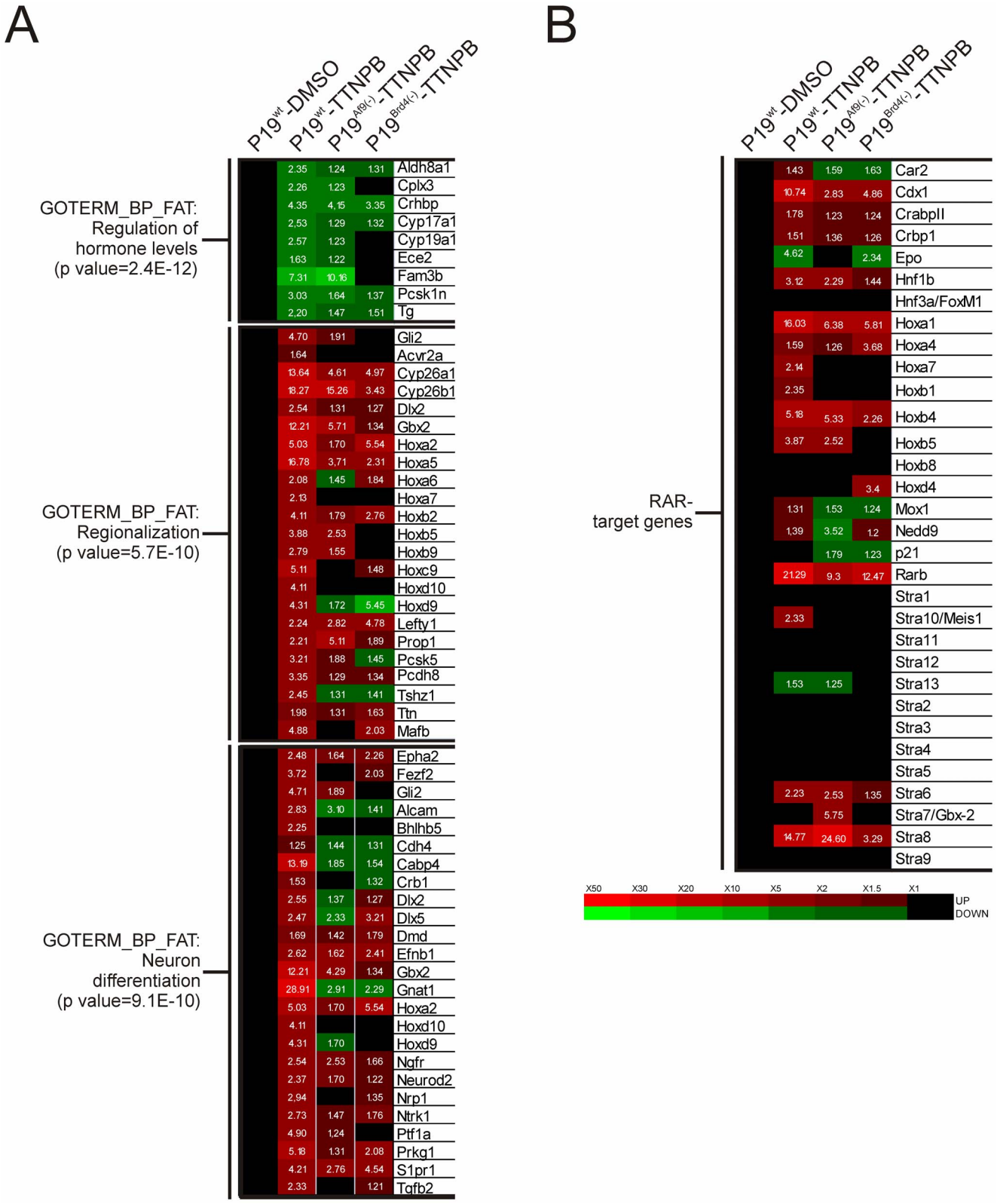

Figure 4. Gene expression level in response to RARα activation in wild type, AF9- and BRD4-deficient backgrounds. (A) Genes exhibiting a fold-change above 1.2 fold in TTNPB-treated P19^{wt} cells were clusterized according to a functional gene ontology classification. Representative functional clusters from the top 10 hits are shown. The basal level in non treated P19^{wt} was arbitrarily set to 1 and is depicted by black boxes. Numbers indicate the fold change ratio of individual genes relative to untreated wt P19 (red: upregulation; green: downregulation; black, no change). (B) Gene expression levels of known atRA-target genes. RA-target genes were selected from the literature and their expression levels were extracted from microarray data. Results are represented as in (A).
doi:10.1371/journal.pone.0064880.g004

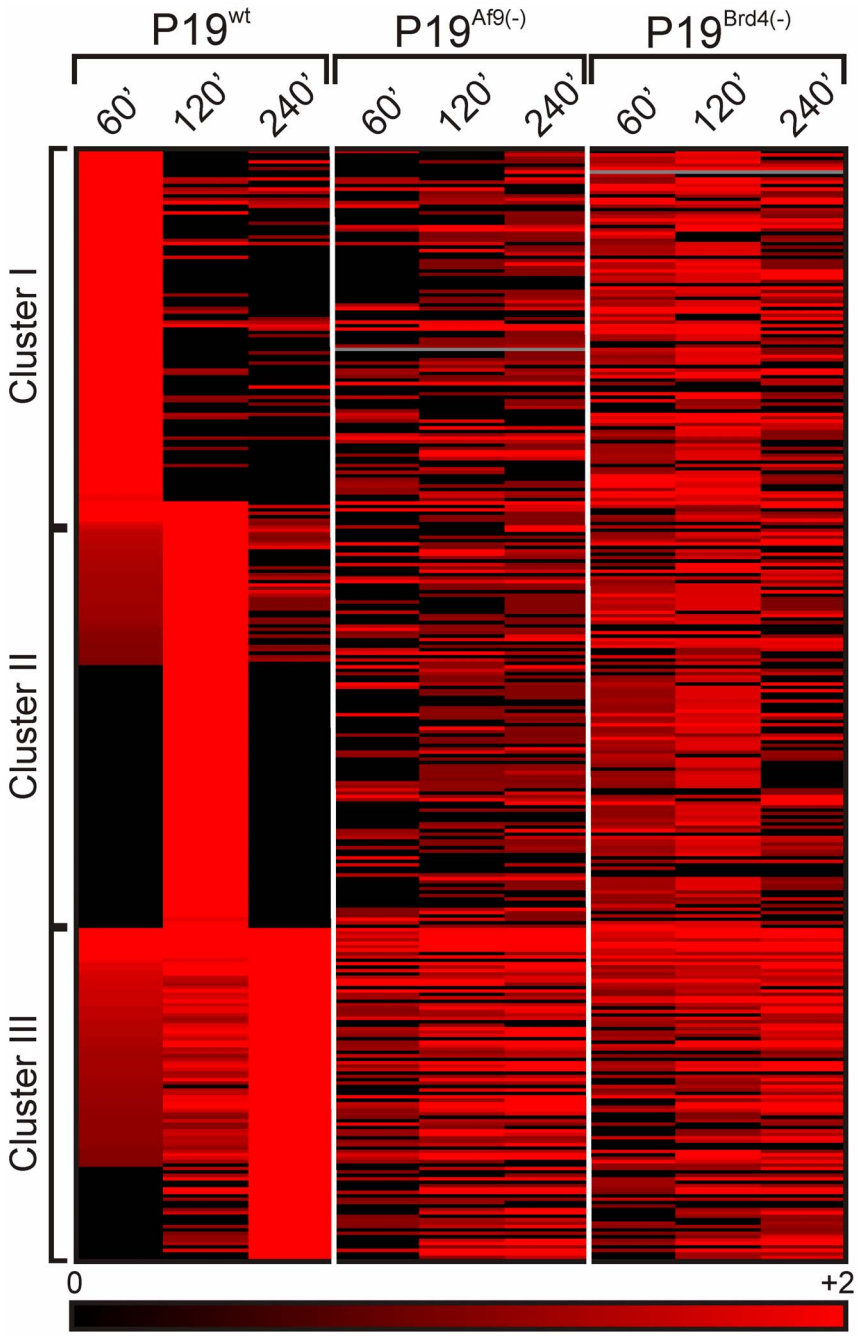

Figure 5. Time-dependent induction of gene expression upon RARα activation in P19^{wt}, P19$^{Af9(-)}$ or P19$^{Brd4(-)}$. Cells were treated with TTNPB for indicated times and gene expression patterns were monitored. Genes induced more than 2-fold and peaking at either 60 minutes, 120 minutes or 240 minutes in the P19^{wt} background were clusterized to define cluster I (peaking at 60 minutes), cluster II (peaking at 120 minutes) and cluster III (peaking at 240 minutes). Associated gene lists were used to generate entity lists in Genespring to follow the expression of these genes in the P19$^{Af9(-)}$ or P19$^{Brd4(-)}$ background. Expression at different times in distinct cellular backgrounds is displayed as a heatmap.
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detected at RAR binding sites. RNApol II accumulated at TSS and exonic regions, as expected from increased mRNA transcripts synthesis (left inserts, Fig. 6). In contrast to AF9- and/or BRD4-dependent genes (clusters C and B respectively), RAR association to either TSS or exonic regions was not detectable for AF9- and BRD4-independent genes (cluster A, *Ccdc88b*, *Cdh18*, *Csn3*), suggesting that these factors are required for RAR recruitment, or at least stable binding, to elongated regions. It also suggested that the retinoid-mediated activation of BRD4 and/or AF9-dependent genes differs significantly from those exhibiting no such dependency, as emphasized by the selective accumulation of RAR at the TSS from BRD4- and AF9-dependent genes.

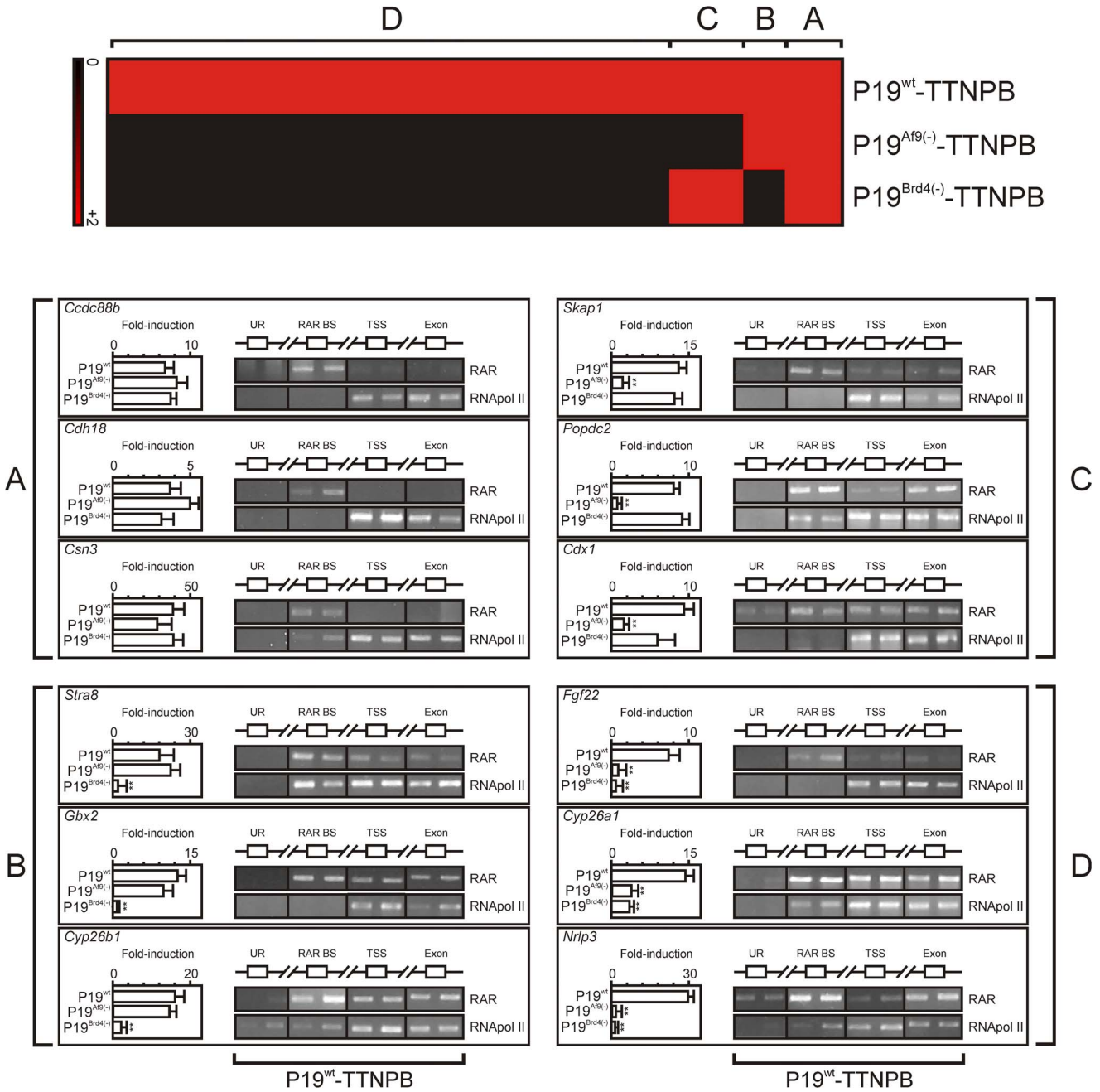

Figure 6. RARα association to transcribed regions in AF9-or BRD4-independent genes. The response of TTNPB-inducible genes (FC>2 after 4 hours) in P19^{wt} was compared to that in P19$^{Af9(-)}$ or P19$^{Brd4(-)}$ in similar conditions. Genes losing their responsiveness to TTNPB (FC<1.2) in either the P19$^{Brd4(-)}$ background (cluster B), the P19$^{Af9(-)}$background (cluster C) or both (cluster D) were identified by microarray data analysis. Genes maintaining an inducibility similar to that observed in P19^{wt} in either the P19$^{Af9(-)}$ or the P19$^{Brd4(-)}$ background were grouped in Cluster A. Genes in each cluster were searched for the occurrence of RAR binding sites on the basis of RAR ChIP-Seq data carried out in mouse ES cells [42]. Three representative genes were selected from each cluster and their inducibility was validated by RT-QPCR in each condition (n = 3, left inset). RARα and RNApol II association to an upstream region (UR), RAR binding site (RAR BS), transcriptional start site region (TSS) and an exon (Exon) was assessed in independent, duplicate ChIP-PCR assays after a 4-hour challenge of P19^{wt} with TTNPB. Input lanes showed an equal loading but were omitted for space purposes.

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AF9 or BRD4 Stabilize RAR Interaction with Transcribed Regions

Given that RAR did not associate to exonic regions of genes from cluster D (ie AF9- and BRD4-independent), we characterized RAR occupancy in the P19$^{Brd4(-)}$ or P19$^{Af9(-)}$ background (Fig. 7). In good agreement with gene expression data, ChIP-QPCR assays revealed that TSS occupancy by either BRD4 or AF9 could be detected at BRD4 and/or AF-dependent genes (clusters B, C and D), but not at the TSS of BRD4- and AF9-independent genes (cluster A). Silencing of *Af9* or of *Brd4* did not modify RAR loading at any of the locations tested in genes from cluster A. RAR loading in AF9-dependent genes (cluster B) was not affected by BRD4

depletion. In contrast, *Af9* knockdown impaired RAR association to exonic regions, whereas binding to the "RAR BS" was not significantly affected. BRD4-dependent genes (cluster C) mirrored this response, since only *Brd4* silencing affected RAR density at exonic sequences. Furthermore, AF9- and BRD4-dependent genes reflected the behavior of the *Rarβ2* gene, for which *Brd4* silencing reduced RAR association to transcribed regions and *Af9* knockdown had an opposite effect. As BRD4 and AF9 were shown to belong to distinct complexes and to interact with RAR DBD, their partitioning was assessed by coimmunoprecipitation of endogenous proteins from P19 cells. These assays showed that while immunoprecipitation of RARα isolated a fraction of BRD4 or of AF9, immunoprecipitation of AF9 was exclusive of that of BRD4, and vice-versa (Fig. S6). These experiments show that either BRD4 or AF9 is sufficient for RAR association to transcribed regions, and suggests that AF9 has an inhibitory role on RAR-BRD4 association.

Discussion

In the current study, mass spectrometry-fingerprinting was used to identify AF-2-independent RARα coactivators. The unexpected interaction of RARα with RARγ led to the identification of BRD4 and AF9 as interacting strongly with the DNA-binding domain of RARα, and to a lesser extent with the N-terminal AF-1 domain in the case of AF9. Using the *Rarβ2* promoter as a paradigm for RAR-mediated transcription, we demonstrate that both proteins displayed RARα coactivating properties, thus functionally and physically connecting RAR to P-TEFb (cyclinT:CDK9), which interacts in an exclusive manner with BRD4 or AF9 [38]. In addition, we observed that RARα can associate to transcribed regions together with RNApol II. CDK9 catalyzes RNApol II CTD Ser2 phosphorylation, allowing the release of paused RNApol II complex for productive RNA elongation by relieving the inhibitory action of the DRB sensitivity-inducing factor DSIF and of the negative elongation factor NELF [reviewed in [36]]. Both the pharmacological inhibition of the P-TEFb kinase CDK9 and AF9 depletion blunted *Rarβ2* gene transcription. RNApol II processivity is controlled by AF9, which directly or indirectly regulates positively CDK9 activity [43]. Thus a decreased RNApol II processivity in *Af9*-depleted P19 cells (Fig. 3E) may account for the increased RAR and RNApol II association to gene body sequences and the observed decreased gene transcription.

BRD4 is known to bind to both components of P-TEFb, cyclinT and cdk9 [27]. It preferentially associates to P-TEFb at the promoter-proximal regions of Ca^{2+}- or of HIV1-regulated genes and is excluded from AFF4-containing complexes [43–47]. BRD4 is however required for the induction of the HIV-1 promoter [8], and show a preferential activity on primary response genes [48]. The negative impact of *Brd4* silencing on some rapidly induced genes in our system (Fig. 5) is in agreement with this observation and suggests that retinoid-controlled transcription may depend

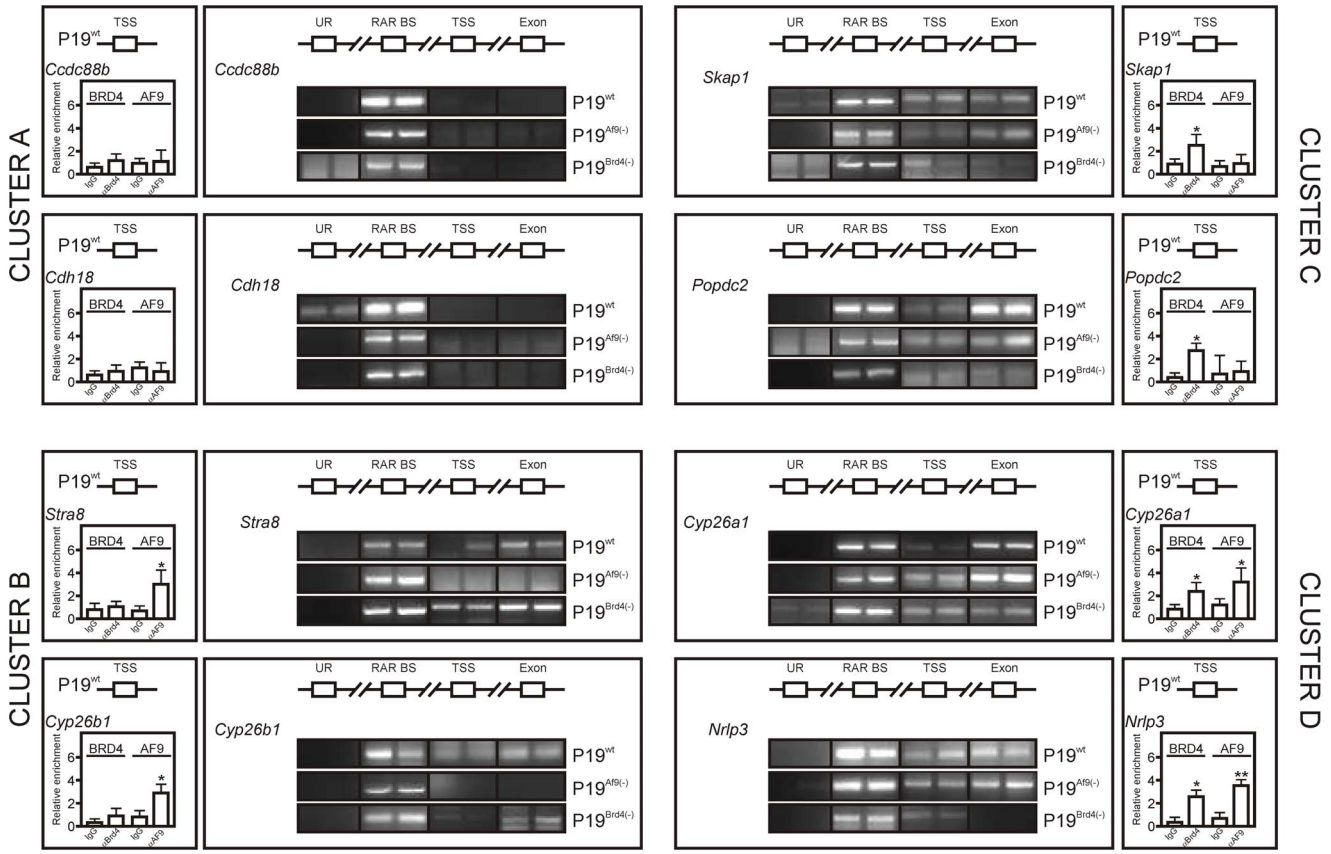

Figure 7. AF9 or BRD4 are required for RAR interaction with exonic regions. Two representative genes from clusters defined in Figure 5 were selected. The loading of AF9 and BRD4 to the TSS was assayed by ChIP-QPCR (n = 2) and results normalized to background values (*Myoglobin* gene) are represented in left insets (bar graphs). The association of RAR to an upstream region (UR), RAR binding site (RAR BS), transcriptional start site region (TSS) and an exon (Exon) was assessed in independent, duplicate ChIP-PCR assays after a 4-hour challenge of $P19^{wt}$, $P19^{Brd4(-)}$ or $P19^{Af9(-)}$ cells with TTNPB. Data are expressed as the mean±SEM (n = 2). *, $p<0.05$; **, $p<0.01$.
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either on the SEC or on the BRD4 version of P-TEFb. Interestingly, BRD4 is required for the rapid induction of a subset of retinoid-regulated genes (our study and [35]), and favors P-TEFb loading due to its affinity for phosphorylated H3Ser10 and acetylated histones H3 and H4 [7,27,49]. Importantly, all of these epigenomic marks are present at the archetypical *Rarβ2* promoter, whose expression is dependent on BRD4 levels (our study and [11]). FRET studies showed convincingly that RARα interacted directly not only with AF9 but also with BRD4 in living cells, suggesting that RARα may constitute a landing pad for BRD4 on retinoid-activated promoters.

An unexpected finding was that some retinoid-regulated genes, including *Rarβ2*, display a similar dependency on BRD4 and AF9 expression. The mutually exclusive binding of P-TEFb to BRD4 and AF9, as well as the similar binding interface on RARα, rules out the co-occurrence of BRD4 and AF9 into the same complex. The observation that AF9 depletion favors BRD4 and RAR recruitment to transcribed regions, yet promotes a decreased transcription efficacy, suggests that BRD4 plays, for this specific cluster (Fig. 6, Cluster D), a moderate role in transcriptional regulation. This is in line with the reported broad distribution, but restricted regulatory role of BRD4 in ES retinoid-regulated genes [35].

The selective recruitment of RAR, but not of RXR, to transcribed regions depending on P-TEFb activity is an unprecedented observation. The functional role of this association is likely to increase transcriptional efficiency, as shown by the loss of rapid gene induction upon *Brd4* or *Af9* knockdown and RAR association to gene bodies. Indeed, the *Hoxb1* gene, which is poorly inducible in our system, is weakly activated in mouse ES cells and does not recruit the SEC complex upon retinoic acid challenge [35]. In contrast, both *Hoxa1* and *Cyp26a1* are strongly induced in both systems and recruit SEC in mouse ES cells. This correlates SEC loading to rapid transcriptional induction, and both AF9 and BRD4 depletion preferentially affected early-induced genes in our model. Our present work, together with published data [50–52], thus show that nuclear receptors can engage physical and functional interaction with the transcriptional elongation machinery, thus providing a mechanistic basis for SEC targeting to conditionally-activated promoters.

In this study, we thus report the following original findings: (i) the P-TEFb interactants BRD4 and AF9 bind directly to and coactivate RAR in living cells; (ii) the activation of the archetypal retinoid-inducible gene *Rarβ2* is dependent on P-TEFb activity; (iii) RAR, but not RXR, associates to transcribed regions of the *Rarβ2* locus in a cdk9-dependent manner and colocalizes with elongating RNApol II; (iv) BRD4 and AF9 facilitate retinoid-induced transcription and exert distinct biological functions in retinoid-mediated neuronal differentiation. Thus monomeric RAR may be involved in promoter-pausing release and possibly transcriptional elongation as shown by our data. Such a dual function has been suggested recently for the transcription factor c-Myc [53]. Whether the RAR/elongation complex serves structural and/or functional functions call for further investigations, which will require to take into account the multiple functions of components of this multimeric complex and the use of simplified transcription systems.

Supporting Information

Figure S1 RAR interaction with isolated RAR domains and functional interference with NAP1L2. (A) Various domains of RARα were expressed as fusion proteins to GST (left panel) and used as baits for ^{35}S-labeled RARα AF-1. Protein bound on beads are visualized by autoradiography, in comparison to 10% input (Input). CB: Coomassie Blue staining of RAR derivatives adsorbed on glutathione-sepharose beads. (B) Coimmunoprecipitation of AF9 and RARα in HeLa cells. HeLa cells were cotransfected with expression vectors coding for either wtRARα (wt) or N-terminally truncated RAR (ΔAF-1) together with an empty pCMV-3×FLAG plasmid (Mock), or pCMV-3×FLAG containing a AF9 cDNA insert. Cell lysates were immunoprecipitated (IP) with an anti-FLAG M2 affinity resin, and immunoprecipitates, as well as cell lysates (Input), were analyzed by western blotting with an anti-RAR antibody. The numbers (ratio) are the ratio of RAR to AF9 detected by western blotting and quantified by densitometric analysis. (C) P19 cells were transfected with the indicated amounts of a NAP1L2 expression vector for 24 hours and *Rarβ2* gene expression level was assayed after a 4-hour treatment with 1 μM atRA, using a Taqman-based RT-QPCR assay. The basal expression level in untreated cells was arbitrarily set to 1 and data are expressed as the mean±SEM (n = 3). *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.
(TIFF)

Figure S2 Retinoic acid induces a neuronal differentiation program in EC P19 cells. (A) Microarray gene expression analysis of RA-stimulated P19 cells. A mRNA expression scatter plot was obtained from gene-level interpretation of microarray data (left panel), out of which the 10 most up- or down-regulated genes were identified (right panel). The two thick green lines in the scatter plot indicate a fold change greater than 2. (B) Genes whose expression was modulated more that 2-fold were clustered using the Gene Ontology functional annotation table [1].
(TIFF)

Figure S3 Basal gene expression in Af9- or Brd4-depleted P19 cells. $P19^{wt}$, $P19^{Brd4(-)}$ or $P19^{Af9(-)}$ cells were treated for 4 hours with 100 nM TTNPB, and mRNAs were extracted and analyzed on Agilent microarrays. Basal expression level was set to 1 in the $P19^{wt}$ background, and genes deregulated by more than 2-fold in both $P19^{Brd4(-)}$ or $P19^{Af9(-)}$ backgrounds were identified using the Genespring software. An entity list consisting of all genes displaying an altered expression in either $P19^{Brd4(-)}$ or $P19^{Af9(-)}$ cells was generated, and their expression level in each background was extracted from microarray data. These expression values, expressed as fold-change over basal in $P19^{wt}$ cells, were processed and used to generate heatmaps with MeV [2]. Genes displaying a FC>5 in the $P19^{Brd4(-)}$ background (top) or in the $P19^{Af9(-)}$ background (bottom) were analyzed using Gene Ontology Functional Annotation Tables and the KEGG database [1]. The most significant terms are indicated. Finally, genes displaying a FC>5 in the $P19^{Brd4(-)}$ background or in the $P19^{Af9(-)}$ background were compared and genes similarly affected by both knockdowns were identified, and subjected to GO FAT and KEGG annotations. These analyses appear in the middle of the figure. Green: down-regulated; red: up-regulated. Raw data are available in Table S3.
(TIFF)

Figure S4 atRA and TTNBP elicits a similar transcriptional program in EC P19 cells. Genes that were modulated with a FC>1.5 at least in one condition (1 μM atRA or 1 μM TTNPB) were identified by data analysis in Genespring v12.0 and the gene list was exported as a text file from this software (available in Table S2). A partial heat map corresponding to upregulated genes (FC>2 in atRA-treated P19) classified by order of induction was generated from this worksheet using MeV and is shown fragmented for visualization purposes from left to right. Up- or

downregulated genes (FC>2) are indicated in red or green respectively.
(TIFF)

Figure S5 Gene expression induction in an Af9- or Brd4-deficient cellular background. (A) Scatter plot representation of the gene expression pattern in wild type P19 (P19 ^{wt}), AF9-depleted P19 or BRD4-depleted P19 (P19$^{Af9(-)}$ or P19$^{Brd4(-)}$ respectively). The two thick green lines in scatter plots indicate a fold change greater than 2. (B) The expression of the neuronal differentiation marker NF160 was assessed by western blot analysis 96 hours after the initiation of neuronal differentiation as previously described [3].
(TIFF)

Figure S6 BRD4 and AF9 binds to RAR as distinct complexes. (A) Western blot of P19 whole cell extracts (100 µg proteins). P19 cells were grown under standard conditions and whole cell extracts were prepared as described in the Materials & Methods section. Proteins were resolved on 4–15% polyacrylamide gels and analyzed by western blotting using an anti-RAR (Santa Cruz, sc551), an anti-Brd4 (Active Motif) or an anti-AF9 (Bethyl A300-595A) antibody. (B) Coimmunoprecipitations assays. RAR was immunoprecipitated with an anti-RAR antibody (Santa-Cruz, sc551). Isolated complexes were resolved by 8% SDS-PAGE and analyzed by western-blotting using an anti-Brd4 (Active Motif) or an anti-AF9 antibody (Bethyl A300-595A). Reciprocal immunoprecipitations were carried out using an anti-Brd4 antibody (Santa Cruz H250) or a mix of anti-AF9 antibodies (Bethyl Labs). Input: 10% of total material (500 µg proteins); IgG: non-immune sera (rabbit); IP: immunoprecipitation with the indicated antibody.
(TIFF)

Table S1 Gene chromosomal coordinates and associated regions amplified by PCR in ChIP experiments. * Taken from [4]. **TSR coordinates are extracted from databases using Genomatix Eldorado. TSR amplicons were defined by extending the identified TSR with +/−50 bp. RARE motif search were carried out using this extended sequence and PCR primers were designed to optimally amplify this sequence using Primer3plus. When multiple potential RARE were detected, primers were designed to encompass all RAREs whenever possible. *** as defined by MathInspector scanning of the sequence using the V$RXRF matrix.
(XLSX)

Table S2 Gene basal expression in the P19^{wt}, P19$^{Brd4(-)}$ or P19$^{Af9(-)}$ background. Genes that were modulated with FC>2 at least in one condition (shAF9 or shBrd4 vs wtP19) were identified by data analysis at the gene level in Genespring v12.0. The gene list was exported as a text file from this software. Gene expression levels in wtP19 were set to arbitrarily set to 1 and gene expression levels observed in either the AF9- or the BRD4-depleted background were expressed relative to this control. A heatmap corresponding to up- or down-regulated genes were generated from this worksheet using MeV and appears in the Supporting information files (Fig. S3).
(XLSX)

Table S3 atRA and TTNPB elicit a similar transcriptional response in P19 EC cells. Genes that were modulated with FC>1.5 at least in one condition (1 µM atRA or 1 µM TTNPB) were identified by data analysis in Genespring v12.0 and the gene list was exported as a text file from this software. Up- or downregulated genes (FC>2) are indicated in red or green respectively. A partial heat map corresponding to upregulated genes was generated from this worksheet using MeV and appears in Figure S3.
(XLSX)

Text S1 References used and cited in the Supporting Information Files.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SF M. Ploton AL AAI RG RM M. Pawlak GS PL BS. Performed the experiments: SF M. Ploton AL AAI RG RM M. Pawlak CR. Analyzed the data: SF CR RM LH BS GS PL. Contributed reagents/materials/analysis tools: CS RG AL AAI. Wrote the paper: SF PL.

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Retinoids and nuclear retinoid receptors in white and brown adipose tissues: physiopathologic aspects

Abstract: Vitamin A, ingested either as retinol or β-carotene from animal- or plant-derived foods, respectively, is a nutrient essential for many biological functions such as embryonic development, vision, immune responses, tissue remodeling, and metabolism. Its main active metabolite is all *trans*-retinoic acid (atRA), which regulates the gene expression through the activation of α, β, and γ isotypes of the nuclear atRA receptor (RAR). More recently, the retinol derivatives were also shown to control the RAR activity, enlightening the interplay between vitamin A metabolism and RAR-mediated transcriptional control. The white and brown adipose tissues regulate the energy homeostasis by providing a dynamic fatty acid storing and oxidizing capacities to the organism, in connection with the other fatty acid-consuming tissues. This concerted interorgan response to the fatty acid fluxes is orchestrated, in part, by the endocrine activity of the adipose tissue depots. The adipose tissues are also a site for synthesizing and storing vitamin A derivatives, which will act as hormonal cues or intracellular to regulate the essential aspects of adipocyte biology. As the agents that prevent adipocyte differentiation, hence, expected to decrease the fat mass, and inducers of uncoupling protein expression, thus, favoring the energy expenditure, the retinoids have prompted many investigations to decipher their roles in adipose tissue pathophysiology, which are summarized in this review.

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Keywords: adipose tissue; metabolism; nuclear receptors; retinoic acid; vitamin A.

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Introduction

Considered for a long time as hormonally inert, simple energy-storing or -dissipating organs, the adipose tissues are regarded today as the important organs involved in energy expenditure and metabolic homeostasis, as well as in endocrine and paracrine signaling. The white and brown adipose tissues (WAT and BAT, respectively) have, indeed, distinct locations and physiological functions. A third category of the adipose tissue, known as the yellow adipose tissue (YAT), is found in the bone marrow, and its functions remain largely unknown.

Very schematically, the WAT and BAT have opposite physiological roles. The WAT, endowed with a low intrinsic oxidative capacity, is specialized in the energy storage under the form of triacylglycerides in the post-prandial state and in the release of energy in the fasting state [1]. The BAT is, in contrast, characterized by a high oxidative capacity and specialized in the adaptive/nonshivering thermogenesis through the mitochondrial oxidation of the stored fatty acids [2]. In vivo, the WAT and BAT are relatively easy to distinguish because of their distinct coloration, cellular morphological differences, and localization in the body. The WAT, the major adipose tissue in the adult, is composed of both subcutaneous and several intra-abdominal depots. The white adipocytes are sphericals cells, whose size depends on the amount of lipid stored in the unilocular lipid droplet of each cell. In contrast, the BAT is composed by the multivacuolar adipocytes displaying a variable diameter, abundant mitochondria, and a dense vascularization giving rise to this brown coloration. Considered a long time to be restricted exclusively to the human neonates and hibernating mammals, and to be nonfunctional or even absent in adults, the BAT has been recently located throughout the neck, supraclavicular, axillary, paravertebral, mediastinal and suprarenal regions in the human adults [3–8]. An area of current and intense investigation is the developmental origin of the BAT and WAT. In vivo lineage tracing and large-scale transcriptomic studies have suggested that, at least in rodents, the BAT occurs as a so-called constitutive BAT, which is located in

the interscapular and perirenal regions, and arising from the Myf5-positive myoblast precursors [9]. The beige or brite adipocytes, residing in the skeletal muscles and WAT depots, are not originating from the Myf5 precursors and are mobilizable brown adipocytes. Recently, the beige cells were identified in the human adipose tissue [10].

In addition to their essential role in energy balance [11], the adipose tissues are active endocrine organs releasing many adipokines that regulate bone metabolism, reproductive, thyroid, and growth hormone axes, immune responses, and angiogenesis [12–14]. The dysregulations of the WAT functions by excessive increase (obesity) or decrease (anorexia) in fat accumulation are reflected by the health alterations, which are surprisingly common such as insulin resistance and type 2 diabetes. Interestingly, the vitamin A status is also correlated to the body thermogenic capacity [15].

Retinoids and adipocyte biology

Retinoid metabolism and storage in adipose tissues

Vitamin A is an essential fat-soluble molecule available from the dietary sources [16]. Vitamin A deficiency (VAD), characterized by impaired vision and growth, is classified as a major risk factor for the increased severity of infectious diseases and associated mortality, thereby, highlighting the critical role of vitamin A [17]. Vitamin A is absorbed in the intestinal lumen either as retinol (ROH) by passive diffusion or as β-carotene through a SR-B1-facilitated transport. The enterocytic β-carotene oxidase converts, by a centric reaction, a fraction of the carotenoids into ROH, which is, in turn, complexed to the cellular retinol-binding protein (CRBP) 2 and further transformed into the retinyl esters (REs) by lecithin:retinol acyltransferase (LRAT). The ROH is effluxed from the enterocytes by the membrane transporter ABCA1, whereas the REs and carotenoids are assembled into the chylomicrons, which are excreted into the lymph [18]. The REs are then converted to ROH, which will be either stored as REs or bound to the ROH-binding protein (RBP)/transthyretin (TTR) and secreted back into circulation to be distributed throughout the body according to the tissue needs [19, 20]. The liver stellate cells are the major site for the RE storage and secretion of RBP-ROH [21]. However, the adipocytes also contain non-negligible amounts of ROH and atRA. Indeed, with an estimated amount ~15%–20% of the total body retinoids in rats, the adipocytes are the second largest storage site of ROH [22–24]. The adipose tissues express all the genes associated with the retinoid uptake and metabolism, such as β-carotene 15,15′-monooxygenase 1 (BCMO1), alcohol dehydrogenase 1 (ADH1), aldehyde dehydrogenases (RALDH1), or with the retinoid mobilization and transport, such as the retinol-binding protein (RBP), cellular retinol-binding protein (CRBP), cellular retinoic acid-binding protein (CRABP), fatty acid-binding proteins FABP, and albumin (Table 1). The knock-out mouse models provided insights into the retinoid metabolism in the WAT. The use of *Lrat*-deficient mice has shown that the RE storage in the WAT is intact in these mice, suggesting that another enzyme, such as diacylglycerol acyltransferase-1 (DGAT1), may account for the RE formation in the adipose tissue [30, 42]. These adipose retinoid stores may be mobilized upon hydrolysis of RE into the ROH by the hormone-sensitive lipase (HSL) [43, 44]. The ROH efflux from the adipocytes, either as a free form or complexed to the RBP, which is expressed and secreted by the adipocytes, is increased by the intracellular cAMP concentrations [45]. Intriguingly, the ROH intestinal uptake and release from the adipocytes follow the mechanisms reminiscent of cholesterol and lipid mobilization in these cell types.

Alcohol dehydrogenase 1 (ADH1) and aldehyde dehydrogenases (RALDH), which convert reversibly ROH to retinaldehyde (RALD) and irreversibly RALD to RA, respectively, are expressed in the adipocytes. Accordingly, several ROH isomers (including all-*trans*, 11-*cis*, 13-*cis*, 9,13-di-*cis*, 9-*cis*, and 11–13-di-*cis* ROH), RALD, and several isomers of RA (all-*trans*, 9-*cis*, and 13-*cis* RA) are found in the adipocyte fraction of the WAT [33, 46, 47]. The WAT and BAT exhibit ROH and RA levels similar to that found in the liver and other RA-responsive tissues [22, 46, 48, 49]. Sima et al. characterized a mixture of the ROH isomers in both the subcutaneous and visceral adipose tissues in which the gene expression studies were suggestive of a high demand for RA production in the visceral fat [41]. Recently, 9-*cis* RA, which remained undetected in the previous studies because of its instability and low concentration, was identified in the rat pancreatic tissue and shown to act on the glucose-stimulated insulin secretion [50]. As 9-*cis* ROH is detected in the WAT, which exhibits high all-*trans* and 9-*cis* retinal oxidation rates [41], it is reasonable to assume that the 9-*cis* RA may occur in the WAT. This, however, has yet to be formally proven.

The adipose tissues are, thus, dynamically involved in retinoid absorption, storage, mobilization and transport. The retinoids stored in the WAT can be mobilized in time of dietary vitamin A insufficiency [42]. More recently, *Raldh1* gene deletion in mice was shown to induce the RALD accumulation in the WAT and to confer resistance

Table 1 The genes expressed by the adipose tissues and involved in the retinoid metabolism and transport.

Enzyme	Symbol	Function	References
Lipoprotein lipase		Facilitates the uptake of retinoids	[25]
β-carotene monoxygenase type 1	BCMO1	Conversion of B-carotene to retinaldehyde	[26]
			[27]
Acyl-CoA:retinol transferase	ARAT	ROH esterification	[28, 29]
Diacylglycerol acyltransferase type 1	DGAT1	Major acyl-CoA retinol acyltransferase (ARAT) in the skin, where it acts to maintain retinoid homeostasis and prevent retinoid toxicity leading to skin and hair disorders	[30, 31]
Aldehyde dehydrogenase	ADH1	ROH oxidation	[32]
Retinaldehyde dehydrogenase	RALDH1	Retinoic acid synthesis	[33]
Protein transport			
Retinoid-binding protein	RBP	A plasma transport protein for retinol	[34]
Cellular retinol-binding proteins	CRBP	ROH protection from oxidation and isomerization, facilitate ROH esterification in RE	[35–37]
Cellular retinoic acid-binding proteins	CRABP	Intracellular lipid binding protein, Retinoic acid binding, delivering RA into nucleus	[38]
Fatty acid-binding proteins	FABP	Cellular retinoid binding protein	[38, 39]
		Role in regulating the cellular redox state	[40]
Membrane receptor			
	STRA6	RBP4 receptor	[41]

to the high-fat diet (HFD)-induced obesity, probably by interfering, at least in part, with the adipocyte differentiation program through the inhibition of PPARγ- and RXR-mediated responses [33]. The RALD turned out to be an efficient RAR activator, switching on a browning transcriptional program in the WAT [51]. Taken together, these data establish a crucial role of vitamin A-converting enzymes and of their products in the adipose tissue functions and differentiation.

The liver pathological conditions have a profound deleterious effect on the hepatic retinoid content, accompanied by a concomitant increase in the extrahepatic retinoid levels, and more specifically in the kidney and adipose tissue [50, 52, 53]. The effects on the retinoid metabolism and homeostasis have, indeed, been reported in the humans with alcoholic steatohepatitis (ASH) as well as in patients exhibiting the distinct stages of non-alcoholic fatty acid liver disease (NAFLD) progression (for review, see [54]). The mechanism underlying this storage shift remains, however, elusive [28, 29, 55].

The nuclear receptors RAR and RXR in adipose tissues

Generalities

From a transcriptional perspective, RA is considered to be the active molecule as both the all-*trans* and 9-*cis* RA isomers initiate the transcriptional responses through the activation of the nuclear receptors RARs and RXRs. The other nuclear receptors, such as PPARβ/δ and TR4, are also activated by atRA [56, 57]. The RARs and RXRs are ligand-inducible transcription factors belonging to the steroid hormone nuclear receptor superfamily. The RARs are activated by the RA isomers (atRA, 9 *cis*-RA, RALD), whereas RXR is activated by the 9-*cis* RA. At least two distinct isoforms generated by the differential promoter usage and/or alternative splicing have been described for the α, β, and γ isotypes of RAR and RXR, which are encoded by separate genes [58–60]. The observations from the mice deficient for a specific RAR or RXR isotype suggested a functional redundancy between these receptors and also revealed non-interchangeable roles in development and morphogenesis [61].

The RARs and RXRs are expressed in the adipose tissues [62, 63], and their expression pattern is conserved in the several mammalian species. The WAT and BAT display distinct relative expression of each isotype. The RARα, RARγ, RXRα, and RXRβ are highly expressed in the WAT depots, whereas RARβ and RXRγ are predominantly detected in the BAT [64]. This characteristic expression pattern suggest specific functions for the RAR and RXR isoforms in the WAT and BAT biology, which have not been investigated so far with the exception of RXRα (see below).

The RAR:RXR heterodimers bind to specific retinoic acid response elements (RAREs), which were initially

found in the promoter region of the target genes. These early studies also showed that the RAREs are typically composed of two direct repeats (DR) of the core motif 5′-PuG(G/T)TCA and separated by 1, 2, or 5 bp (referred to as DR1, DR2, or DR5, respectively) [65]. Such an architecture was recently confirmed at the genome-wide level by scanning the mouse ES cell genome for the RAR binding sites using a ChIP-sequencing approach [66]. Of note, a similar study carried out in the mouse embryonal carcinoma F9 cells, however, extended the RAR-binding repertoire to DR0, DR8, and IR0 (inverted repeat 0) elements [67]. Although many of the genes associated to the identified RAREs are involved in cellular differentiation, reflecting a general interest of the scientific community for the retinoid-controlled developmental effects, it is worth noting here that the retinoic acid receptors also regulates the expression of the several genes regulating lipid or glucose metabolism including phosphonenolpyruvate carboxykinase (PEPCK), steraoyl-CoA desaturase 1 (SCD1), uncoupling protein 1 and 3 (UCP1&3), and hormone-sensitive lipase (HSL). It is worth noting here that RXR, either as a homodimeric complex or integrated in the heterodimeric complexes with the other NRs including PPAR, VDR, LXR, FXR, can also control the transcription of the metabolic genes through the ligation of the retinoids or other endogenous lipids [68–70].

The ChIP-seq studies also showed that RAR bind mostly in the intronic or promoter-distal intergenic regions and showed a strong overlap in the RAR isotype-binding sites. Although this conclusion was initially drawn from the cellular models in which the RARs were overexpressed as the C-terminal GFP fusion proteins [71], this overlap was also observed in a study characterizing the genomic binding sites of the endogenous RAR and RXR in the F9 cells [72], thereby, corroborating the functional studies in which both isoforms showed at least partial functional redundancy. Interestingly, the other reports [71, 73] showed that RARα and the estrogen receptor α (ERα) can occupy the overlapping genomic regions, pointing to a yet unsuspected cooperation between these two nuclear receptors. Although both studies have been carried out using an estrogen-dependent breast cancer cell line (MCF-7), this observation is of potential interest for the adipose tissue biology, as the estrogens play a critical role in the control of body fat distribution and metabolic activity [74].

The RARs function as ligand-dependent transcriptional regulators [70, 75] for which the RXRs serve as the obligate dimerization partners [76]. The RARs and RXRs contain several distinct functional domains, of which the DNA-binding domain (DBD) and the two transcriptional activation functions (AFs) have been extensively studied both from a structural and functional perspective. The two AFs include the poorly conserved, ligand-independent N-terminal (AF-1) domain and the C-terminal region known also as the ligand-binding domain (LBD) [77]. Whereas the AF1-mediated transcription is essentially controlled through the posttranslational modifications and involves the recruitment of the general transcription initiation and elongation factors [78, 79], the AF2-mediated transcription is the result of the ligand-induced conformational changes in the C-terminus domain of the receptor. These structural transitions create a protein-protein interaction interface that recognizes the LXXLL-motif containing the polypeptides, also called the nuclear receptor box (NR box) [80–82]. A plethora of transcriptional coregulators ([77] for review), coactivators, and corepressors, are directly recruited through this NR box to the RAR AF2 in response to the retinoids [83, 84]. The basic mechanism for switching on the transcription by RAR:RXR dimers and the network of interactions with coregulatory protein complexes have been reviewed recently [85].

In addition to these classical genomic effects, the RARs induce nongenomic responses by activating several kinase signaling cascades. Indeed, several studies showed that RA rapidly activates p38MAPK, p42/p44MAPK, and the downstream protein kinase MSK1 [86–90]. Although the consequences of p38 MAPK activation on glucose uptake by the WAT remain controversial [91], this raises the possibility that the atRA might control the adipocyte functions through the nongenomic effects.

Finally, the RARs have been reported to associate with PI3- or Src kinases located in the cytosol or in the plasma membrane [92, 93]. This RA-induced kinase network leads to the dynamic and coordinated phosphorylation of RAR, coregulators, and histones allowing a precise tuning of the RAR cofactor recruitment, of chromatin remodeling, and of the docking of the transcriptional machinery [85]. The retinoic acid receptors and retinoids are, thus, important players in regulating the nuclear and cytoplasmic signaling pathways whose modulation leads to the finely orchestrated transcriptional responses.

Retinoic acid receptors and adipocyte differentiation

A large body of data attests to the critical role of the retinoids and of their receptors in adipocyte differentiation. The mouse embryonic stem cells are committed to the adipocyte lineage upon RARβ activation [94]. On the one hand, however, most reports point to an inhibitory action of the retinoids on stem cell commitment to the adipocyte

lineage and on adipocyte differentiation when administrated at the early stage of the differentiation process in vitro [95, 96], and conversely, the retinoids potentiate osteoblastic differentiation [97]. Using the mouse multipotent mesenchymal C3H10T1/2 cell line, it was shown that RA cooperates with BMP2 to channel the cells toward the osteoblastic lineage [98]. This mechanism involves a RA-mediated increased expression of Smad3, which, through the inhibition of C/EBPβ, induces the expression of the osteoblast master regulator runt-related transcription factor 2 (Runx2) and inhibit PPARγ expression [99–101]. Furthermore, atRA is able to blunt the BMP4-mediated C3H10T1/2 commitment toward the adipocyte lineage by downregulating the Smad1–5-8/p38MAPK signaling pathway [102]. Using the precommitted fibroblastic cell line 3T3-L1, atRA was shown to activate the CRABPII-RARγ pathway and to prevent the differentiation of 3T3-L1 fibroblasts into adipocytes by increasing the expression of the adipogenesis inhibitors such as Pref-1, Sox9, and KLF2 [103]. On the other hand, the gene inactivation of RXRα confers resistance to the dietary and chemically induced obesity and blunts the fasting-induced lipolysis [104]. These results also suggested that RXRα is required for adipocyte differentiation, in agreement with the reported differentiating effect of the oxime rexinoids in the 3T3-L1 model [105].

Similar to atRA, the ROH blunts the adipocytic differentiation of the human and bovine precursor cells [106, 107]. Interestingly, the ROH complexed to the plasma transporter RBP (RBP-ROH) induces little effect in the preadipocytes, but leads to the recruitment and activation of JAK2 and STAT5 when bound to the membrane receptor STRA6, expressed in the mature adipocytes. The upregulation of the expression of the STAT target genes by RBP-ROH, including the suppressor of cytokine signaling 3 (SOCS3), inhibits the cytokine signaling mediated by the JAK/STAT pathway and increases triglyceride accumulation in a STRA6-dependent manner [108].

The adipogenesis is also accompanied by a significant modulation of nuclear receptor expression, also depending on the used model. The nondifferentiated 3T3-L1 preadipocytes primarily express RARα and RARγ. The adipogenic signals lead to a dramatically decreased expression of RARγ and to the increased expression of PPARβ/δ, whereas the RARα levels are nearly constant. RARβ is not detectable both in the pre- and mature adipocytes [95, 109–113]. The expression levels of the retinoid receptor mRNAs in the subcutaneous WAT are decreased in the obese patients, whereas PPARγ is higher in the obese cohort [114, 115]. Taken together, these data suggest that, from a pharmacological perspective, the activation of a selective subset of retinoid receptors through the use of the isotype-selective ligands may lead to the predictable outcome on adipocyte differentiation.

Retinoids and adipocyte functions

In addition to blocking the adipocyte differentiation process, the retinoids exert a control on the mature adipocyte functions. Quite remarkably, the retinoids are strong inducers of *UCP-1* expression in a variety of models, implying that they may increase energy expenditure. In agreement, the atRA-treated 3T3-L1 adipocytes show reduced TG levels and increased expression of gene involved in oxidative metabolism [116]. Indeed, *UCP-1* is a direct target gene for RAR [117–121], and in addition, atRA has been shown to exert a direct effect on the proton transport by UCP-1 [122]. An alternative explanation is based on the decreased RAR and increased PPARδ/β expression in the mature adipocytes. PPARδ/β is known to control lipid and glucose metabolism, which, thus could, upon atRA-induced activation, enhance the lipid oxidation and deplete the adipocyte lipid stores. Illustrating this possibility, a 5-week treatment by RA led to decreased adiposity and hepatic steatosis in high fat-fed mice, but the potential toxic effects of atRA were not evaluated in this study [111]. Taken together, these studies and others [123] indicate that atRA favors, both in vivo and in vitro, the acquisition of the BAT-like properties in the WAT. In the fetal rat brown adipocytes, atRA activates p44/p42 and p38MAPK and increases *UCP-1* expression in a p38-dependent manner [124, 125]. On the other hand, RXR activation specifically activates the *UCP-2* expression [126]. Collectively, these data show that the retinoids might increase the thermogenic capacity of the WAT and BAT by increasing the expression of the uncoupling protein genes.

Retinoid metabolism and adipocyte differentiation

The data described above investigate the effect of the exogenous retinoids on the adipocyte differentiation, raising the question of the actual contribution of the endogenous retinoids to this process. β-carotene 15,15′-monooxygenase 1 (Bcmo1) converts β-carotene by centric cleavage to RALD. The Bcmo1-deficient mice are dyslipidemic and susceptible to diet-induced obesity. Bcmo1 expression is induced during 3T3-L1 differentiation and is necessary for β-carotene conversion to atRA [27], pointing to a role of the metabolic conversion of the retinoid precursors in adipocyte biology. β-carotene can also

undergo asymmetric cleavage yielding the apocarotenals. The β-apo-14′-carotenal (apo14), but not the structurally related apocarotenals, represses PPARγ and RXR activation, hence, there is adipogenesis [127]. RALD is further converted to atRA by (the R)ALDHs. Surprisingly, the *Raldh1a1* knockdown blunts adipogenesis in vitro, and the gain-of-function experiments suggested that the endogenous production of RA is required for adipocyte maturation [128]. The endogenous RA production turned out to regulate the *Zfp423* expression in vitro, a transcription factor required for *Pparγ* expression. Finally, *Raldh1a1*$^{-/-}$ mice exhibited the decreased visceral fat accumulation, suggesting that RALD or RA might control the adipose tissue functions in a depot-specific manner. These data, therefore, question the relevance of the in vitro studies using high concentrations of exogenous atRA (1–10 μM), which led to conclude to an inhibitory role of the retinoid signaling pathway on adipogenesis.

Metabolic diseases and retinoid metabolism

Obesity is an excess accumulation of the adipose tissue and is a strong risk factor for the metabolic abnormalities such as type 2 diabetes [129, 130]. As the adipose tissues are metabolically active and physiologically relevant storage sites of retinoids, it is not surprising that retinoid metabolism is altered in the mouse models of obesity and insulin resistance. The expression of the enzymes controlling the RALD levels, and in particular RALDH1 (or ALDH1a1), which is the major enzyme expressed during adipocyte differentiation in vitro and is predominantly expressed in fat depots, is indeed disturbed in the ob/ob mice [128]. Obesity in mice is highly associated with the decreased RALD levels in the adipose tissues [33].

Several studies have reported a severely increased RBP4 synthesis in the adipose tissue from obese mice and humans [131–133]. The increase of serum RBP level, reported in the obese subjects and type 2 diabetic subjects, is linked to the visceral fat volume [133]. In this study, it was shown that the *RBP4* mRNA levels are correlated with the adipocyte size. Moreover, the serum transthyretin levels are inversely correlated with the glucose transporter *GLUT4* mRNA levels. The ratio of RBP to ROH is, however, a better predictive proxy of type 2 diabetes than the RBP level. A good correlation has, indeed, been established between the RBP4 to ROH ratio and type 2 diabetes in a cohort of 233 individuals exhibiting various grades of progression toward the overt type 2 diabetes [134]. However, the limited information is available on the rate of vitamin A metabolism in diseases such as diabetes, in which vitamin A transport is enhanced by an elevation in the plasma RBP4.

Pharmacological alteration of metabolism by retinoids and prospective use in metabolic diseases

The retinoids are prescribed in specific and limited therapeutic applications including leukemia and the skin disorders [15, 135]. The preventive and therapeutic antitumor effect of vitamin A, thus, of retinoids in the treatment of the precancerous lesions, were reported in the early 1960s [136, 137]. The identification and the development of the retinoids with an improved risk:benefit ratio has been a challenge in the 1980/1990s [138–141]. Despite the promising prospects in oncology, their clinical use is still in its infancy mostly because of high toxicity, as the systemic retinoids are associated with the adverse events including the dreaded retinoic acid syndrome or RAS [142], pseudotumor cerebri characterized by the increased intracranial pressure [143], or the other manifestations such as the decreased bone mineral density, inflammatory bowel diseases and psychological disorders. However, the retinoids are still drawing attention in this field, and the others such as the neurodegenerative [144], cardiac [145], and metabolic diseases. As mentioned above, the metabolic effects of the retinoids are well described in a number of in vitro and in vivo models and have also been reported in humans [146]. Indeed, the patients under retinoid therapy display increased transaminases, hypertriglyceridemia, and hyper LDL-cholesterolemia [147, 148]. However, the impact on the lipid metabolism is highly variable among the patients and call for a stringent follow-up before after the initiation of treatment. The RA dose-response studies revealed that the high doses of RA (>269 mg/m²/day) promote massive, limitating grade 3 hypertriglyceridemia (>10-fold increase), while the lower doses (45 mg/m²/day) led to a moderate twofold increase in the blood triglycerides, as observed with a retinoic acid analo [149–151].

Promoting the BAT-like characteristics may represent a therapeutic strategy to combat obesity, and its complications [152] and the retinoids, much like the other factors determining a BAT-like phenotype have received considerable attention [9, 153, 154]. The synthesis and evaluation of the retinoids in the metabolic diseases have

been intense [155, 156]. Realizing that the safe and specific modulation of the RARs by the retinoids is a daunting prospect, many investigators explored the possibility of selective activation of the RXR heterodimers. While beyond the scope of this review, it is worth noting here that the RXR ligands (rexinoids) usually exhibit hypertriglyceridemic effects mostly through the activation of the RXR/LXR heterodimer [157], a deleterious effect, which seems to be minored when coadministrating RXR and the insulin-sensitizing PPARγ ligands [69, 158, 159]. In contrast, the rexinoids may synergize with the retinoids to increase hypertriglyceridemia, at least in rats [160]. The design of the novel heterodimer-specific rexinoids might, thus, provide an alternative strategy in treating the metabolic disorders [161].

Expert opinion

The retinoids or retinoid analogs are widely used via the topical or systemic administration to treat the skin or neoplastic disorders, respectively, thus, exploiting the general antiproliferative, prodifferentiating properties of these compounds. In contrast, the exogenous retinoids block adipocyte differentiation and promote the expression of the mitochondrial uncoupling protein 1 in the mature white adipocytes, hence, pointing to the potential applications in anti-obesity therapy. The atRA treatment of a variety of rodent models of genetically or diet-induced obesity induced, in most cases, a reduction in body weight and decreased adiposity, as well as the increased body temperature, in line with the in vitro observations. PPARβ/δ, which displays significant affinity for atRA and regulates the genes involved in the lipid and glucose homeostasis, may also participate in the RA-induced metabolic effects as suggested by the use of RAR and PPARβ/δ ligands. Teasing out how these two nuclear receptors actually participate to the retinoid-induced metabolic effects will require the use of tissue-specific PPARβ/δ and of the RAR-deficient mouse lines and the careful assessments of the retinoid-induced metabolic effects. It is worth noting here that the nongenomic, or more specifically the receptor-independent effects such as retinoylation or protein-kinase activation, will not be addressed using such a strategy, and the identification, thereof, by a proteomic approach may be worth considering. However, such a systematic approach is long and costly, and the search for the heterodimer-specific compounds may be an alternative strategy. The design of such ligands has been reported with the description of the rexinoids with the glucose-normalizing effects in the rodents, but again, a very careful characterization of the binding specificity of these rexinoids by mass spectrometry or other physical methods is mandatory prior to drawing any conclusion about their (potential) biological activity. The management of the overweight and obese patients is currently limited to the lifestyle interventions and bariatric surgery. A drug-based therapy would be of interest in controlling the increased adiposity, although there is much debate about whether fighting obesity or its metabolic consequences such as fatty liver diseases is more appropriate. The adipose tissue expandability is likely to be a mechanism protecting the peripheral tissues from the ectopic lipid accumulation, and the inadequate use of drugs such as the retinoid derivatives, most of which still displaying significant teratogenic and metabolic adverse effects in rodents and humans, may lead to public health problems. The design of the selective modulators of retinoid receptors, exhibiting appropriate tissue- and even cell-specific gene modulatory properties, would be an asset in this and other therapeutic fields.

Outlook

The current view of the nuclear receptor-mediated therapy is still focused on a "single-hit" approach, according to which very specific ligands ideally act on a single tissue or organ to promote the expression of a defined subset of genes. While this approach is probably tenable in anti-cancer therapy, correcting the metabolic abnormalities requires eliciting highly integrated responses in multiple organs and involves chronic treatments. A combined approach, involving the genome-wide and targeted proteomics, as well as a detailed understanding of the compound pharmacokinetics and biodisponibility is mandatory to rationally design and evaluate the novel molecules usable for fighting obesity. The technologies necessary for these investigations are available, with the advent of the new-generation sequencing, mass spectrometry proteomics, and imaging, which will yield a comprehensive view of the retinoid/rexinoid action in model organisms.

Highlights

- The effects of the exogenous retinoids in the adipose tissues are mediated through distinct nuclear receptors.
- The vitamin A metabolites have distinct abilities to modulate the activity of the nuclear receptors controlling the adipose tissue physiology.

- The exogenous retinoids may promote the browning of the white adipocyte.
- The exogenous retinoids decrease adiposity in the rodent models of obesity.
- The retinoids exert ill-defined nongenomic effects, which may contribute to the observed effects.
- The retinoids have a deleterious impact on the metabolic parameters and have not been evaluated for their toxicity in chronic treatments.
- The RAR- or heterodimer-specific ligands, which can minimize the unwanted side effects, can be designed.
- The drug-mediated intervention with the retinoic acid metabolism blocking agents (RAMBA) should be tested as an alternative approach to modulate the adipose tissue functions.

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Non-classical transcriptional regulation of *HLA-G*: an update

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Abstract

Human leucocyte antigen-G (HLA-G) plays a key role in maternal–foetal tolerance and allotransplantation acceptance and is also implicated in tumour escape from the immune system. The modulation of HLA-G expression can prove to be very important to therapeutic goals in some pregnancy complications, transplantation, cancer and possibly autoimmune diseases. In spite of substantial similarities with classical *HLA*-class I genes, HLA-G is characterized by a restricted tissue-specific expression in non-pathological situations. HLA-G expression is mainly controlled at the transcriptional level by a unique gene promoter when compared with classical *HLA*-class I genes, and at the post-transcriptional level including alternative splicing, mRNA stability, translation and protein transport to the cell surface. We focus on the characteristics of the *HLA-G* gene promoter and the factors which are involved in *HLA-G* transcriptional modulation. They take part in epigenetic mechanisms that control key functions of the *HLA-G* gene in the regulation of immune tolerance.

Keywords: HLA-G • transcription factors • epigenetics • gene regulation

Introduction

Human leucocyte antigen-G (HLA-G) is encoded by the major histocompatibility complex with biological and structural properties associated with a specific function in immune tolerance [1]. HLA-G was first characterized as a protein associated with β_2-microglobulin expressed in the BeWo choriocarcinoma cell line [2] and later as an array of five 37- to 39-kD isoforms in the cytotrophoblasts of placenta [3, 4]. During its 20-year history, HLA-G has been shown to be of crucial importance in the success of implantation and in foetal–maternal symbiosis during human pregnancy [5, 6]. Beyond this perfect example of successful physiological tolerance to semi-allografts, HLA-G has been demonstrated to contribute greatly to the protection of transplanted organs such as heart [7] and kidney/liver [8, 9] allografts against rejection. Evidence has been accumulated showing that HLA-G expression, which is very restricted in non-pathological conditions, may also be a strategy used by malignant tumours [10] and virus [11, 12] to escape the host's immune surveillance. HLA-G exerts these major functions by inhibiting NK and T-lymphocyte-mediated cytotoxicity as well as a proliferative allogenic response [13]. This inhibition is mediated through direct binding to the

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inhibitory receptors ILT-2 (LILRB1/CD85j) [14], ILT-4 (LILRB2/CD85d) [15] and KIR2DL4 (CD158d) [16]. The HLA-G protective effect can occur in the presence of a few HLA-G-expressing cells, by cell-to-cell contact-dependent uptake of HLA-G (trogocytosis) from APC and tumoral cells to T and NK cells, respectively [17, 18]. This process acts through effector cells made to act as suppressor cells locally and temporarily. Finally, ILT2, ILT3, ILT4 and KIR2DL4 expression is up-regulated by HLA-G in antigen-presenting, NK and T cells, suggesting that up-regulation of inhibitory receptors in immune cells might increase their activation thresholds and participate in immune escape mechanisms [19].

The *HLA-G* gene was cloned in 1987 [20] and maps on the short arm of chromosome 6 in the p21.31 region. It presents a gene structure consisting of eight exons and seven introns with approximately 86% similarities with the consensus sequence of the *HLA-A*, *HLA-B* and *HLA-C* genes. Exon 1 encodes the signal peptide, exons 2, 3 and 4 encode the α_1, α_2 and α_3 extracellular domains, respectively, and exon 5 encodes the transmembrane domain. Nonetheless, a stop codon in the second codon of exon 6 results in a shorter cytoplasmic tail region in comparison with classical HLA-class I molecules. A low amount of gene polymorphism is found since only 42 alleles are listed by the WHO Nomenclature Committee for factors of the HLA System (http://www.anthonynolan.org.uk), the *G*010101* group (five alleles) being the predominant one, with frequency varying from 32% to 83% in Japanese, Caucasian and African populations [21]. *HLA-G* alleles are essentially characterized by variations in promoter, 3′UT regions, introns and synonymous substitution in exons, low amino acid changes defining 15 protein variants only [22–25].

An alternative splicing of the primary transcript generates the membrane-bound isoforms HLA-G1 (complete molecule), HLA-G2 (minus exon 3), HLA-G3 (minus exons 3–4), HLA-G4 (minus exon 4) and the soluble isoforms HLA-G5 (soluble HLA-G1 counterpart), HLA-G6 (soluble HLA-G2 counterpart) and HLA-G7 (α_1 domain) (39 to 17 kD) [26–28]. Recent crystallography studies have validated the HLA-G1 heterotrimeric structure with its heavy chain non-covalently associated with β_2m and a nonamer peptide [29]. The loading of high-affinity peptides (KIPAQFYIL) prevents retrieval of the molecule and results in increased cell surface expression of HLA-G1 [30]. Soluble isoforms are encoded by transcripts in which intron 2 (G7) [28] or intron 4 (G5 and G6) [31, 32] are retained and are translated until a premature stop codon that prevents the translation from exon 3 and 5, respectively. Soluble HLA-G1 also may be generated by proteolytic shedding (sHLA-G1) [33], which is likely to be regulated by NF-κB activation [34]. Besides, it has been demonstrated that HLA-G1 and HLA-G5 may also be produced as β_2m-free heavy chains and more importantly as disulphide-bonded homodimers [35–37] and that the ILT-2 and ILT-4 binding sites of HLA-G dimers are more accessible than those of HLA-G monomers [38].

Under non-pathological conditions, HLA-G expression occurs during pregnancy primarily in the pre-implanted embryo [6, 39, 40] and at the maternal–foetal interface on extravillous invasive cytotrophoblasts [3, 4]. A number of other extrafoetal cells also express HLA-G, including amnion epithelial cells [41, 42] and endothelial cells of foetal blood vessels in the placenta [43]. Over the past few years, the HLA-G expression pattern was extended to a few healthy adult tissues in immune privileged sites, namely thymus [44, 45], cornea [46], pancreas [47] and the proximal nail matrix [48]. In addition, HLA-G protein may be produced by human decidual stromal cells [49], monocytes [50], keratinocytes [51] and erythroblasts from primitive to definitive haematopoiesis [52]. In contrast, restrictive expression of HLA-G is abrogated under pathological conditions, with up-regulation observed in grafted organs, inflammatory and autoimmune diseases, and viral aggressions [1]. In particular, more than 1000 malignant lesions have been analysed for the HLA-G expression and definitely demonstrate that HLA-G is switched on in numerous tumours [53]. Interestingly, expression frequency varies according to the tumour type, ranging from less than 30% in tumour lesions such as lung carcinoma [54, 55] and breast carcinoma [56, 57] to at least 80% in tumour lesions such as pancreatic ductal carcinoma, biliary cancer [58] and oesophageal squamous cell carcinoma [59]. Nonetheless, HLA-G1 cell surface expression and HLA-G transcripts may be lost along long-term *in vitro* propagation [60, 61], suggesting that not only HLA-G expression is under the control of genetics but also micro-environmental factors.

On top of the expression regulation of antigen-processing machinery components, post-translational mechanisms such as mRNA stability or protein translation are important for HLA-G expression, in particular during invasiveness of cytotrophoblasts [62]. In this situation, the recent identification of HLA-G-specific miRNAs may be assumed in these mechanisms [24]. On the other hand, a key level of regulation is undoubtedly *HLA-G* gene transcription, since high amounts of *HLA-G* transcripts are observed in cells expressing HLA-G protein, while very low amounts or absence of *HLA-G* transcripts are observed in cells where HLA-G protein is not detected [63–66]. *HLA-G* transcription also exhibits temporal regulation observed during the course of gestation [67], with high levels of *HLA-G* mRNA in first- and second-trimester trophoblasts, whereas a reduced level of *HLA-G* mRNA is observed in term cells [3, 4]. This spatiotemporal pattern of *HLA-G* transcriptional regulation is still partially elucidated and is dependent on transcriptional factors and specific cis-regulatory elements located within a non-classical *HLA-G* gene promoter [68, 69]. We propose herein to present this in detail.

HLA-G gene promoter region: regulatory sites and binding factors

The HLA-G promoter is unique among the *HLA* genes [67] with a divergent proximal region when compared to the other HLA, a trophoblast-specific regulatory element located at −1.2 kb from exon 1 [70] and specific regulatory elements. It is worth noting that in the published data, some findings are less valid then others depending on the method used in evaluating regulatory

Table 1 Promoter of *HLA-G* gene and associated transcriptional factors

Response element	Location (bp upstream of ATG)	Factors	Methods used for validation	References
CRE/TRE	−1380/−1370; −934; −770	CREB1, ATF-1, c-jun	EMSA, reporter gene, ChIP	[96]
RRE	−1356; −142/−133; −53	RREB-1	EMSA, reporter gene, ChIP	(Flajollet *et al.* unpublished data)
ISRE	−744	IRF-1	EMSA, reporter gene	[100]
HSE	−459/−454	HSF-1	EMSA	[103]
P50, SP1	−187/−171; −166	P50, SP1	EMSA	[72]
X1 box	−124	RFX5	EMSA (negative using ChIP)	[87, 88]
CAAT	−71	CTF		
TCTAAA	−44	TFII		
PRE	−37	PR	EMSA, reporter gene, DNA fragment-binding ELISA	[105]

element location and function. Indeed, the analysis could have been performed *in vitro* (*e.g.* electrophoretic mobility shift assays [EMSAs] and reporter gene assays), *in situ* (*e.g.* chromatin immunoprecipitation [ChIP] and RNA interference) or *in vivo* (*e.g.* transgenic mice), a criterion that should be taken into account in evaluating the impact of the results (Table 1). The *HLA-G* promoter also exhibits a pattern of variations characterized by two divergent lineages, which is consistent with balancing selection. This is probably related to highly regulated expression favouring high- and low-expressing promoters under temporally and/or spatially varying selective pressures [22]. Variations in 3′UTR that could influence RNA stability and/or translation have also been identified [24]. These observations strongly suggest that the *HLA-G* gene polymorphisms should now be considered a very pertinent parameter in the understanding of *HLA-G* gene regulation and more particularly in some *HLA-G*-associated diseases.

The atypical proximal promoter region of the *HLA-G* gene among classical *HLA* class I genes

Classical *HLA*-class I gene promoters contain two main regulatory modules, namely enhancer A/interferon (IFN)-stimulated response element (ISRE) and SXY boxes, located within the 220 bp 5′ of the gene initiation codon (ATG). These two distinct cis-acting elements contribute to the constitutive and inducible level of MHC class I genes, the SXY module being shared by MHC class II genes [71]. The most upstream module contains the enhancer A with κB2 and κB1, two palindromic binding sites for the NF-κB/rel family members and a Sp1 transcription factor site [72, 73]. This module is also composed of a response element localized −180 bp from the ATG (consensus sequence AGTTTCNNTTCT) that may bind factors of the interferon regulatory factor (IRF) family such as IRF-1, IFN consensus sequence binding protein (gene activation), IFN-stimulated gene factor (ISGF)-3 and IRF-2 (repressors) [73]. Additionally, E-box elements can be found in the upstream HLA-class I module and are binding sites for upstream stimulatory factor (USF)-i and USF-2 [73].

The downstream SXY module was first demonstrated to be crucial in the regulation of HLA-class II expression [74]. It comprises the X1, X2 (site α) boxes and Y box (an inverted CCAAT-binding site, also named enhancer B), bound by the multiprotein complex RFX (RFX5, -AP, -ANK/B) [75–78], X2-BP/ATF/cAMP response element-binding (CREB) [79] and NF-Y [80] factors, respectively. All these factors cooperate to allow the formation of a stable multiprotein complex and the binding of the class II transactivator (CIITA), which mediates constitutive and IFN-γ-induced expression of HLA-class I molecules [81–85]. S box function is not fully understood and could possibly play a role in promoter architecture [86].

Like classical *HLA*-class I promoters, *HLA-G* promoter exhibits a CCAAT box and an unusual TATA element, TCTTAA, controlling basal regulation. A transcriptional initiation site located 25 bp downstream of TATA is conserved in *HLA-G*. Nonetheless, a second putative initiation site of *HLA-G* transcription has been reported 51 bp upstream of TATA (CTCACTCCC) (http://www.ncbi.nlm.nih.gov/), but the functionality of both sites has not yet been demonstrated. On the other hand, a modified enhancer A and a deleted ISRE render the *HLA-G* gene promoter unresponsive to NF-κB [72] and IFN-γ [73]. The p50 homeodomain subunit of NF-κB displays a strong binding affinity to the two κB sites *in vitro*, but this subunit without p65/relA does not possess a transactivation function [72]. In addition, the upstream region encompassing the SXY module only contains conserved S and X1 sequences and despite its binding capacity *in vitro*, the intact X1 box is unresponsive to the RFX5 factor *in situ* [87, 88]. The absence of RFX5 binding and the presence of nucleotide

variations in X2-Y boxes explain the absence of a higher-order complex and the lack of CIITA recruitment at the *HLA-G* promoter *in situ* [88] and in transfection experiments with CIITA expression plasmid alone or in co-transfection experiments with luciferase reporter constructs containing the *HLA-G* promoter fragment [69, 86]. In comparison with non-classical HLA-class I genes, *HLA-E* and *HLA-F*, *HLA-G* promoter, with its unique characteristics, is the odd one out [68] since the HLA-E promoter is induced through the SXY module even if it is not regulated by enhancer A and ISRE, and *HLA-F* promoter exhibits extensive similarities with those of classical HLA-class I genes [69]. Furthermore, a putative negative regulatory element located in exon 1 of HLA class I genes, whose activity can be modulated by hormones [89], is also deleted in the *HLA-G* gene and might, in part, explain why *HLA-G* is expressed in human trophoblasts, whereas HLA class I genes are not.

Finally, Monarch 1, a factor belonging to the CATERPILLER gene family, which is expressed primarily by myeloid-monocytic cells, is the only factor found to enhance both classical HLA-class I and *HLA-G* genes. More particularly, Monarch-1 was demonstrated to enhance the *HLA-B* promoter but the regulatory target elements have not been identified to date [90].

Alternative regulatory elements within the *HLA-G* gene promoter

To investigate alternative transactivation pathways to the conserved MHC class I regulatory routes, one of the strategies commonly used was to perform transient transfections with luciferase reporter constructs containing *HLA-G* promoter fragments of different lengths. This identified a negative regulatory sequence within the *HLA-G* promoter fragment extending 450 bp from the ATG and a slightly induced activity of intron 2-containing construct in JEG-3 choriocarcinoma cells [91]. Other major strategies were the use of *HLA-G* transgenic mice into which *HLA-G* transgenes of different lengths were introduced, *in silico* analysis of the *HLA-G* gene promoter sequence, and the identification of the target sites of specific modulatory agents present in the placental or tumoral micro-environment. From these analyses emerged regions identified as putative key components of the *HLA-G* gene regulation. However, precise target sites and/or transcription factors have not always been clarified, as is the case for IFN-γ, leukaemia inhibitory factor (LIF) and hypoxia.

The locus control region

A positive regulatory region of the *HLA-G* gene was found that contained in a 244-bp HindIII/EcoR1 fragment located −1.2 kb from the ATG, using *HLA-G* transgenic mice [70, 92]. This fragment was demonstrated to be critical for spatio-temporal expression of HLA-G mRNAs by using a *HLA-G* transgene including the entire coding region, 1 kb of the 3′ flanking region and 1.2 or 1.4 kb of the 5′ flanking region (5.7- and 6.0-kb fragment, respectively). Indeed, the presence of the 244-bp HindIII/EcoR1 region was associated with a tissue-specific pattern of HLA-G expression in spongiotrophoblasts and mesenchymal cells in placenta and in thymus. HLA-G transcription obtained with the 6.0-kb fragment parallels that seen in human extraembryonic tissues during the course of gestation, reaching the highest levels of expression in trophoblast at day 12.5 and then decreasing progressively before parturition. The distal 244-bp fragment that is required for tissue-specific expression of HLA-G presents a similarity in function with a locus control region (LCR). In agreement with this, sequence analysis of the regulatory fragment has revealed similarities to important elements for the activity of the H3S region of the β-globin LCR [93] such as the TATA symmetrically surrounded by GGGTGG and the putative AP1-binding site [94]. Moreover, DNA binding assays with nuclear extracts from HLA-G^+ and HLA-G^- cells revealed the formation of several complexes in this region. Some of them are specific of HLA-G expression status, whereas others are shared complexes [65, 95]. One of these shared complexes has been identified in an independent study by Van den Elsen's group and corresponds to the DNA binding of ATF1/CREB1/c-jun on the CRE/TRE (cyclic AMP-response element/TPA-response element) located at position −1380/−1370 in the putative HLA-G LCR [96]. ChIP assay demonstrated the *in situ* binding of CREB-1 and c-jun to this region [96].

cAMP response element/TPA response element

The computer-assisted search for alternative putative regulatory elements in the promoter had led Van den Elsen's group to localize two additional functional CRE/TRE elements dispersed through the promoter region at positions −934 and −770 from the ATG [96]. EMSAs demonstrated that CRE/TRE_{-934} and CRE/TRE_{-770} bind CREB1 and ATF1/CREB1 factors, respectively. Promoter activity assays and mutagenesis studies revealed the crucial role of the three CREs for the basal level of *HLA-G* promoter activity and its transactivation, with the most important contribution of $CRE_{-1380/-1370}$ within putative LCR. In accordance with this, transient transfection of the CREB repressor ICER (inducing cAMP early repressor) inhibits the CREB-induced transactivation of the *HLA-G* gene's 1438-bp promoter, while enhanced transactivation occurs with the co-activators CBP/P300 (CREB binding Protein). Notably, CREB, CBP/P300 and *HLA-G* are co-expressed in extravillous cytotrophoblasts. Nonetheless, CREB association to the *HLA-G* gene promoter *in situ* was also observed in HLA-G^- cell lines, which strongly suggests that tissue-specific expression of HLA-G involves additional regulation mechanisms, including epigenetics.

Interferon-stimulated response element

The cascade of events initiated by IFNs involves the activation of JAK/STAT transduction pathways and the transactivation of the gene promoter with the ISRE and IFN-γ activation site (GAS). Despite a non-conserved HLA class I ISRE in the proximal promoter of HLA-G, several investigations have revealed that the *HLA-G* gene is responsive to up-regulation following treatment with IFN-α, IFN-β and IFN-γ [97, 98]. Enhancement of steady-state levels of *HLA-G* mRNA upon IFN treatments was observed in

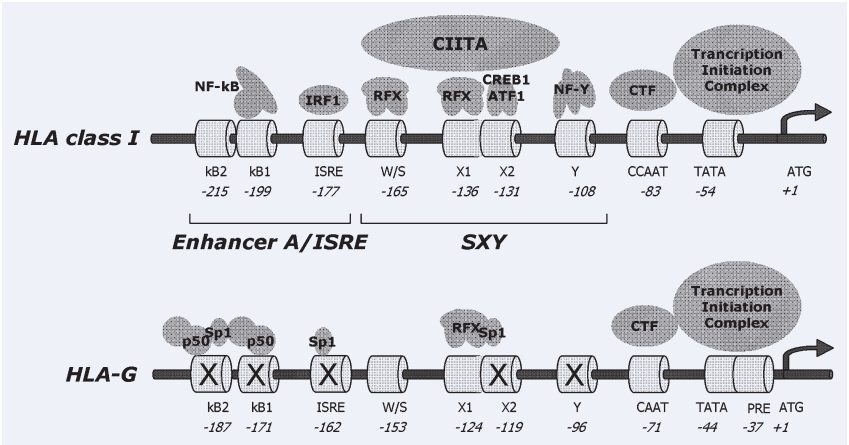

Fig. 1 HLA-class I and *HLA-G* gene promoter: cis-regulatory sequences and their interacting factors. Boxes with X within HLA-G promoter indicate that mutations prevent binding of classical HLA-class I transacting factors.

several cell types such as trophoblast cell lines [97], blood cells (monocytes and macrophage cell lines) [50] and glioblastoma cell lines [99]. In particular, IFN-β enhances the levels of *HLA-G* transcripts in trophoblast explants, amnion and thymus-derived epithelial cells [100]. However, it is a general rule that a basal *HLA-G* transcriptional level is required for IFN-induced up-regulation of *HLA-G* mRNAs [97] and then up-regulation of HLA-G cell surface expression.

Computer-assisted searches within the *HLA-G* promoter sequence led our group to identify an ISRE motif, which is highly homologous to the consensus ISRE. It is located at position −744 bp upstream of ATG, beside a GAS-like element (−734) previously shown to be unable to interact with a GAS-binding complex [98]. We demonstrated that the HLA-G ISRE is a binding site for IFN-response factor-1 (IRF-1) and transactivates HLA-G expression following IFN-β treatment [100]. Despite a weak induction in JEG-3 cells [96, 100], the activity of *HLA-G* promoter was clearly significant in the thymic epithelial cell LT-TEC2 [100].

Although IRF-1 binds to HLA-G ISRE, no transactivation effect in response to IFN-γ was observed in luciferase assays using the 1.4-kb *HLA-G* promoter [91, 96]. Besides, the use of a model system consisting of mouse fibroblasts transfected with a 6.0-kb fragment containing the whole *HLA-G* gene demonstrated the presence of elements that respond to IFN-γ [101]. Consequently, other regulatory pathways or IFN-γ responsive elements should be located outside the 1.4-kb promoter region with the *HLA-G* gene and/or the 3′UT region.

Heat shock element

Stress-induced proteins have been implicated in balancing immune responses during various diseases [102]. This prompted us to evaluate the effect of stress on *HLA-G* gene expression in the M8 (melanoma) and T98G (glioblastoma) HLA-G$^-$ cell lines using heat shock at 42°C or arsenite treatment for 2 hrs. Stress induced an increase in the level of *HLA-G* mRNA with a specificity compared to other HLA class I transcripts. Interestingly, HLA-G6 transcript was induced prior to the other *HLA-G* transcripts, suggesting tight control of *HLA-G* alternative splicing. The study also identified a heat shock element (HSE) within the *HLA-G* promoter at position −459/−454 that is defined as a repetition of the pentanucleotide NGAAN arranged in alternating orientation. The HSE seems to be functional since it binds heat shock factor-1 (HSF-1) *in vitro* by EMSAs [103]. Nonetheless, additional functional analysis using a reporter gene under the control of *HLA-G* promoter with wild-type or mutated HSE would be necessary to improve *HLA-G* HSE functionality.

Progesterone response element

Progesterone is an essential steroid to maintain pregnancy and has been suggested to be an important immune modulator during this time. That is why Librach's group investigated the potential effects of progesterone on *HLA-G* gene expression, revealing that at 10, 100 and 1000 ng/ml progesterone enhanced *HLA-G* mRNA expression in JEG-3 cells by 2.36-, 10.53-, and 17.58-fold, respectively, as compared to controls [104]. More recently, this group demonstrated that the *HLA-G* gene promoter is up-regulated by progesterone through a specific binding site for the progesterone receptor (PR) complex [105]. The identified progesterone response element (PRE)-like sequence is a 15-bp non-classical consensus core sequence that has 60% homology to the wild-type mouse mammary virus (MMTV) PRE and a weaker affinity for PR complexes than MMTV-PRE, probably because of variations in the fixed half site of steroid hormone response elements. This PRE is located −37 bp from the ATG and overlaps the HLA-G TATA box, but the authors do not exclude the presence of other PREs in the *HLA-G* promoter region. Indeed the chloramphenicol acetyltransferase reporter gene assay was not performed with the scrambled HLA-G PRE site and although the authors stipulated that PRE is specific of the *HLA-G* promoter, very similar sequences can be found at the same location in the HLA-class I promoter.

Leukaemia inhibitory factor target site

LIF is a pleiotropic cytokine that is expressed at the maternal–foetal interface and plays an essential part in embryo implantation

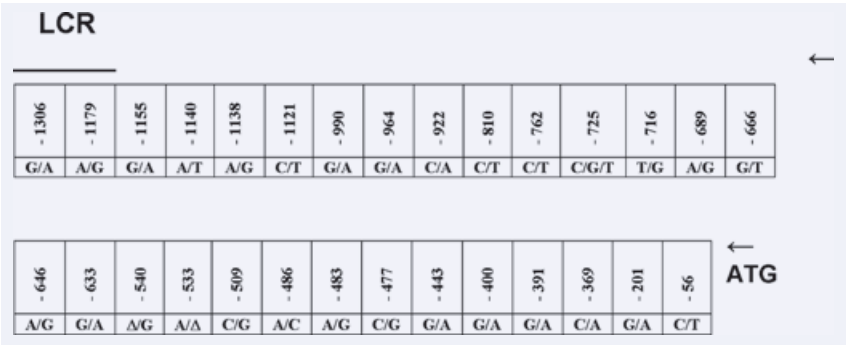

Fig. 2 SNPs along the 1.4 kb of the *HLA-G* gene promoter sequence. ATG: initiation codon; LCR: locus control region; Δ: deletion.

and in mediating interactions between maternal decidual leucocytes and trophoblasts [106, 107]. Upon 72 hrs stimulation with LIF, an up to 3.6-fold elevation of HLA-G mRNA has been demonstrated with JEG-3 choriocarcinoma cells. Luciferase reporter gene assays demonstrated that the stimulation of transcription was driven by a 890-bp promoter fragment of the 5′ *HLA-G* gene flanking region [108]. Nonetheless, precise target site(s) have not yet been identified. Moreover, endoplasmic reticulum aminopeptidase-1 (ERAP1) is also induced by LIF and plays a role in presenting antigenic peptides to HLA-G and then mediates HLA-G cell surface expression [109].

Ras response elements

To further identify factors involved in the regulation of *HLA-G* gene expression, our group recently developed a specific proteomic approach to characterize proteins differentially bound to the proximal and distal *HLA-G* gene promoter. Biotinylated, double-stranded *HLA-G* promoter fragments were incubated with nuclear protein extracts of HLA-G$^+$ and HLA-G$^-$ cells for transcriptional activity and isolated with streptavidin-coated magnetic beads. This DNA-affinity strategy was followed by a 2D separation and the proteins of interest were analysed using mass spectrometry. This allowed us to identify the zinc finger protein Ras responsive element binding 1 (RREB-1) [110] that is capable of binding three Ras response elements (RREs) along the *HLA-G* gene promoter.

We demonstrated that RREB-1 is involved in the repression of *HLA-G* transcriptional activity, acting through the recruitment of factors such as histone deacetylase 1 (HDAC1) and C-terminal binding protein (CtBP) [111] implicated in chromatin remodelling (Flajollet *et al.*, unpublished data).

Sequence polymorphism within the *HLA-G* gene promoter and the 3′UT region

Evidence has been accumulated showing that the *HLA-G* gene polymorphism is involved in the regulation of the *HLA-G* gene transcriptional activity. In particular, some *HLA-G* allelic variants are associated with differences in the pattern of *HLA-G* mRNA isoforms and *HLA-G* mRNA levels [112, 113]. On the one hand, polymorphism in the 3′UT region such as the absence or presence of 14 bp of 'exon 8' has been studied more extensively. The presence of the 14 bp is associated with low levels of mRNA expression [113, 114] and mediates or is involved in the out-splicing of the first 92 b of exon 8 [115]. These transcripts were shown to be more stable than the complete RNA [116]. Moreover, a C/G single nucleotide polymorphism (SNP) at +3142 bp in the *HLA-G* mRNA has recently been demonstrated to influence the targeting of three microRNAs [24]. On the other hand, an effort has been made to extend the analysis of *HLA-G* gene variations to the promoter region. To date, 29 SNPs have been identified [22, 23, 117] within this region (Fig. 2) and we cannot exclude that in some cases polymorphism on the promoter may be in linkage disequilibrium with 3′UT variants and that some of them could influence alternative splicing [118]. Interestingly, many of the polymorphisms either coincide with or are closed to the known regulatory elements and thus may affect the binding of the corresponding regulatory factors (Fig. 2).

Modulation of *HLA-G* transcription by micro-environmental factors with unidentified target sites

The restricted expression of *HLA-G* in physiological conditions and its up-regulation in pathological situations reveals a significant correlation of *HLA-G* transcription with biochemical environment [49]. This is strongly supported by the fact that *HLA-G* expression and transcription may be specifically down-regulated or even lost during long-term culture of biopsy-derived cancer cells [60, 61].

Variations of environmental factors such as growth factor, cytokines, hormones, physical conditions and stress dramatically occur during pregnancy as well as during inflammation, viral infection and cancer. Interestingly, numerous micro-environmental factors and molecular circuits are shared by placental and pathological situations such as cancer [119]; some of them have been demonstrated to be involved in *HLA-G* gene transcription. However, with the exception of IFNs and progesterone, the mechanisms by which most of these key regulatory molecules exert control on the *HLA-G* gene transcription, either directly on the *HLA-G* promoter or not, need further exploration.

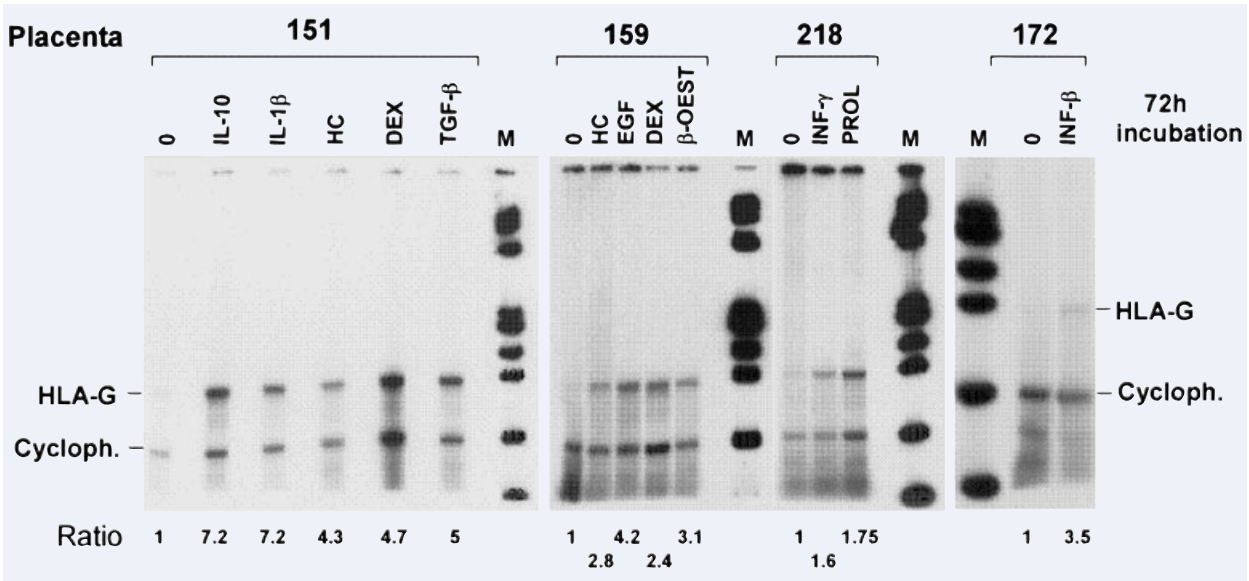

Fig. 3 RNAse protection assay analysis of *HLA-G* mRNA levels in four trophoblast organ explants (namely 151, 159, 218, 172) of first-trimester gestation incubated 72 hrs without (0) or with micro-environmental factors (IL-10: 2 ng/ml; IL-1β: 17 pg/ml; HC: 10^{-8} mol/l hydrocortisone; DEX: 10^{-7} mol/l dexamethasone; TGF-β: 10 ng/ml; EGF: 20 ng/ml; β-OEST: 10^{-7} mol/l β-oestradiol; IFN-γ: 100 U/ml; PROL: 10^{-10} mol/L prolactine; IFN-β: 1000 U/ml). The HLA-G template used for the riboprobe was obtained by PCR amplification of *HLA-G* genomic fragment located in the 3′UT region as previously described [124]. Ratio indicates the values obtained for HLA-G signals normalized to the constitutively expressed cyclophilin (Cycloph.) signal compared with untreated trophoblasts (assigned as value of 1). M: molecular weight size marker.

Cytokines, growth factors and hormones

Placenta is the main HLA-G-producing site that releases and/or is in contact with a variety of cytokines, both anti-inflammatory (IL-10, IL-4, IL-5, IL-6) and pro-inflammatory (tumor necrosis factor [TNF]-α, IL-1β, IL-2), and transforming growth factors (TGF-β), granulocyte macrophage colony stimulating factor [GM-CSF], granulocyte colony stimulating factor [G-CSF], colony-stimulating factor [CSF-1], LIF and epidermal growth factor [EGF]). Autocrine and paracrine mechanisms occur through specific cytokine receptors. Cytokines are crucial for successful embryo implantation and contributes to the maternal metabolic changes necessary to accommodate the increased energy needs of the foetus. Pregnancy is associated with dynamic changes in cytokine levels and ratios and is mainly characterized by an increase in the concentration of cytokines in the second half of pregnancy, with many changes orchestrated around IL-12 [120, 121]. Pregnancy also affects hormones in the body, mostly because of the effects of hormones produced by the placenta. In particular, human chorionic gonadotrophin, produced by the developing placenta, stimulates the ovaries to produce the oestrogen and progesterone needed to sustain pregnancy. By the fourth month of pregnancy, the placenta takes over from the ovaries as the main producer of oestrogen and progesterone [122]. These hormones are involved in womb changes to make room for the growing baby. Other hormones come into play that help the womb to contract during and after labour (oxytocin) as well as stimulate the production and release of breast milk (prolactin).

Our group suggested that these molecules might influence *HLA-G* gene expression, so we investigated the effect of a part of the network of cytokines and hormones involved in the placental or shared tumoral micro-environment on the *HLA-G* transcription of cultured trophoblast explants (Fig. 3). As a result, we observed that the amounts of *HLA-G* transcripts increased approximately 7.2-fold following treatment with IL-10 [123] and IL-1β, and from approximately 1.6- to fivefold with glucocorticoid hormones [124], TGF-β, EGF, β-oestradiol, prolactin and IFNs in comparison with *HLA-G* transcript levels in untreated trophoblasts. Besides, IL-10, a major suppressor of the immune response and inflammation [125], was demonstrated to increase HLA-G mRNA and cell surface protein expression by monocytes [123] and renal cell carcinoma (RCC) cell lines [126], *HLA-G* mRNA and soluble HLA-G protein by mononuclear cells from patients suffering from non-Hodgkin lymphomas (T-NHL) [127], acute myeloblastic leukaemia (AML) and acute lymphoblastic leukaemia (B-ALL), and HLA-G protein cell surface expression by decidual stromal cells [49] and the FON melanoma cell line [60]. Notably, reduced placental IL-10 production occurs in human pathological pregnancies such as in pre-eclampsia [128] in which a defect in HLA-G transcription is observed [114, 129]. Likewise, IL-10 production in cancer is associated with *HLA-G* expression [130] and the IL-10 homolog produced by cytomegalovirus (CMV) can up-regulate HLA-G protein expression at the monocyte cell surface [131]. On the other hand, GM-CSF treatment combined with IFN-γ and/or IL-2 has been shown to enhance both *HLA-G* mRNA and soluble

HLA-G protein in mononuclear cells from patients suffering from T-NHL [127], AML and T-ALL [132]. Moreover, cell surface expression on the U937 monohistiocyte cell line was demonstrated, but the effect on the *HLA-G* transcription was not investigated [133, 134] (Table 2).

Yet, although preliminary data show a clear potential effect of cytokines, growth factors, and hormone on the modulation of *HLA-G* transcription, some of them require additional investigation and it is likely that a combination of agents might be an interesting and pertinent avenue of research. Moreover, the molecular mechanisms responsible for enhancing *HLA-G* transcription by almost all the modulators described, either alone or in combination, remain to be elucidated. In particular, they could act through promoters of transacting factors instead of direct interaction with the *HLA-G* gene promoter and they also might be involved in RNA stability. Whatever the mechanisms at work, the efficiency of IL-10 or IFN treatment requires basal transcriptional activity to enhance *HLA-G* gene activity. A hypoxic environment is one possible candidate factor to reverse *HLA-G* gene repression.

Hypoxia

Maintenance of oxygen (O_2) homeostasis is critical for the maintenance of life. Hypoxia induces a series of adaptive physiological responses observed in biological processes such as maternal-foetal interactions and cancer, since it is associated with cell proliferation. Hypoxia followed by normoxia regulates the depth of the trophoblastic invasion and the vascular remodelling of the uterine tissues [135]. Tumoral hypoxia (up to 50–60% of solid tumours) arises as a result of an imbalance between the supply and consumption of O_2 [136]. In response to hypoxic conditions and to restore pO_2 homeostasis, cells instantaneously express a key factor, the hypoxia-inducible factor (HIF), which activates transcription of over 70 genes controlling glycolysis, glucose transport, cell survival and death, cell adhesion, angiogenesis and erythropoiesis [137, 138]. HIF is a heterodimer composed of constitutively expressed HIF-1β and inducibly expressed HIF-1α subunits. Under normoxic conditions (21% O_2), HIF-1α is hydroxylated and targeted by the van Hippel–Lindau tumour-suppressor protein, which upon synthesis causes its rapid degradation by the ubiquitin-26S proteasome pathways. With a low O_2 level ($<$6%) hydroxylation in HIF-1α is inhibited, resulting in a translocation of the subunit into the nucleus, a dimerization with HIF-1β, and binding to the hypoxia responsive element (HRE) (RCGTG consensus sequence) on the promoter of target genes [139]. Besides, HIF is able to interact with the transcriptional co-activators CBP/P300, SRC1, TIF2, which potentiate transactivation [137, 140].

Stress conditions such as heat shock or arsenite treatment were demonstrated to induce *HLA-G* gene transcription in HLA-G$^-$ cells. The effect of hypoxic stress on the *HLA-G* transcription was first assessed by Ferrone's group using the iron chelator desferrioxamine (DFX) to stabilize HIF-1α in HLA-G$^-$ melanoma cells. They found that DFX treatment induces *HLA-G* gene transcription in seven of 13 melanoma cell lines. The effect is dose- and time-dependent and approximatively 16-fold lower than the level of constitutive mRNA in the JEG-3 choriocarcinoma cell line [141]. Our group confirmed these results with the M8 melanoma cell line, thus supporting hypoxia as a candidate micro-environmental factor to reverse *HLA-G* gene repression [142]. The effect on the *HLA-G* mRNA up-regulation was further observed with undifferentiated cytotrophoblasts isolated from first-trimester placenta cultured with different concentrations (20%, 8%, 2%) of O_2 [143] and with Raji (Burkitt's B lymphoma) cells [144]. The computer search analysis of the *HLA-G* gene promoter sequence identified a putative consensus HRE located $-$243 bp upstream of the ATG. Whether this HRE is functional is still unknown [141].

As for the effect of hypoxia on HLA-G cell surface expression, results differ depending on the cell type or culture conditions. It is likely that post-transcriptional mechanisms may be involved since the lack of HLA-G translation can be observed in cells with hypoxia-induced mRNA [141, 142]. On top of that, the down-regulation of HLA-G expression is reported in cells expressing HLA-G at the cell surface following exposure to low O_2 concentration [145] or DFX [142].

Chromatin remodelling at the *HLA-G* gene locus

The epigenetic control of gene promoters is a critical mechanism in transcriptional regulation since it determines the accessibility and recruitment of regulatory factors to the DNA. Epigenetic modifications involve DNA methylation and histone tail modifications such as acetylation, phosphorylation, methylation, ubiquitylation and sumoylation [146]. Epigenetic processes control implantation, placentation, organ formation and foetal growth. In particular, there is a stepwise decline in DNA methylation from fertilization until the morula stage [147] and this correlates with the activation of *HLA-G* gene transcription [39, 40]. Alteration in epigenetics may contribute to pathological situations such as pre-eclampsia [148] and are widely recognized as contributing to tumorigenesis [149].

Evidence for HLA-G silencing by a DNA methylation process was reported first by Le Bouteiller's group in the HLA-G$^-$ choriocarcinoma JAR cell line [150, 151]. The use of demethylating agents such as 5-azacytidine and 5-aza-2′-deoxycitidine (5-Aza-dC) further demonstrated that the repression of *HLA-G* gene activity in cultured cell lines of various origins is reversed by demethylating treatment [144, 150, 152–155] and is maintained at least 5 days [153]. This treatment may also enhance steady state levels of *HLA-G* mRNA [60, 153] and it directly induces HLA-G protein expression in JAR, Raji (Burkitt's B lymphoma, LCL721.221 (lymphoblastoid B cell) [152], OCM-1A (melanoma) [154] and RCC cells [155] as well as human leukaemia cell lines [156]. The HLA-G inhibitory process seems to be independent of the expression of other HLA-class I and HLA-class II, which are detected differentially according to the cell line. By direct sequencing of bisulphite-treated DNA, one study found no correlation

Table 2 *HLA-G* transcriptional effectors and effect on HLA-G expression

Effector molecules	Modulation of *HLA-G* gene expression				References
	mRNA	Protein			
		Flow cytometry	Western blot	ELISA/ELIspot	
Growth factors/cytokines					
EGF	↑ . Trophoblasts	nd	nd	nd	Present paper
GM-CSF + IFN-γ	↑/= . Mononuclear cells from T-NHL, AML,T-ALL	↑ . U937 (histiocytic lymphoma)	↑ . Mononuclear cells from T-NHL	↑/= . Mononuclear cells^{m} from AML, B-ALL, T-ALL, T-NHL = . PBMC	[127] [132–134]
IL-1 β	↑ . Trophoblasts	nd	nd	nd	Present paper
IL-2 + IFN-γ	↑ . Mononuclear cells from T-NHL	↑ . U937	nd	↑ . Mononuclear cells from T-NHL	[127] [133]
IL-2 + IFN-γ + GM-CSF	↑ . Mononuclear cells from T-NHL	nd	↑ . Mononuclear cells from T-NHL	↑ Mononuclear cells^{m} from B-ALL, T-NHL = . PBMC	[127]
IL-10	↑ . Trophoblasts . Monocytes ↑/= . Mononuclear cells from AML, B-ALL, T-NHL . RCC cell lines	↑ . Monocytes . Decidual stromal cells ↑/= . RCC cell lines = . FON (melanoma)	↑ . Mononuclear cells from T-NHL . Decidual stromal cells	↑ . JEG-3 ↑/= . Mononuclear cells from AML, T-NHL = . PBMC	[49] [60] [123] [126, 127] [132] [162]
IFN-α	↑ . JEG-3 . U937 . Blood monocytes = . JAR	↑ . U937 .THP-1 (acute monocytic leukaemia) = . JEG-3	nd	↑ . JEG-3 . Serum of treated patient with melanoma	[50] [162, 163]
IFN-β	↑ . JEG-3 . U937 . Blood monocytes .Thymic epithelial cells . Amniotic epithelial cells = . JAR	↑ .Thymic epithelial cells . Amniotic epithelial cells ↑ . U937 . THP-1 = . JEG-3	nd	↑ . JEG-3	[50] [99] [162]

Continued

Table 2 Continued

Effector molecules	Modulation of *HLA-G* gene expression				References
	mRNA	Protein			
		Flow cytometry	Western blot	ELISA/ELIspot	
IFN-γ	↑ . JEG-3	↑ . U937	↑ . Decidual stromal cells	↑ . JEG-3	[49, 50]
	. U937 . Blood monocytes . THP-1	. Blood monocytes . THP-1		= . PBMC^{m} . Mononuclear cells^{m} from AML, B-ALL, T-ALL	[60] [123] [126] [162] [164]
		. HL-60 (acute promyelocytic leukaemia)			
	. RCC cell lines . Glioblastoma cell lines	. Decidual stromal cells . FON (melanoma)			
	= . JAR	. RCC cell lines = . JEG-3			
LIF	↑ . JEG-3	↑ . JEG-3 = . FON	nd	↑ . JEG-3	[60] [108] [109]
TGF-β	↑ . Trophoblasts	↓ . FON	nd	nd	Present paper [60]
TNF-α/PMA	= . JEG-3	↓ . JEG-3 . FON	↑ . JEG-3 . FON	↑ . M8-HLA-G1	[34]
		. M8-HLA-G1 (Melanoma transfectant)	. M8-HLA-G1		
Hormones					
β-oestradiol	↑ . Trophoblasts	nd	nd	nd	
Progesterone	↑ . JEG-3	nd	↑ . JEG-3 .Cytotrophoblasts	↑ . JEG-3 . Trophoblasts	[104, 105] Present paper
Progesterone +cAMP	nd	↑ . Decidual stromal cells	↑ . Decidual stromal cells	nd	[49]
Glucocorticoids	↑ . Trophoblasts	nd	nd	nd	Present paper
Prolactin	↑ . Trophoblasts	nd	nd	nd	Present paper
Stress					
Arsenite	↑ . M8 [a] (melanoma) . T98G [a] (glioblastoma)	= . M8 . T98G	nd	nd	[103]

Continued

Table 2 Continued

Effector molecules	Modulation of *HLA-G* gene expression				References
	mRNA	Protein			
		Flow cytometry	Western blot	ELISA/ELIspot	
Heat shock	↑ . M8 [a] . T98G [a] . JAR . Raji	= . M8 . T98G	nd	nd	[103] [141] [144]
Hypoxia/DFX/CoCl2	↑ . Extravillous cytotrophoblasts ↑ . Melanoma cells (M8 [a], 1074mel [a]) . Raji [a] (Burkitt's B lymphoma) = . JAR	↓ . HTR-8/SVneo on matrigel (first-trimester cytotrophoblast) . FON . JEG-3	↓ . HTR-8/SVneo on matrigel	nd	[142] [144, 145] [165]
Epigenetic treatments					
5-azacytidine/5-aza-2′deoxycytidine	↑ . JAR [a] . FON . Melanoma cell lines (OCM-1a[a], M8 [a], FON) . Glioblastoma (U87MG, LN-229, LN-428) . B,T and myelo-monocytic Leukaemia cell lines. . BG-1 [a] (ovarian cancer cells) ↑/= . RCC [a] cell lines ↑ . Tera-2 (lung embryonic carcinoma)	↑ . JAR . FON . Raji . LCL721.221 (lymphoblastoid B cells) = . NKL (NK cell leukaemia) . KG1a (acute myelogenous leukaemia) . M8	↑ . JAR . Raji . LCL721.221 = . NKL . KG1a . M8	↑/= . RCC cell lines	[60] [126] [144] [150] [152–154] [156] [158]
NaBu/TSA/VA	↑ . M8 . JAR . Raji	nd	nd	nd	[144] [152]

ALL: acute lymphoblastic leukaemia; AML: acute myeloblastic leukaemia; DFX: desferrioxamine; NHL: Non-Hodgkin lymphoma; PBMC: peripheral blood mononuclear cells and VA: valproic acid.
[a]: Raise HLA-G gene repression.
[m]: Mean of several experiments.
↑: up-regulation (↑ : low).
↓: down-regulation (↓ : low).
=: no effect.
↑/=: up-regulation or no effect.
↓/=: down-regulation or no effect.
nd: not determined.

between *HLA-G* gene transcriptional activity and methylation of 63 CpG islands at the *HLA-G* locus in blood cells expressing ($CD2^+$ lymphocytes) or not expressing ($CD34^+$ haematopoietic cells) *HLA-G* mRNA [157]. Nonetheless, recent data focussing on the promoter region covering 450 bp 5′ from the ATG (19 CpG sites) strongly argue for a cis-acting CpG methylation associated with *HLA-G* gene silencing [153, 154, 158]. Moreover, the analysis of chromatin remodelling at the *HLA-G* locus performed with histone deacetylase inhibitors (HDAC), trichostatin A (TSA) and sodium butyrate (NaBu) treatments, demonstrated the *HLA-G* gene activation in M8 (melanoma), JAR and Raji cells, despite a lower mRNA level in comparison with 5-Aza-dC treatment [144, 152]. To back this up, the analysis of H3 and H4 histone acetylation by ChIP of the proximal and distal *HLA-G* gene promoter showed the presence of hyperacetylated histones in $HLA\text{-}G^+$ cells (FON and JEG-3 cells), while hypoacetylated histones were predominant in $HLA\text{-}G^-$ cells (M8 and JAR cells) [153]. Thus, the acetylation of lysine residues of H3 and H4 renders the chromatin in a permissive state, arguing in favour of *HLA-G* gene expression.

Concluding remarks

The present data highlight the complexity of the regulation of non-classical *HLA-G* transcriptional activity, which is likely to be associated with the tight control of HLA-G function participating in immune tolerance. Several regulatory elements have now been identified, but some of them need further functional validation using reporter genes and interference RNA tools. The demonstration of their presence or absence *in situ* using ChIP assays is also required since the chromatin environment is crucial for binding and function. Cytokines and micro-environmental agents act on *HLA-G* gene expression in a cell-specific manner and may have pleiotropic activities. From this point of view, the medical relevance of the current findings on the HLA-G regulation should be carefully evaluated. In this regard, the development of animal models would make it possible to validate *in vivo* both the concept that HLA-G is a key component in immunoregulation and the molecular mechanisms modulating its expression. Moreover, an emerging point of view is that the impact of *HLA-G* gene polymorphism and temporal data on regulatory processes are needed to understand modifications at the *HLA-G* locus, particularly epigenetic changes following micro-environmental signals during placentation and various pathological situations.

Considering all the regulatory mechanisms known to date, they support a coherent regulatory model of *HLA-G* gene expression based on those previously proposed by Ferrone's group [159]: HLA-G is generally not expressed under non-pathological conditions *in vivo*, probably because the promoter is inactivated by DNA methylation and at least histone hypoacetylation. During *in vivo* proliferative processes, cells will be exposed to stress (*e.g.* hypoxia) and undergo epigenetic changes such as DNA demethylation and histone acetylation, leading to opened chromatin and accessibility to transcription factors. It is likely that sequence variations at specific transcription factor target sites influence the level of response. Upon gene activation, cytokines and hormones of the micro-environment will enhance the amounts of *HLA-G* transcripts and then protein expression. The micro-environment could also induce antigen-processing machinery components, thus contributing to the transport and stabilization of HLA-G molecules at the cell surface. TNF-α may enhance intracytoplasmic HLA-G cell content and may enhance HLA-G1 proteolytic shedding following NF-κB activation. Upon adaptation to tissue culture *in vitro*, cells may not be exposed to the same stressful conditions and *HLA-G* transcription may be changed. Thus long-term growth *in vitro* may subsequently lead to methylation and hypoacetylation of the *HLA-G* promoter and silence the gene.

Finally, the fact that demethylation treatment may activate *HLA-G* receptor genes [160, 161] should be considered in cancer therapy using treatments blocking HDAC and/or reversing DNA methylation to enhance tumour suppressor genes. These treatments might favour the enhancement of both *HLA-G* at the cell surface of tumoral cells, and KIR protein expression at the cell surface of tumour infiltrating lymphocytes might thus favour tumour escape. Therefore, in addition to the crucial need to better understand HLA-G function, extensive studies on the control of *HLA-G* gene expression are fundamental to developing non-deleterious therapeutic strategies.

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RREB-1 Is a Transcriptional Repressor of HLA-G[1]

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The nonclassical HLA-G is a molecule specifically involved in immune tolerance with highly restricted tissue distribution in healthy conditions. Yet it is overexpressed in numerous tumors and in allografts with better acceptance. Major mechanisms involved in regulation of *HLA-G* transcription are still poorly described. Thus, to characterize these mechanisms we have developed a specific proteomic approach to identify proteins that bind differentially to the *HLA-G* gene promoter by promoter pull-down assay followed by spectrometry mass analysis. Among specific binding factors, we focused on RREB-1, a *ras*-responsive element binding protein 1. We demonstrated that RREB-1 represses *HLA-G* transcriptional activity and binds three *ras* response elements within the *HLA-G* promoter. RREB-1 protein, specifically in HLA-G-negative cells, interacts with subunits of CtBP complex implicated in chromatin remodeling. This demonstration is the first of a repressor factor of *HLA-G* transcriptional activity taking part in *HLA-G* repression by epigenetic mechanisms. *The Journal of Immunology,* **2009, 183: 6948–6959.**

The HLA-G molecule is a nonclassical HLA class I molecule proposed to play an important role in immune tolerance, particularly in the maternal immunological accommodation of the fetus (1). Despite a mounting number of publications, data on HLA-G function and tissue distribution have described both well-established features and unresolved questions (2).

Evidences have been accumulated showing that HLA-G inhibits the NK (3) and T lymphocyte-mediated direct cytotoxicity (4). It also inhibits the proliferative allogenic response (5) through interaction with inhibitory receptors ILT2/CD85j/LILRB1 (6) and ILT4/CD85d/LILRB2 (7). Whether KIR2DL4/CD158d (8) is expressed in vivo and can bind HLA-G is still unclear (2).

In nonpathological conditions, the expression of HLA-G proteins is restricted. HLA-G is detected in a few fetal tissues, which include cytotrophoblasts and amnion of the placenta (9, 10) and a few healthy adult tissues, including thymus (11), cornea (12), pancreas (13), and erythroblasts (14). Nonetheless, because all HLA-I molecules share 80% amino acid, cross-reactivity to the other HLA by HLA-G Abs has been hypothesized for studies in which biochemical analysis to identify the 39-kD H chain of HLA-G was not done. However, the surface expression of HLA-G expression in vivo is consistently and reproducibly detected in extravillous trophoblasts with all anti-HLA-G Ab and by varied techniques. HLA-G expression is also reported in pathological conditions. HLA-G is found in numerous tumors and then would favor malignant cell development by impairing antitumor immunity (15, 16). However, it is of note that there is controversy about some of these findings based on means of detecting specific HLA-G expression. HLA-G is also observed in biopsy samples and sera of patients undergoing transplantation and is associated with a reduced number of rejection episodes (17, 18). Notably, the presence of stable HLA-G protein expression is correlated with high levels of *HLA-G* transcripts, whereas its absence in almost all nonpathological situations is correlated with low level or absence of *HLA-G* transcripts (19, 20). Thus, the understanding of molecular mechanisms that regulate *HLA-G* transcription is crucial.

HLA-G gene promoter structure and mRNA have been previously published (21–23) and are reported in the NCBI database. *HLA-G* gene consists of exon 1 (5′-ATG) encoding the signal peptide, exons 2, 3, and 4 encoding α1, α2, and α3 extracellular domains, respectively and exon 5 encoding transmembrane domain and a very short cytoplasmic tail together with exon 6. A major feature of HLA-G is the alternative splicing of primary transcript that deletes specific exons or retains introns, thus giving rise to seven proteins isoforms (24–26). Promoter regions have been initially defined by comparison with classical *HLA-I* gene promoters (27, 28) include transcriptional control of classical *HLA* class I genes (HLA-A, HLA-B, and HLA-C) and β_2-microglogulin genes, which are mediated by a conserved *cis*-acting regulatory element scattered through the region named "proximal promoter." These major regulatory elements include CCAAT and unusual TATA (TCTTAA) elements controlling basal regulation and a transcriptional initiation site located 25-bp downstream of TATA, that are conserved in HLA-G. Nonetheless, a second putative initiation site of HLA-G transcription can be observed 51-bp upstream of TATA (CTCACTCCC), but its functionality is not currently demonstrated. Other regulatory elements are enhancer A, IFN-stimulated response element, and the SXY module, which is crucial for the constitutive and CIITA-mediated HLA class I gene transactivation (27, 29). HLA class I-acting regulatory elements (except S1 and X1 boxes) are disrupted within the *HLA-G* gene proximal promoter region rendering this gene unresponsive to NF-κB, IFN regulatory factor 1, and CIITA-mediated induction pathways (30, 31).

HLA-G gene transcriptional machinery has been poorly described even if some of the mechanisms that govern *HLA-G* transcriptional activity have been identified (23). Using HLA-G

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transgenic mice a putative locus control region (LCR)[3] has been found located −1.2 kb from the ATG translation start site (20, 32–34) (named "distal promoter" region). A few *HLA-G* promoter binding factors and their target sites have been characterized such as CREB1 (28), IFN regulatory factor 1 (35), heat shock transcription factor 1 (36), or progesterone receptor (37). Microenvironmental parameters have been shown to play a key role in the modulation of *HLA-G* gene, including stress conditions such as heat shock (36) or hypoxia (38), cytokines such as IFN (35, 39), IL-10 (40), leukemia inhibitory factor (41), GM-CSF (42), and hormones such as glucocorticoids (43) or progesterone (44). However, for most of them the mechanism of action remains unknown. Recently, we provided evidence for *cis*-acting epigenetic mechanisms like CpG methylation and histone deacetylation involved in *HLA-G* gene response (45, 46). The recruitment of repressor regulatory mechanisms in HLA-G negative cells and the possible presence of negative regulatory factors have been considered (20, 30, 34) but no corepressor factor has yet been determined.

In an attempt to better understand the regulatory mechanisms that participate in physiological and pathological expression of HLA-G, we have developed a strategy to isolate and characterize protein complexes implied in *HLA-G* gene transcriptional machinery. We used a comparative proteomic approach to identify proteins that bind differentially to the *HLA-G* gene promoter by promoter pull-down assay, followed by spectrometry mass analysis. This search led to the identification of *ras*-responsive element binding protein 1 (RREB-1) in protein extracts from HLA-G-negative cells. We provide evidence of the role of RREB-1 in repression of *HLA-G* transcription through the recruitment of C-terminal binding protein (CtBP)1, CtBP2, or both and histone deacetylase (HDAC)1, which are subunits of CtBP complex, a corepressor involved in chromatin remodeling. Ours results further propose that repressive activity of RREB-1 on *HLA-G* promoter may be regulated by posttranslational modifications that govern association with CtBP.

Materials and Methods

Plasmids

The plasmids encoding for RREB-1 were a gift from A. Leiter (University of Massachusetts Medical School, Worcester, MA). The p1400 Luciferase (1438 bp of *HLA-G* promoter) and p500 Luciferase (500 bp of *HLA-G* promoter) reporter gene have been previously described (46). Mutated *ras*-responsive element (RRE) sites were introduced within the p1400 luciferase using complementary mutated oligonucleotides carrying the same mutations as in EMSA. Site-directed mutagenesis of the three RREB-1 binding sites was performed by MilleGen. Mutated plasmid p1400 RRE-mut-Luc was verified by sequencing.

Human cell line culture

The chroriocarcinoma cell line JEG-3 (American Type Culture Collection) and HeLa cell line were grown in DMEM supplemented with Glutamax-I, 4500 mg/L glucose (Invitrogen), 10% FCS (BioWhittaker), 1000 U of penicillin, and 10 μg/ml streptomycin. The melanoma cell line M8 and the Burkitt's lymphoma B cell line Raji were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 10% FCS (Biowhittaker), 1000 U of penicillin, and 10 μg/ml streptomycin. All cultures were maintained at 37°C and 5% CO_2.

Whole cell extracts obtained by freeze-thaw cycles

Cells (~50 million) were pelleted, washed with ice cold PBS, and resuspended in two packed cell volumes of a buffer containing 50 mM HEPES (pH 7.9), 400 nM KCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 2 mM DTT, 5 μg/ml leupeptin (Sigma-Aldrich), 1 mM PMSF (Roche Diagnostics), 0.5 mM Na_3VO_4, and 0.5 mM sodium fluoride and protease inhibitor mix (Complete; Roche Diagnostics). Cell extracts were obtained by three successive freeze-thaw cycles, snap frozen in liquid nitrogen and stored at −80°C. Protein extracts were cleared by centrifugation and stored at −80°C.

Promoter pull down assays

Promoter pull-down assays were performed as described (47) (Fig. 1*A*). Briefly, promoter templates biotinylated at the 5′ end of the upper strand were generated by PCR using primers HLA-G (forward) 5′-biot-CCCGCGTTGGGGATTCTCTC and (reverse) 5′-ATGAGTCCGGGTGG GTGAGC. Specificity of PCR products (20 pmol) was verified by sequencing (Genoscreen). Templates were coupled to 900 μg of streptavidin-coated magnetic beads (Streptavidin Paramagnetic Particle; Promega). Whole cell extracts (7 mg, 400–600 μl) from Raji or JEG cells were first preincubated for 30 min at 4°C with 1500 μg of paramagnetic beads (non-specific binding on beads) in a buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 9 mM $MgCl_2$, 0.1 mM EDTA, 0.01% Nonidet P-40, 0.15 mg/ml polydeoxyinosinic-polydeoxycytidylic acid (Amersham Biosciences), heat denaturated salmon sperm DNA (Invitrogen), and protease inhibitor mix (Complete; Roche Diagnostics). After discarding of beads, the supernatant was incubated with DNA template in the same buffer for over 2 h at 4°C with agitation. Beads were washed three times with the same buffer containing 1 mg/ml BSA. Proteins were eluted with 140 μl of buffer containing 9 M deionized urea (Bio-Rad), 4.4% CHAPS (Sigma-Aldrich), 5 mM tributylphosphine (Sigma-Aldrich), 0.22% Bio-Lyte 3–10 (Bio-Rad), and traces of bromophenol blue. After 1 h of incubation at room temperature, beads were discarded.

Two-dimensional gel electrophoresis

The supernatant was directly applied to IPG-Strip (pH 3–10; Bio-Rad) by 14–16 h rehydration at 20°C. Proteins were focused successively on Protean IEF Cell (Bio-Rad) during two steps: a desalting period because the current raise to 250 V, followed by a rapid ramping to 8000 V for 10,000 Volthours without exceeding 50 μA per IPG-Strip. Focused IPG strips were equilibrated for 15 min in a solution (6 M urea, 2% SDS, 20% glycerol, 375 mM Tris-HCl (pH 8.8), and 2% DTT) and then for an additional 15 min in the same solution except that DTT was replaced by 2.5% iodoacetamide (Bio-Rad) and trace bromophenol blue. After equilibration, two-dimensional SDS-PAGE was performed on Ettan DALT II system.

Gel scanning and image analysis

After SDS-PAGE, the gel was colored with Sypro ruby solution (Bio-Rad) overnight after 30 min fixing in 40% ethanol-10% acetic acid solution, 1 h in 10% ethanol-7% acetic acid, followed by 1 h in ultrapure water. Gel picture was captured on UV plate with a camera imager (Appligen) (Fig. 1, *B* and *C*) and analyzed, and relevant spots were excised.

Protein identification by MALDI-TOF

Selected differential protein spots were excised from preparative gels reduced in 1-mm^3 fragments and in-gel trypsin digestion was performed. The gels spots were washed during 5 min with two-distillation water and destained with a fresh solution containing 100 mM NH_4HCO_3 in 50% acetonitrile at 37°C for 5 min. After being dried with a speed-vac, gel fragments were incubated in digestion solution consisting of 100 mM NH_4CO_3, and 10 mM DTT at 56°C for 30 min, then rinsed with acetonitrile for 15 min, and incubated in 100 mM NH_4HCO_3, 55 mM iodoacetamide in the dark for an additional 20 min at 37°C before being dehydrated in acetonitrile and dryed with speed-vac. Fragments were rehydrated 40 min on ice in digestion solution contained 25 mM NH_4CO_3, 0.04% $CaCl_2$, 0.01% TFA, and 6 μg/ml trypsin-modified Sequencing Grade (Roche) for 2 h at 37°C. Action of the trypsin was inactivated with formic acid. The tryptic peptide mixture was extracted by a 10 min sonication and purified with Millipore ZIP-TIP C18 column (Millipore) following suppliers instructions. The purified tryptic peptide mixture was resuspended in 3 μl of 1% formic acid. A 0.5 μl of the mixture was mixed with one volume of solution containing saturated CCA (α-cyano-4-hydroxycinnamic; Sigma-Aldrich) as solution in 50:50 TFA 0.1% to acetonitril) or 2,5 dihydroxybenzoic acid (Sigma-Aldrich) as solution in 9:1 TFA 0.1% to ethanol. The 1 μl of the mixture CCA matrix was loaded on a stainless steel plate, air-dried, and analyzed with mass spectrometer. The standard peptide mixture (des-arg bradykinin, angiotensin, neurotensin, ACTH 18–39, ACTH 7–38) was spotted at the same time to correct the machine. Analysis were performed in positive mode with reflectron, using a 20,000 V acceleration tension, a 200 ns held extraction using a mean of 350 laser pulses on a mass range between 650 and 4000 Da.

[3] Abbreviations used in this paper: LCR, locus control region; CtBP, C-terminal binding protein; RRE, *ras*-responsive element; RREB, *ras*-responsive element binding protein; siRNA, small interference RNA; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation.

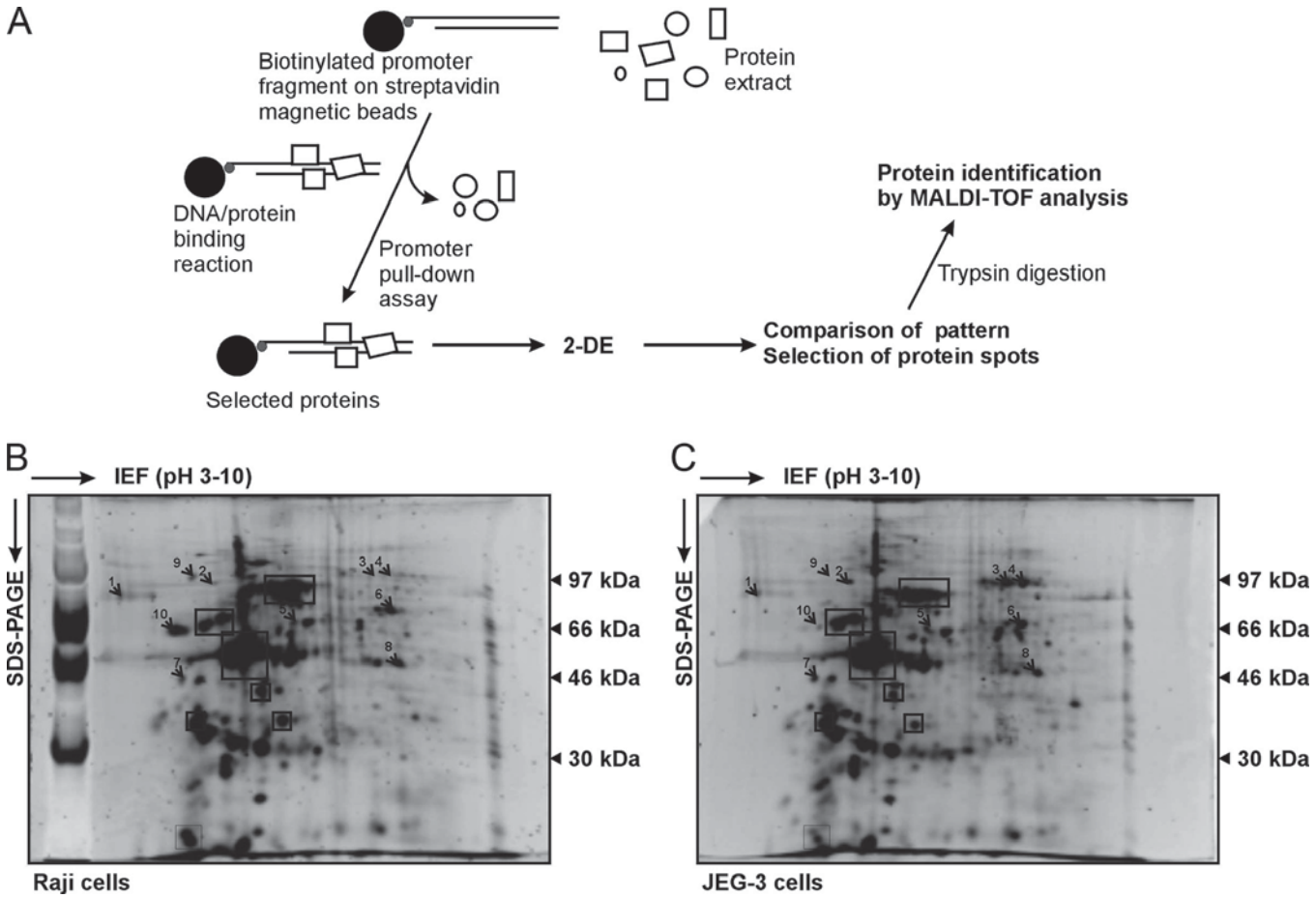

FIGURE 1. Representative two-dimensional gel electrophoresis (2-DE) pattern of protein extracts selected on HLA-G proximal promoter by pull-down assay. *A*, Strategy developed for the identification of *HLA-G* promoter binding proteins by combining proteomic to DNA affinity. *B* and *C*, Two-dimensional gel electrophoresis obtained with Raji protein extracts (negative HLA-G transcription) on the *left* (*B*) and JEG-3 protein extracts (positive HLA-G transcription) on the *right* (*C*) in at least two independent experiments. Protein extracts were used in promoter pull-down assays conducted with 170 bp fragment of *HLA-G* proximal promoter fragment (location of primers are in Fig. 4). Gels were stained with Sypro ruby solution using isoelectric focusing (IEF). Spots in boxes are the ones found at the same locations in gels obtained with control experiments using magnetic beads without DNA fragment to identify nonspecific protein binding to the matrix. Specific protein spots are indicated (arrowhead with number). MALDI-TOF analysis of spot 7 and spot 10 identified NF-Y and RREB-1 (Table I) factors, respectively.

Mass spectra were recorded in the reflectron positive mode of a MALDI-TOF mass spectrometer (DE-STRE Voyager; Applera Applied Biosystems) by summing 350 laser shots with an acceleration voltage of 20 kV, 200-ns delay. MALDI-TOF peptides mass maps were processed using the peptide mass fingerprinting analysis software ProFound (129.85.19.192/prowl-cgi/ProFound.exe). The NCBInr and Swissprot databases were used for the searches with several passes of searching with different limitations for each spots. In general, all spots were searched with methionine oxidation, no limitation for isoelectric point, and with the following parameters to find the best match: two missed cut cleavages, limited to the "mammal category", or a set with or without 50% of total molecular mass, alkylation partial chemical modifications, protonization charge, and 0.15 Da mass precision.

Transient transfection and luciferase activity assays

One day before transfection, cells were plated in 6-well plates with 50–60% confluence. Each well was transfected using Exgen 500 reagent (Fermentas) according to the manufacturer's instructions, with a DNA mixture including 1 μg of firefly luciferase reporter gene containing *HLA-G* promoter, 50 ng of a control plasmid tk-luciferase (tk-Renilla; Promega) and the indicated concentration of pCDNA-RREB-1. After 24 h of transfection, cells were harvested and luciferase activity was assayed with Bright-Glo luciferase assays System and *Renilla* luciferase assays system (Promega) using a luminometer (Lumar LB 9507; EG & G Berthold). Firefly luciferase values were normalized to those of *Renilla* luciferase values. Assays were run in duplicate, triplicate or more (two, three, or more independent experiments, respectively, each was performed as technical duplicate). In experiments in which RNA silencing analysis was performed, luciferase activity was assayed 48 h following small interference RNA (siRNA) transfection. In such a situation cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, first with siRNA and second (t = 24 h) with recombinant plasmids containing reporter genes.

RNA silencing

For RNA interference experiments, we used ON-TARGET plus SMART pool siRNA oligonucleotides (Dharmacon), a predesigned siRNA reagent against different splice variants of RREB-1. A siRNA control encoding scrambled sequence was used as control. siRNA were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Real-time RT-PCR analysis

RNA extraction, DNase I treatment, reverse transcription and real-time RT-PCR, were conducted as previously described (45, 46). Duplex PCR was performed with GAPDH as an endogenous control, HLA-G specific primers selected to amplify all alternative forms of HLA-G transcripts and a HLA-G-specific probe located in exon 5.

Cellular extracts preparation, immunoprecipitation, and Western blot

Cellular extract preparation and Western blot analyses were conducted as previously described (34). Immunoprecipitation assays were performed as described (48). Immunodetections were performed using the following Abs: anti-RREB-1 from Rockland; anti-HDAC1 (H-51), anti-CtBP (E-12

Table I. *Identification of human RREB-1 peptides (spot 10) by mass matching and MS/MS*[a]

Mass (*D*)			
Observed	Expected	Predicted Sequence	RREB-1 Peptide
1,005,420	1,005,428	NQETKEEK	37–44
120,538	1,200,546	SSYNCPLCEK	45–54
1,320,572	1,320,662	ISQAWCETNLR	278–288
1,522,574	1,522,762	KADEVFHCPVCFK	184–196
2,266,000	2,266,08	CTVCGQSFTTNGNMHRHMK	108–126

[a] Protein of spot 10 was excised and subjected to in-gel trypsin digestion. The observed molecular mass of peptides was compared with expected molecular mass of RREB-1 (by NCBInr and SwissProt databases). Human RREB-1 peptides were identified by mass matching and tandem mass spectrometry (MS/MS).

detects CtBP1 and CtBP2), and control rabbit IgG from Santa Cruz Biotechnology; anti-acetyl lysine (AB3879) from Chemicon; and monoclonal anti-α tubulin from Sigma-Aldrich.

EMSA analysis

The 2 μl of nuclear extracts were incubated with γ-^{32}P double-strand oligonucleotide probe (1–2 ng, 10^5 cpm) encompassing the RREs of *HLA-G* promoter, 2 μg of polydeoxyinosinic-polydeoxycytidylic acid (Amersham Biosciences) in binding buffer containing 40 mM KCL, 20 mM HEPES (pH 7.5), 0.1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM DTT, and 0.4% Ficoll. The following oligonucleotides (Eurogentec) were used for probes: HE-443 5′-GGAACTTGAGAGAGGACCGAAGAGAAGCA and 5′-GCTTCTCTTCGGTCCTCTCTCAAGTTCC) (49); RRE-53 5′-AGCGTCGCCGCGGTCCTGGTTCTAAAG and 5′-CTTTAGAACC AGGACCGCGGCGACGCT); RRE-142 5′-AACCTGTGTCGGGTCC TTCTTCCTGGATACTCACCG and 5′-CGGTGAGTATCCAGGAAG AAGGACCCGACACAGGTT); and RRE-1356 5′-CTGACTCATAGT AGCAGGACCACTATAGAGAG and 5′-CTCTCTATAGTGGTCCTG CTACTATGAGTCAG). The following oligonucleotides with mutated RRE sites were: RRE-53mut AGCGTCGCCGCAAAAAAGGTTCTA AAG and CTTTAGAACCTTTTTTGCGGCGACGCT; RRE-142mut AA CCTGTGTCGAAAAAATCTAAAAAAATACTCACCG and CGGTGA GTATTTTTTTAGATTTTTTCGACACAGGTT; and RRE-1356mut CT GACTCATAGTAGTAGGTGTTTTTTTAGAGAGandCTCTCTAAAAAA CACCTACTACTATGAGTCAG). Competition experiments were performed using 100-fold molar excess of cold probe. Complexes were separated by electrophoresis for 1–2 h at 200 V in 5% polyacrylamide gel (29:1 acrylamide to bisacrylamide ratio).

Combined Western blotting and EMSA

The assay WEMSA combines Western blotting and EMSA analyses (50). Nuclear extracts and reaction mixtures are prepared as described for EMSA, except for the use of unlabeled oligonucleotides. After incubation for 30 min at room temperature, reaction mixtures were separated by nondenaturing PAGE, exactly as done for EMSA. The separated DNA-protein complexes were then transferred to nitrocellulose membranes and probed with anti-RREB-1, anti-HDAC1, and anti-CtBP Abs by Western blotting as described below.

Chromatin immunoprecipitation assays (ChIP)

ChIP assays were performed as previously described (51) using anti-RREB-1 from Rockland; anti-acetylated histone H3 (06-599) and anti-phosphorylated Ser^{10} histone H3 (07-081) from Upstate Biotechnology Associates; and anti-RNApolII (C-21) and anti-HDAC1 (H-51) from Santa Cruz Biotechnology. Briefly, formaldehyde cross-linked chromatin was sonicated and immunoprecipitated with either no Ab or the indicated Ab. ChIP analyses were performed at least in triplicate using distinct DNA preparations. Immunoprecipitated DNA was purified and quantified by regular PCR. The *HLA-G* promoter targeted sequences (−187; +4) and (−1438; −1185) were amplified using the following primers: 5′-CCCG CGTTGGGGATTCTCTC-3′, 5′-AGAGGGTTCGGGGCGCCATGAC CAC-3′ and 5′-AAGCTTCACAAGAATGAGGTGGAGCCACTGG-3′, 5′-TAAACCTAAGAGTTCCGCTGCT-3′, respectively.

Results

Identification of proteins that bind to the HLA-G proximal promoter by two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry analysis

To isolate and identify factors associated with the *HLA-G* gene promoter and implied in HLA-G expression, we initiated a comparative proteomic study of elements bound to the *HLA-G* gene promoter using protein extracts from HLA-G-positive JEG-3 cells and HLA-G-negative Raji cells. JEG-3 choriocarcinoma is a well-documented HLA-G-positive cellular model, commonly used in numerous studies on HLA-G expression. Raji (Burkitt's B lymphoma) was selected as a second model instead of the HLA-G-negative choriocarcinoma JAR because we first hypothesized that cell differences compared with JEG-3 would be more marked at the *HLA-G* gene promoter using cells both having strong different cellular origin and absence of *HLA-G* gene transcription. Moreover previous analysis of CpG methylation at the *HLA-G* gene promoter strongly suggested that *HLA-G* gene repression in JAR was principally governed by *cis*-acting DNA methylation (46).

Biotinylated, doubled stranded HLA-G proximal promoter fragments (170 bp) (see Fig. 4) obtained by PCR on human genomic DNA were verified by DNA sequencing (data not shown), and then incubated with protein extracts of HLA-G-positive and HLA-G-negative cells. DNA affinity strategy was followed by two-dimensional gel electrophoresis separation, and proteins of interest were then digested by trypsin and analyzed by MALDI-TOF (Fig. 1*A*). Representative two-dimensional gel electrophoresis results obtained from at least two independent experiments reveal both differential and shared DNA binding proteins by comparing HLA-G-positive (Fig. 1*C*) and HLA-G-negative (Fig. 1*B*) cell patterns. Among the shared proteins, some of them were considered nonspecific binding factors (surrounded spots) because they were also revealed by two-dimensional gel electrophoresis analysis performed with magnetic beads alone (data not shown). Mass matching analysis allowed the identification of one shared specific factor (spot 7, Fig. 1, *B* and *C*), which corresponds to subunit C of NF-Y, a factor that binds the Y box (ATTGG) and CAAT-box motifs (52). The later target site is present within the HLA-G bait fragment. In contrast, by analyzing the presence or absence and the intensity of protein spots in two-dimensional patterns obtained with HLA-G-positive and HLA-G-negative cells, we focused on spot 10 that exhibits great intensity in Raji cells only. MALDI-TOF mass spectrometry analysis revealed that the matched peptides cover 20% of the RREB-1 protein sequence. A complete list of RREB-1 peptides identified by mass matching is shown in Table I. Based on protein functions using

information from the database of Swiss-Prot websites, this protein was associated with transcriptional activity, making RREB-1 a good candidate for *HLA-G* gene regulation. Main published data indicate that RREB-1 is a Kruppel-type C_2H_2 zinc finger DNA-binding protein, originally isolated from a human medullary thyroid carcinoma cell line, which was proposed to be involved in mediating a *ras*-driven differentiation program (53). RREB-1 was first identified as a 756 aa protein containing four zinc fingers generated by alternative splicing of FinB gene mRNA (54, 55). RREB-1 binds to the RRE (49, 53) and was demonstrated to regulate the expression of several genes (54–58).

RREB-1 exerts a repressive activity on the HLA-G *promoter in HLA-G-negative cells*

To further characterize RREB-1 implication in the *HLA-G* gene transcriptional regulation, transient transfection assays were performed in the HLA-G-negative HeLa cell line first. Such a cellular model is commonly used in transfection experiments, notably to analyze *HLA-G* promoter activity (20, 30, 34). Real-time RT-PCR analysis showed that steady state levels of HLA-G mRNA in HeLa cells were very low ($\sim$1.5 10^{-5}) compared with HLA-G-positive choriocarcinoma JEG-3 cells (assigned a value of 1) (data not shown). Transfection experiments were done in triplicate using a luciferase reporter construct driven by promoter fragment encompassing either 1.4 or 0.5 kb upstream to the start codon of the *HLA-G* gene. In the absence of transfected RREB-1 we, respectively, observed a 12-fold (mean of two independent experiments) and a 5-fold (one experiment) increase in luciferase activity in comparison to the promoter-less control vector (data not shown). Moreover, as demonstrated in Fig. 2 for both plasmid constructs, cotransfection experiments performed with increasing doses of pCDNA-RREB1 plasmid resulted in the decrease of the reporter gene expression in a dose-dependent manner compared with cells transfected with empty pCDNA3 plasmid only. To further investigate the repressor effect of RREB-1, we followed a RNA interference approach using specific RREB-1 siRNA (vs control siRNA) transfected in HeLa cells 48 h before the analysis of luciferase activity. RREB-1 protein was decreased by 60–70% when assayed by Western blot analysis of whole cell extracts (*inset*, Fig. 2*B*). We repeated the luciferase experiments with 1.4-kb *HLA-G* promoter construction in HeLa cells in the presence of siRNA against RREB-1 mRNA. The transcriptional activity of the luciferase reporter gene was enhanced (2-fold) in RREB-1-depleted cells compared with control cells, and the enhancement was still observed in the presence of pCDNA3-RREB-1 (Fig. 2*B*). To investigate more the *HLA-G* promoter activity in response to RREB-1 in different cell types, we performed luciferase reporter experiments with 1.4-kb promoter fragment and 0.5 μg of pCDNA3-RREB-1 both in melanoma M8, another HLA-G-negative cell line (45, 46), and in the HLA-G-positive choriocarcinoma JEG-3 cells. As expected luciferase gene expression in control conditions was higher in JEG-3 cells than in M8 cells reflecting differences in the transcriptional capacity of the cell lines. We observed a significant 23% mean decrease ($p < 0.05$) in the luciferase reporter gene expression in M8 cells compared with M8 cells transfected with empty pCDNA3 plasmid, whereas the mean decrease was only 6% with JEG-3 cells (Fig. 3*A*).

Moreover, to evaluate the effect of RREB-1 on endogenous *HLA-G* expression we investigated the amounts of *HLA-G* mRNA in HLA-G-negative M8 cells in the presence or absence of specific RREB-1 siRNA. In agreement with luciferase experiments we observed that the amounts of *HLA-G* mRNA was significantly enhanced (over 13-fold) after RREB-1 siRNA treatment compared with M8 cells treated with siRNA control encoding scrambled sequence, thus backing up the role of RREB-1 in the observed *HLA-G* gene repression (Fig. 3*B*).

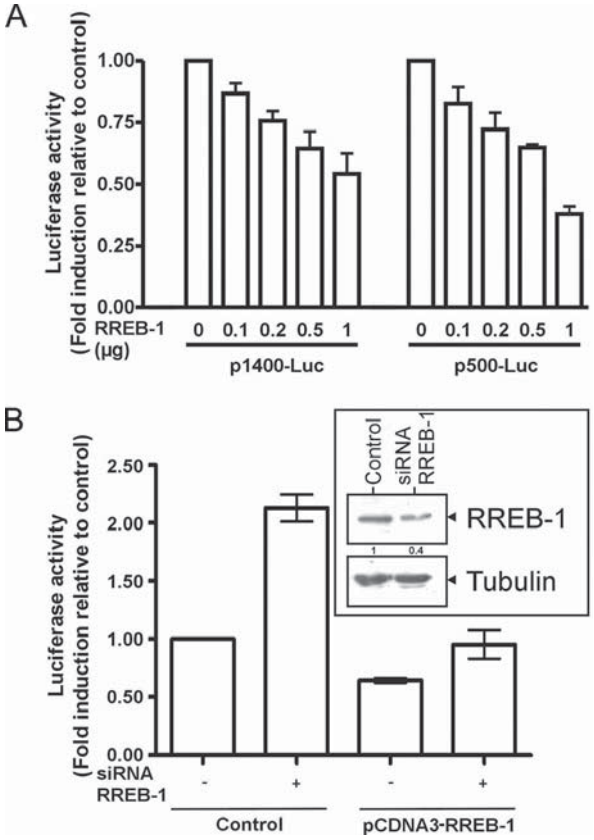

FIGURE 2. Transcriptional repressive activity of RREB-1 on *HLA-G* gene promoter in HeLa cells as determined by reporter luciferase plasmid transient transfection. *A*, Luciferase activity of p1400-Luc (1438 bp of *HLA-G* promoter) and p500-Luc (500 bp of *HLA-G* promoter) contructs in the presence of different doses of RREB-1 expression plasmid (0, 0.1, 0.2, 0.5, 1 μg). *B*, p1400-luciferase activity in the presence (+) or not (−) of RREB-1 siRNA with (pCDNA3 RREB-1) or without (control) RREB-1 transfection plasmid (0.5 μg). *A* and *B*, Empty pCDNA3 was added to make the total amount of DNA equal in all transfections. Normalized with *Renilla* luciferase, all data were expressed as a fold induction over the relative control (empty pCDNA3 plasmid), which was arbitrarily set to 1. Data represent mean $\pm$ SD of determinations from three independent experiments. Representative Western blot analysis (*inset*) shows efficiency of RREB-1 siRNA. The ratio corresponds to the amount of RREB-1 protein normalized to tubulin protein. A value of 1 is assigned to the RREB-1 constitutive level.

Because two-dimensional gel electrophoresis analysis showed a differential profile for RREB-1 spot between cells expressing (JEG-3) or not (Raji) *HLA-G* transcripts (Fig. 1) we wondered whether the expression of RREB-1 was specific of HLA-G-negative cells only. We thus performed a Western blot analysis using anti-RREB-1 polyclonal Ab with nuclear extracts of Raji, and melanoma M8, compared with HLA-G-positive JEG-3 cells. Unexpectedly, we noted no significant differences in the expression levels of RREB-1 regardless of the cell types we analyzed (Fig. 3*C*). This may suggest that RREB-1 from JEG-3, which was not observed in two-dimensional gel electrophoresis pattern of JEG-3 at the position of spot 10, cannot bind proximal *HLA-G* promoter or exhibit posttranslational modifications such as phosphorylation or acetylation events affecting isoelectric point. Taken together, these results demonstrate that RREB-1 may act as a potential repressor factor of *HLA-G* transcription depending on cellular context.

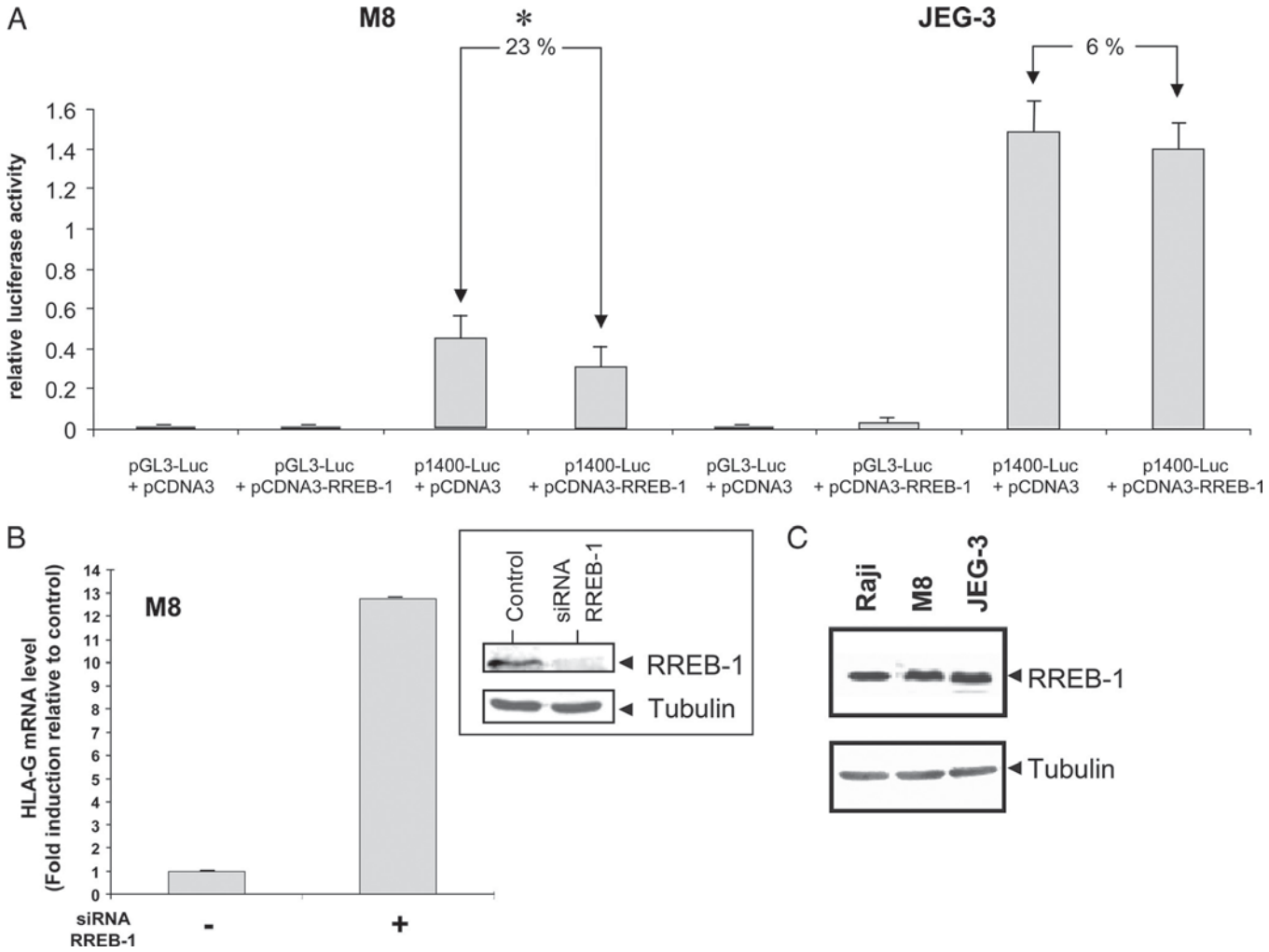

FIGURE 3. RREB-1 activity on *HLA-G* gene promoter in M8 melanoma and JEG-3 choriocarcinoma cells as determined by reporter luciferase plasmid transient transfection and RREB-1 effect on HLA-G mRNA amounts in M8 cells. *A*, Luciferase activity of p1400-Luc (1438 bp of *HLA-G* promoter) construct in the presence of 0.5 μg of RREB-1 expression plasmid (pCDNA3-RREB1) or mock plasmid (pCDNA3) in M8 cells. Results are mean ± SEM of four independent experiments and in JEG-3 cells mean ± SEM of two independent experiments. Percentage in *A* and *B* indicates the mean decrease in luciferase activity obtained in the presence of RREB-1 overexpression. *, $p < 0.05$ by two-way ANOVA test. *B*, HLA-G specific real–time RT-PCR analysis in M8 cells in the absence (−) or presence (+) of siRNA targeting RREB-1 as a mean of two independent experiments. Steady state levels of *HLA-G* mRNA levels in control M8 cells are assigned a value of 1 and were analyzed in the presence of a scramble siRNA used in Fig. 2. Representative Western blot analysis (*inset*) shows efficiency of RREB-1 siRNA. *C*, Western blot analysis of whole cell lysates for RREB-1 expression in Raji, M8, and JEG-3 cells is shown. Tubulin was revealed to control the protein loading.

HLA-G *promoter region contains three functional RREB-1 target sites*

The implication of RREB-1 protein in the modulation of *HLA-G* gene promoter activity prompted us to search for potential binding RRE sequences within the *HLA-G* gene promoter. We investigated the presence of the consensus DNA binding site CCCCACCATC-CCC obtained using the casting method (53) and the consensus ttk-related site GGTCCT corresponding to the binding site for LZ321, both sites were shown to bind RREB-1 with equal affinity (49). We found three potential RREB-1 target sites along the 1.4-kb promoter sequence upstream to ATG (Fig. 4): two putative binding sites are localized into the proximal promoter, one in position −70 to −44 that contains only one GGTCCT motif (RRE-53), and the second site in position −159 to −124 that contains one direct and one inverted GGTCCT motif (RRE-142). A third target site related to RREB-1 consensus binding sequence was found within the putative LCR (distal promoter), in position −1380 to −1346 (RRE-1356).

To investigate whether these HLA-G RRE sequences were direct targets for RREB-1, we performed EMSA first with Raji nuclear extracts and ^{32}P-labeled oligonucleotides corresponding to HLA-G probes RRE-53, RRE-142, and RRE-1356 (Fig. 5*A*). Binding patterns were compared with the one of a control pattern obtained with RREB-1 and HE-443 probe (49) that reveal major specific complexes a. EMSA obtained with the three HLA-G RRE probes reveals a strong binding activity compared with the probe alone, with the presence of major DNA-protein complexes migrating at a similar positions than the control complexes a for each probes and a slower migrating complex b exhibiting lower intensity (Fig. 5*B*). The binding DNA complexes were abolished in competition experiments using a 100-fold excess of cold RRE-site competitor (HE-443) (Fig. 5, *lanes 8*, *13*, and *18*) or the unlabeled probe itself (Fig. 5, *lanes 4*, *9*, *14*, and *19*). The specificity of RREB-1 DNA-binding was further confirmed as previously done (55) using nuclear extracts from Raji cells that were either immunodepleted with an Ab directed against

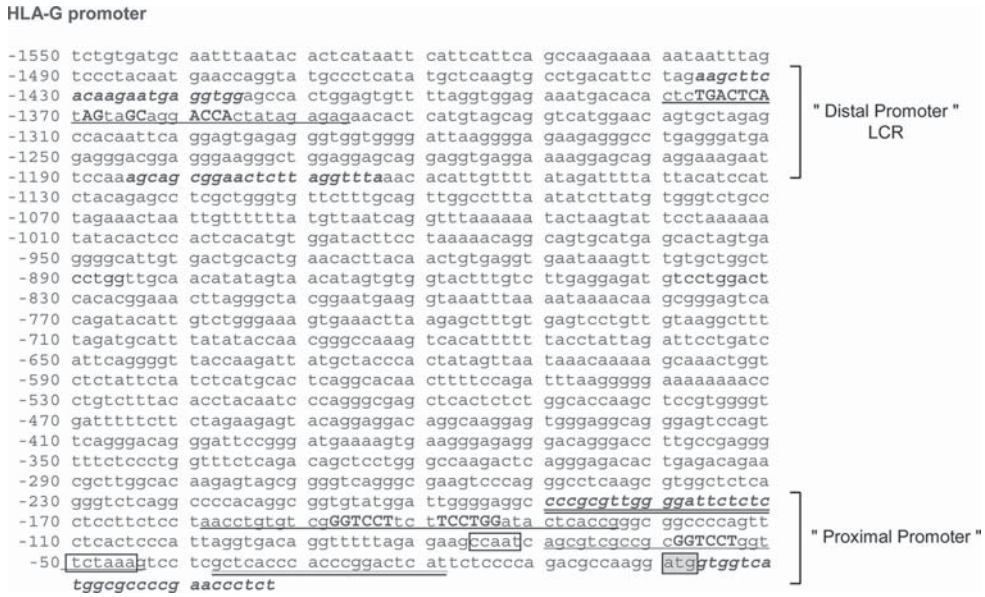

FIGURE 4. HLA-G 1.4-kb promoter region contains three RREB-1 target sites. Nucleotide sequence of the *HLA-G* gene promoter is from ATG (+1 to −1550 bp). Locations of RREB-1 target sites are indicated by nucleotides (bold and upper capital letters) from 5′ to 3′ (RRE-1356, RRE-142, RRE-53 in Fig. 5). Sequences corresponding to oligonucleotide probes used for EMSA analysis (underlined), sequences corresponding to PCR primers used to generate promoter pull-down assay (double-underlined), and sequences corresponding to PCR primers used to amplify "proximal" and "distal" promoter (LCR) (bold and italic letters) are shown. CAAT and TATA sequences and ATG initiation site are boxed.

RREB-1 or immunodepleted with rabbit IgG to control nonspecific loss of protein. In such a condition, DNA binding of RREB-1-depleted extracts was very weak or absent compared with the one we obtained using nonimmunoprecipitated fractions, thus giving a first demonstration for specific RREB-1 binding to the three HLA-G RREs (Fig. 5*C*). Interestingly, EMSA patterns exhibit a few differences according to the oligonucleotide probe used (RRE-53, RRE-142, RRE-1356). This suggests that other factors than RREB-1 may contribute to the observed pattern as well. In agreement with this, we found a band that was present in the RRE-142 pattern even after immunodepletion with the RREB-1 Ab (arrows, Fig. 5*C*). For example it is of note that the RRE-142 oligonucleotide contains the conserved HLA-G regulatory X1 box that was previously demonstrated to associate RFX factors in vitro (30, 31).

To further evaluate the specificity of RREB-1 binding to the *HLA-G* RREs we performed competition experiments with labeled RRE oligonucleotide probes and 100-fold excess of unlabeled competitor oligonucleotides in which the RRE binding site has been mutated (RREmut) or not. These experiments were done both with nuclear extracts from Raji and M8 melanoma cells, which are negatives for *HLA-G* transcriptional activity. In accordance with previous results, EMSA revealed that most of complexes were displaced by addition of an excess of cold wild-type RRE oligonucleotides, whereas an excess of cold mutated RRE oligonucleotides did not significantly compete with probes (see supplemental Fig. 1).[4] In addition, EMSA profiles obtained with nuclear extracts from HLA-G-negative cells were compared with EMSA profile from HLA-G-positive JEG-3 cells (Fig. 6). By mean of this data, we noted that complexes a are always present in both cell types whatever HLA-G RRE probes used, whereas complex b is formed with all probes in HLA-G-negative cells and only with RRE-1356 probe (LCR location) incubated with JEG-3 extracts.

[4] The online version of this article contains supplemental material.

Next, to assess more the functionality of the *HLA-G* RREs we investigated the effect of RREB-1 overexpression using a luciferase reporter construct driven by a mutated 1.4 kb *HLA-G* promoter fragment (p1400 RREmut-Luc) in which the three RREB-1 binding sites have been scrambled. Cotransfection experiments performed in HeLa and M8 cells with 0.5 μg of pCDNA3-RREB-1 (Fig. 7) showed that mutations of RRE sites preventing the binding of RREB-1 impaired the previously observed down-regulation of *HLA-G* promoter activity in Figs. 2 and 3.

Finally, to confirm that the RREB-1 binding sites identified by EMSA are able to recruit RREB-1 in living cells, we performed ChIP assays both with M8 and JEG-3 cells (Fig. 8*A*). As expected, RREB-1 proteins were tethered to the proximal and distal (LCR) promoter (Fig. 4) of HLA-G-negative cells. No precipitation was detected in an irrelevant region of *HLA-G* by RREB-1 Ab (data not shown). Interestingly RREB-1 was only found associated with the *HLA-G* distal promoter (LCR) in JEG-3 cells, a result agreeing on two-dimensional gel electrophoresis analysis in which spot 10 (RREB-1) was absent in gels obtained with JEG-3 protein extracts and proximal *HLA-G* promoter that contains RRE-53 and RRE-142 sequences (Fig. 4). This result is also in accordance with the fact that complex b is exclusively observed with RRE-1356 oligonucleotide probe (within LCR) in EMSA performed with JEG-3 cell proteins.

RREB-1 can associate HDAC1 and CtBP components on the HLA-G *gene promoter*

We first wondered whether RREB-1 binding to the *HLA-G* promoter was associated or not with chromatin modifications involved in the repression of transcriptional activity. By analyzing M8 and JEG-3 cells we found that HDAC1 binding to the *HLA-G* gene promoter is restricted to M8 cells. In agreement on our previous results (46), we confirmed that histone H3 are constitutively acetylated and phosphorylated in JEG-3 cells, providing a chromatinized environment that allow the recruitment of RNA polymerase II on the transcription start site of *HLA-G* gene. In contrast, H3 histones are neither acetylated nor phosphorylated in M8 cells

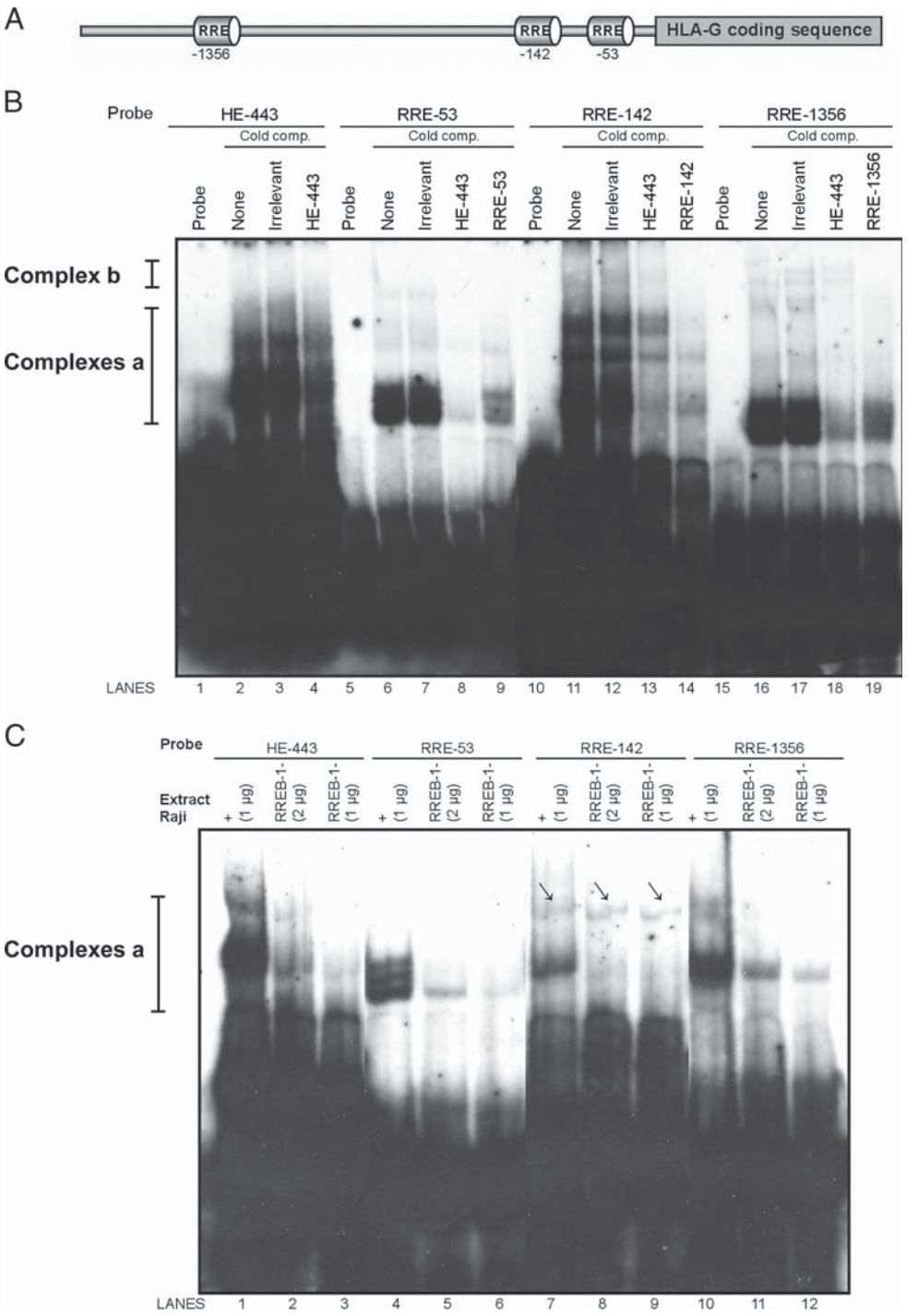

FIGURE 5. EMSA validation of RREB-1-DNA binding to the *HLA-G* promoter RRE sites with Raji cell nuclear extracts. *A*, Location of EMSA oligonucleotide probes covers RRE-53, RRE-142, and RRE-1356 motifs along the *HLA-G* gene promoter (see also Fig. 4). *B*, EMSA performed without extracts (Probe) (*lanes 1*, *5*, *10*, and *15*) or with 2 μg of Raji cell nuclear extracts incubated with ^{32}P-labeled *HLA-G* probes in the absence (None) (*lanes 2*, *6*, *11*, and *16*) or in the presence of a 400-fold molar excess of unlabeled competitors. RRE-53, RRE-142, and RRE-1356 fragments (*lanes 9*, *14*, and *19*, respectively), irrelevant fragment that differs from RRE (*lanes 3*, *7*, *12*, and *17*), and HE-443, which is a RREB-1 DNA binding control with GGTCCT motif (*lanes 4*, *8*, *13*, and *18*). Complexes a indicates the major specific RREB-1 binding activity. Complex b is analyzed in Fig. 7. Arrows designate factors that differ from RREB-1. *C*, Depletion EMSA experiments with Ab-directed against RREB-1. Nuclear extracts from Raji cells were either RREB-1-depleted (RREB-1 −) or not (control IgG +) and subsequently used in EMSA experiments with ^{32}P-labeled HLA-G probes HE-443, RRE-53, RRE-142, and RRE-1356. The amount of nuclear extracts used is indicated (1 μg or 2 μg).

(Fig. 8*B*). These data suggest that deacetylation of H3 in M8 cells and the no-occupancy by RNA polII on the *HLA-G* promoter may be related to the recruitment of HDAC1 and may be associated with the interaction with RREB-1.

Because RREB-1 was previously detected in a CtBP corepressor complex we thus performed combined Western blotting and EMSA and supershift experiments with anti-RREB-1 Ab and Abs against relevant subunits of CtBP, namely anti-HDAC1 and anti-CtBP1/2 (59). Using M8 nuclear extracts we observed that immunodetection of EMSA gel conducted with RRE-53 oligonucleotide probes and blotted to a nitrocellulose membrane (WEMSA) with each Abs revealed a smear and discrete bands migrating at the same position than those revealed with labeled RRE-53 probe. These results confirmed the presence of RREB-1 in complexes a and complex b and strongly suggest that HDAC1 and CtBP1/2 may participate in RREB-1 complexes (Fig. 9*A*). In combined Western blotting and EMSA experiments it is noticeable that on top of discrete bands we observed smears testifying to the presence of HDAC1 and CtBP in a multitude of complexes in the cells. Nonetheless discrete bands indicate that HDAC1, CtBP1/2, and RREB1 are privileged partners (59). These results are in agreement with supershift experiments performed with RRE-53 probe and anti-CtBP1/2 and anti-HDAC1 Abs (Fig. 9*B*).

HDAC1 and CtBP1/2 are preferentially associated with RREB1 in M8 HLA-G-negative cells

To test whether HDAC1 and CtBP1/2 are interacting with RREB-1, we performed an immunoprecipitation assay using M8

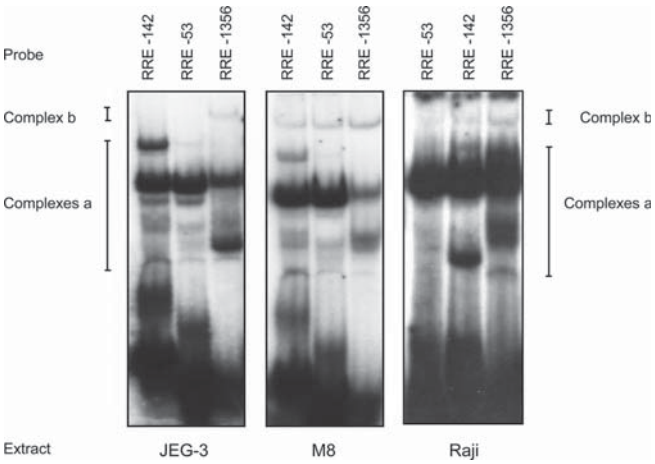

FIGURE 6. RREB-1 binding on HLA-G-RREs in EMSA performed with JEG-3, M8, and Raji protein extracts. Comparison of representative EMSA performed with M8 (melanoma), Raji (Burkitt's lymphoma), and JEG-3 (choriocarcinoma) cell extracts incubated with HLA-G probes RRE-53, RRE-142 and RRE-1356 in JEG-3 and M8 and in Raji in three independent experiments. Complexes a and complex b revealed with Raji cells in Fig. 5 are observed with M8 and JEG-3 cell extracts but complex b in JEG-3 EMSA is formed with RRE-1356 probe only.

and JEG-3 nuclear extracts, HDAC1 and CtBP1/2 specific Abs. Western blot analysis targeting RREB-1 shows that HDAC1 and CtBP1/2 are present in RREB-1 containing complex (Fig. 10*A*) and more particularly in M8 cells in comparison with JEG-3 cells (RREB-1 band at ~80–90 kD is of the predicted molecular

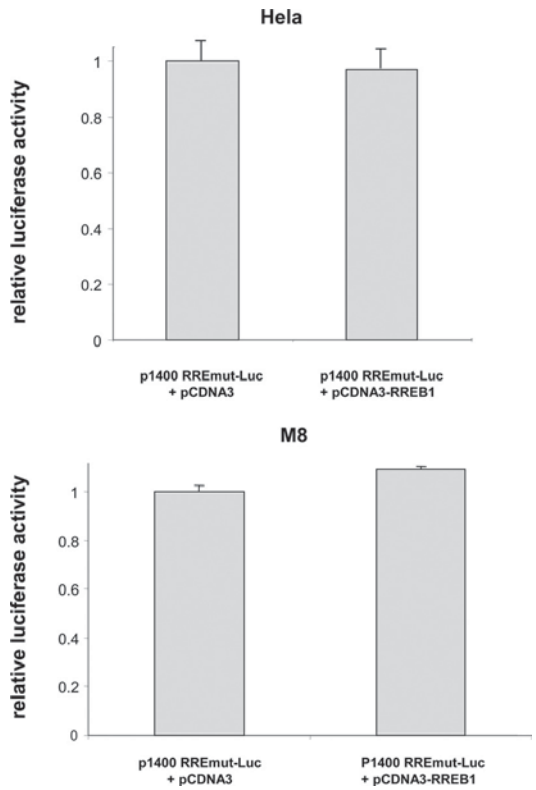

FIGURE 7. Scrambled *HLA-G* RREs affect down-regulation of *HLA-G* promoter activity in response to exogenous RREB-1. Luciferase activity of p1400 RREmut-Luc (1438 bp of *HLA-G* promoter with mutated RREs) in the presence of 0.5 μg of RREB-1 expression plasmid or mock plasmid (assigned a level of 1) in HeLa cells as mean ± SEM of two independent experiments and in M8 melanoma cells as mean ± SEM of three independent experiments.

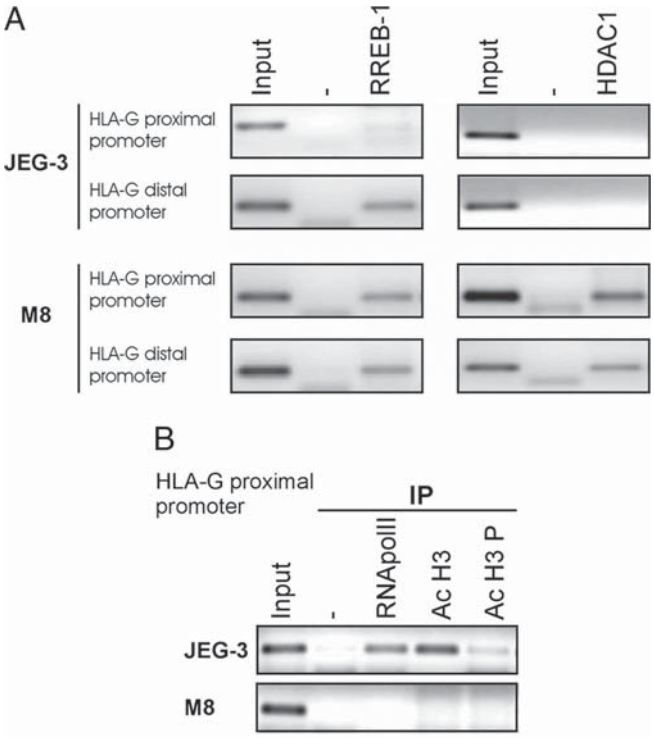

FIGURE 8. In situ binding of RREB-1 or HDAC1 to the *HLA-G* promoter in a repressive and active-type chromatin. *A* and *B*, ChIP performed with JEG-3 (HLA-G +) and M8 (HLA-G −) cells using anti-RREB-1 and anti-HDAC1 Abs on distal and proximal promoter regions (*A*) Abs targeting RNA polymerase II (RNApolII), acetylated histone H3 (AcH3), and phosphorylated histone H3 (AcH3 P) on proximal promoter region (*B*). Immunoprecipitated *HLA-G* promoter regions are analyzed on agarose gels by semiquantitative HLA-G-specific PCRs targeting proximal and distal *HLA-G* promoter. Input chromatin (Input) used as PCR control and IgG (−) are shown. The absence of RREB-1 and HDAC1 binding observed in JEG-3 cells and the absence of RNA polymerase II, acetylated histone H3, and phosphorylated histone H3 binding in M8 cells validate the specificity of Abs used in ChIP assays.

weight). In addition, RREB-1 acetylation has been tested in both cellular types by RREB-1 immunoprecipitation followed by Western blot using anti-acetylated lysine Ab. RREB-1 acetylation was clearly predominant in HLA-G-positive JEG-3 cells (Fig. 10*B*).

Discussion

Previous studies have revealed very little shared functional regulatory sequences and binding factors between *HLA-G* and *HLA class I* genes (31) and only a few factors modulating *HLA-G* gene expression have been identified until now (18, 23). We thus devised a strategy to isolate unidentified factor candidates implied in the transcriptional regulation of the *HLA-G* gene. This original approach combining *HLA-G* promoter pull-down experiments and mass spectrometry, allowed prediction of RREB-1 (53), a zinc finger transcription factor also named FinB (54), LZ321 (49), and Zep-1. Because the factor is expressed ubiquitously in human tissues outside the adult brain (54, 60), its presence in all cell types analyzed, namely Raji, M8, and JEG-3, is understandable at first sight.

Looking for RREB-1 function on the *HLA-G* gene transcription and associated mechanisms, we highlighted that overexpression and selective inhibition of expression of RREB-1 protein affect transcriptional activity driven by *HLA-G* promoter according to a repressive activity in HLA-G-negative cells. According to this,

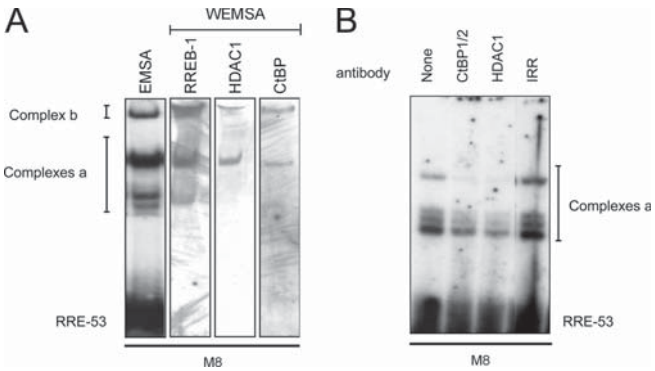

FIGURE 9. RREB-1 can associate HDAC1 and CtBP components on the *HLA-G* promoter. *A*, Combined Western blotting and EMSA analysis of M8 protein extracts with RRE-53 (EMSA), anti-RREB-1, anti-HDAC1, and anti-CtBP Abs. *B*, Representative EMSA showing supershift analysis with M8 nuclear extracts and RRE-53-labeled oligonucleotide probe following protein incubation with anti-CtBP1/2, anti-HDAC1, and irrelevant Abs (IRR). Nuclear extracts without Abs are shown (None).

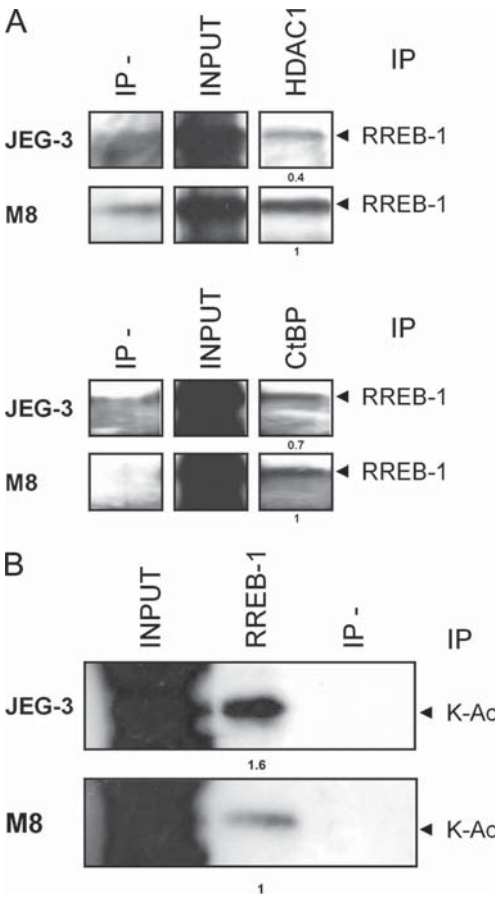

FIGURE 10. Criteria of RREB-1, HDAC1, and CtBP1/2 complex formation in JEG-3 and M8 cells. *A*, Representative Western blot analysis of immunoprecipitated proteins from M8 and JEG-3 whole cell extracts with either anti-HDAC1 or anti-CtBP1/2 Abs and revealed with anti-RREB-1 Ab from two independent experiments. A 1% whole protein extract (Input) or no Ab (IP-) is shown. *B*, Immunoprecipitation of RREB-1 protein in M8 and JEG-3 cells extracts followed by Western blot analysis using anti-K-acetylated Ab from four independent experiments. Ratios correspond to the amount of RREB-1 (*A*) and K-acetylated RREB-1 (*B*) proteins in JEG-3 compared with M8. Value 1 is assigned to the RREB-1 constitutive level in M8.

RREB-1 has been described to be implied in transcriptional repression of several genes such as p16INK4a (56) angiotensinogen (57), and prostate-specific Ag (58). Interestingly, RREB-1 was also described as a coactivator of calcitonin (53), c-erbB2 (54), and secretin (55) genes, thus confirming that its normal cellular function is dependent on cellular context or probably also on promoter of genes. In agreement on that we found that RREB-1 repressive activity on the *HLA-G* promoter was reduced or absent in HLA-G-positive JEG-3 cells.

RREB-1 targets three binding sites identified dispersed through the *HLA-G* promoter and demonstrated not only to bind RREB-1 in vitro but also to drive the down-regulation of *HLA-G* promoter activity measured by luciferase reporter gene experiments. RRE-53 and RRE-142 are located within the proximal *HLA-G* promoter and respectively contain one and two GGTCCT sequences to which LZ321 was shown to bind (49). A third RRE corresponding to the consensus sequence derived by the method of cyclic amplification and selection of target (53) has been found at position −1356 in the distal *HLA-G* promoter region which was proposed to be a putative locus control region, previously associated with the high regulation of HLA-G tissue-specific expression in transgenic mouse (32, 33). Interestingly we observed by EMSA that RREB-1 is constitutively bound to the three *HLA-G* promoter RREs in cells expressing or not HLA-G. However two-dimensional gel electrophoresis pattern obtained with HLA-G-positive JEG-3 cell extracts and proximal promoter region revealed that the spot 10 corresponding to RREB-1 in Raji was absent. A plausible explanation would be that RREB-1 binding activity to the *HLA-G* promoter requires posttranslational modifications changing the physicochemical criteria of the protein (molecular mass and isoelectric point), as previously reported for numerous proteins (61) to exert its function. Presumably any change would be to isoelectric point as molecular mass changes have not been detected on Western blots. Moreover, a marked RREB-1 acetylation in HLA-G-positive JEG-3 cells in comparison with HLA-G-negative M8 cells strongly argues for that.

In agreement on the two-dimensional gel electrophoresis analysis, ChIP assays revealed the RREB-1 binding to the proximal *HLA-G* promoter (contains RRE-53 and RRE-142) in HLA-G-negative cells only. In contrast, EMSA obtained with JEG-3 and HLA-G-negative cell protein extracts were very similar and thus seemed contradictory results. Nevertheless we noted that complex b was not formed with JEG-3 extracts and probes containing proximal promoter RRE binding sites. Therefore, complex b could involve specific RREB-1 modifications or combination of factors responsible in the two-dimensional gel electrophoresis and ChIP-specific patterns. A second explanation would be that factors involved both in complexes a and b could not associate the *HLA-G* proximal promoter in JEG-3 chromatin environment. In this regard it is noteworthy that the location of RRE-53 site is very close to the TATA box and might be in the way of transcription initiation complex. In agreement, ChIP analysis did not found RNA pol II at the *HLA-G* promoter in HLA-G-negative M8 cells, whereas it was found at the *HLA-G* promoter in HLA-G-positive JEG-3 cells. In addition, the fact that promoter response to RREB-1 transfection was low or absent in JEG-3 cells clearly support such a hypothesis.

It is also noteworthy that we observed the distal promoter occupancy by RREB-1 in HLA-G-positive cells. One possible explanation of this result would be that RREB-1 binding to the LCR in JEG-3 is regulating the overall levels of *HLA-G* transcripts in JEG-3 cells, which is lower than the one observed in

the melanoma cell line FON (46). Another explanation would be that RREB-1 in JEG-3 exhibited specific modifications acting on its repressive activity. In such a condition specific RREB-1 modifications in JEG-3 cells might explain that the effect of RREB-1 transfection on the luciferase reporter gene expression was low in this cell type.

Indeed, the present work underlined the formation of a multiprotein complex present predominantly in HLA-G negative cells. A combined Western blotting and EMSA assay has revealed that this complex is constituted of RREB-1, the histone deacetylase HDAC1 and CtBP1/2. We also demonstrated by immunoprecipitate assays that RREB-1 interacts with HDAC1 and preferentially coimmunoprecipitates both with HDAC1 and CtBP1/2 in HLA-G-negative cells. Of great interest is that RREB-1, HDAC1, and CtBP1/2 are members of the nuclear protein complex CtBP core complex with CoREST, EHMT, ZEB1/2, or ZnF217 (59). CtBP is assembled by CtBP1 or CtBP2 and contains various enzymatic elements principally implied in modifications of histone (deacetylation and methylation) (62), and particularly histone H3 Lys^9 (59). Given that both HDAC and histone methyltransferase activities are associated with CtBP complex, it can facilitate stepwise, coordinated, enzyme reactions that convert an active chromatin structure to a repressive state. CtBP has been implicated in the repression of several genes (63) by interaction with DNA binding factors such as E1A or RIP140. Therefore we can hypothesize that the repression function of RREB-1 also would be mediated by the corepressor CtBP. On the contrary we found that RREB-1 is more acetylated in HLA-G-positive cells than in HLA-G-negative cells, a posttranscriptional modification previously implicated in the disruption of CtBP with E1A or RIP140 and mediated by CBP/p300 or pCAF (64–66). Therefore we can speculate that CtBP recruitment via RREB-1 would be dependent on CBP/p300. We are currently examining such a mechanism. It would be compatible with the role of CREB, CBP and P300 in HLA-G transactivation and facilitated by multiple CRE/TRE sites in the promoter region of HLA-G (28) and flanking CtBP binding sites.

Finally, we and other groups have demonstrated that DNA methylation is an important mechanism in repression of HLA-G expression (46, 67). The present work thus suggests that RREB-1 and DNA methylation at the *HLA-G* locus are complementary mechanisms. We can also hypothesize that both mechanisms are closely involved in HLA-G transcriptional repression. Indeed, according to the present ChIP experiments, RREB-1 binding to the *HLA-G* promoter might be dependent or might be associated to the methylation status of the proximal promoter. Nonetheless EMSA and reporter luciferase experiments suggest that DNA methylation is not the necessary condition for the RREB-1 binding and repressive activity in M8 cells. However, we cannot exclude the fact that DNA methylation might enhance RREB-1 binding and thus repressive activity.

In conclusion, the proteomic approach used in this study allowed to define the first potential transcriptional repressor of HLA-G, which is RREB-1. Our results highlight the implication of RREB-1 in the transcriptional regulation of *HLA-G* gene and expand our understanding of the molecular mechanisms involved in this process. In particular we showed that nonacetylated RREB-1 recruits elements of CtBP complex implied in chromatin remodeling. Posttranscriptional modifications of RREB-1 thus appear to be key events in the regulation of HLA-G and remain to be investigated. These aspects would be useful in developing new strategies to modulate HLA-G expression in cancer therapies and transplantation.

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Disclosures

The authors have no financial conflict of interest.

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Increased Adipogenesis in Cultured Embryonic Chondrocytes and in Adult Bone Marrow of Dominant Negative Erg Transgenic Mice

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Abstract

In monolayer culture, primary articular chondrocytes have an intrinsic tendency to lose their phenotype during expansion. The molecular events underlying this chondrocyte dedifferentiation are still largely unknown. Several transcription factors are important for chondrocyte differentiation. The Ets transcription factor family may be involved in skeletal development. One family member, the *Erg* gene, is mainly expressed during cartilage formation. To further investigate the potential role of Erg in the maintenance of the chondrocyte phenotype, we isolated and cultured chondrocytes from the rib cartilage of embryos of transgenic mice that express a dominant negative form of Erg (DN-Erg) during cartilage formation. DN-Erg expression in chondrocytes cultured for up to 20 days did not affect the early dedifferentiation usually observed in cultured chondrocytes. However, lipid droplets accumulated in DN-Erg chondrocytes, suggesting adipocyte emergence. Transcriptomic analysis using a DNA microarray, validated by quantitative RT-PCR, revealed strong differential gene expression, with a decrease in chondrogenesis-related markers and an increase in adipogenesis-related gene expression in cultured DN-Erg chondrocytes. These results indicate that Erg is involved in either maintaining the chondrogenic phenotype *in vitro* or in cell fate orientation. Along with the *in vitro* studies, we compared adipocyte presence in wild-type and transgenic mice skeletons. Histological investigations revealed an increase in the number of adipocytes in the bone marrow of adult DN-Erg mice even though no adipocytes were detected in embryonic cartilage or bone. These findings suggest that the Ets transcription factor family may contribute to the homeostatic balance in skeleton cell plasticity.

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Introduction

Chondrocytes, osteoblasts, fibroblasts, adipocytes and skeletal myoblasts are highly specific cell types derived from multipotent mesenchymal stem cells (MSC) through a specific differentiation pathway [1,2,3,4,5]. Although MSCs are fully committed to a developmental lineage, several studies have shown that MSC-derived cells can switch to another cell lineage or return to an uncommitted developmental stage [6,7,8,9,10]. Likewise, the signalling molecules and pathways leading to transdifferentiation (i.e. lineage reprogramming) remain poorly defined.

Chondrogenesis is a tightly regulated process that is initiated by the condensation of committed MSCs, followed by differentiation into chondrocytes and the expression of cartilage-specific markers [11,12,13]. Each specific differentiation program of MSC-derived cell types is harmoniously and dynamically controlled by several specific signal transduction (cytokines, growth factors and extra-cellular matrix molecules) and transcription factors [14,15]. The osteochondrogenic state is regulated by two master transcription factors: Sox9 (Sex determining region Y-box9) and Runx2 (Runt-related transcription factor 2) essential for the determination and maturation of chondrocytes and osteoblasts, respectively [16,17]. Several molecular players have been identified, including the transcription factors NF-κB, C/EBPβ, ETS, Runx2, and hypoxia-inducible factor-2α, all of which are involved in cartilage formation [18,19,20]. Among them, we and others have shown that the *Ets-related gene* (*Erg*) is expressed during the earliest events of skeletal formation and is associated with precartilaginous condensation and chondrogenic differentiation [21,22,23,24]. The Erg transcription factor belongs to the ETS family of DNA-binding proteins [25]. Several members of the ETS family are involved in a variety of cellular and developmental processes. In skeletal formation, the *Erg* gene is the earliest ETS member family expressed in cartilage during embryonic development followed by *Fli1*, *Ets-2* and *Pea3* in a lesser extend [24,26]. This family of

transcriptional regulators shares a highly conserved 85 amino-acid DNA-binding domain (ETS domain) that specifically recognises DNA over an 11 bp sequence centred around a consensus core sequence, 5′-GGAA/T-3′ [27].

To explore the roles Erg may play in the chondrogenesis process or in chondrogenic maturation, we established a transgenic mouse model that overexpresses a dominant negative fragment of the Erg protein (DN-Erg), specifically restricted to the ETS domain. The transgene construct is specifically expressed in chondrocytes during cartilage formation in embryos because it is under the control of the *collagen II* (*Col2a1*) promoter and competes with endogenous wild-type Erg protein functions. However, since other ETS family genes, such as *Fli1*, *Ets2* and *Pea3* are also expressed, to a lesser extent, in cartilage, we cannot rule out that the binding of these transcription factors is also involved. Transgenic mouse embryos and newborns have no obvious malformations, but clinical early-ageing processes, including hyperlordosis/hyperkyphosis and reduced mobility, are observed during the first 6 months post natum (unpublished data). This manifestation of early ageing indicates that the Erg transcription factor is involved in the regulation of various genes affecting cartilage formation and skeletogenesis.

In this study, to explore the physiological roles of Erg proteins in the maintenance of the chondrocyte phenotype, we isolated chondrocytes from the rib cage of embryos of wild-type (wt) and DN-Erg transgenic mice and cultured them in monolayers. We observed that transgene expression was correlated with the accumulation of lipid droplets in cultured chondrocytes compared to wt. To determine the differentially expressed gene profile during the dedifferentiation process from monolayer-cultured wt and DN-Erg chondrocytes, we used DNA microarray analysis to study the transcriptome modifications in chondrocytes of wt and DN-Erg transgenic mice during culture. Among functional categories accounting for most genes with altered expression in cultured DN-Erg chondrocytes, the adipocyte pathway genes were upregulated.

In addition, because the phenotype of transgenic mice overexpressing DN-Erg was associated with early-ageing skeleton phenotypes and ageing is associated with decreased bone marrow cellularity and increased bone marrow fat, we performed a histological comparison of morphological features of bone marrow from adult mice femur and showed a dramatic increase in adipocytes in DN-Erg transgenic mice.

The data presented here demonstrate that monolayer-cultured DN-Erg chondrocytes spontaneously underwent adipocyte differentiation, suggesting that Erg is involved in the differentiation plasticity of chondrocytes.

Materials and Methods

Cell cultures

Chondrocytes of murine embryos were isolated from the ribs of 18.5 days post-coitum (E18.5) mice according to the protocol described in [28,29]. Mouse care and treatment were conducted in accordance with institutional guidelines in compliance with national law and policies. This study was specifically approves by our local ethics committee (Authorisation no. CEEA 13/2009 issued by the Comite d'Ethique en Experimentation Animale, Nord-Pas-de-Calais). Chondrocytes were seeded in 6-well culture plates at 10^6 cells/well and were grown for 20 days. Cells were cultured in DMEM Nutrient Mixture F-12 Ham (Sigma) supplemented with 10% foetal bovine serum, 1% gentamicin, and 1% glutamine. The culture medium was replaced every two days.

Cytochemical analyses

Alcian blue staining. Cultures were washed with PBS before fixing with methanol for 10 min. After rinsing with PBS, cultures were stained overnight with Alcian blue solution (pH 2.5, 3% glacial acetic acid). Cells were washed three times with glacial acetic acid (4%) and twice with sterile distilled water.

Oil red O staining. Cells were washed twice with PBS and fixed with 10% formalin (pH 7.4) for at least 1 h at room temperature. After washing with 60% isopropanol, the cells were stained for 10 min at room temperature with filtered Oil red O/60% isopropanol solution. The cells were washed twice with distilled water. Red-stained adipocytes were observed under a light microscope.

Histological analysis

Whole skeletons of E18.5 embryos were stained with Alizarin red S and Alcian blue [30]. Ribs and legs of 18.5 day embryos and of 40 week-old mice were dissected, fixed and embedded in paraffin. Paraffin blocks were prepared using standard histological procedures. The resulting serial sections (5–6 μm thickness) were stained with hematoxylin and eosin or Sudan black B, as indicated.

Adipocyte number was quantified by three different observers looking at five different fields per section at least three different mice. The result was expressed as the mean of total adipocyte number per square millimeter of marrow tissue area in the analysed fields.

Immunohistochemistry

According to the provided protocol for immunohistochemistry, sections were demasked by treatment with xylene twice for 5 min at 37°C, rehydrated through graded ethanols and were incubated with rabbit anti-mouse adiponectin Acrp30 (N-20, sc-17044-R from Santa Cruz Biotechnology, dilution 1/100). Antigen retrieval was conducted in sodium citrate buffer pH 6 for 30 minutes at 95°C. A biotinylated goat anti-rabbit IgG antibody (Vector), diluted 1:1000, was used as the secondary antibody with PBS for 1 hour at room temperature. Antigen immunolocalization was analysed using DAB peroxidise substrate (vector labs) according to manufacturer's instructions. Negative controls were realized by omitting primary antibodies. Counterstaining was done with hematoxylin.

RNA preparation

Total RNA was isolated using the Nucleospin RNA II System (Macherey-Nagel GmbH &Co., Düren, Germany) according to the manufacturer's protocol. The RNA was eluted with 50 μl of RNase-free water. RNA integrity and purity were verified using the Agilent Bioanalyzer system (Agilent Technology).

PCR and quantitative real-time PCR (RT-qPCR)

For PCR, 1 μg of total RNA was first reverse-transcribed using Superscript II reverse transcriptase (Invitrogen), random hexamers (Roche), and dNTPs at 42°C for 1 h. For non-quantitative PCR, the High Fidelity PCR Master kit (Roche) was used according to the manufacturer's protocol. Amplification conditions were adjusted to be within the linear range. Quantitative real-time PCR (RT-qPCR) was performed on a LightCycler (Roche Diagnostics) using the LightCycler FastStart DNA Master SYBR Green kit (Roche Diagnostics) according to the manufacturer's instructions. The PCR primers were designed to amplify cDNA fragments ranging in size from 150 to 400 bp and are listed in Table 1. Gene expression levels in each sample were determined using the comparative Ct method (after validation assays for each

Table 1. Primer sequences of genes investigated in this study.

Symbol	Full name	Accession #	Forward (F) and Reverse (R) primer sequence
Adamts5	Disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 5	NM_011782	F: 5′-ATGCAGCCATCCTGTTCAC-3′
			R: 5′-CATTCCCAGGGTGTCACAT-3′
Adipoq	Adiponectin, C1Q and collagen domain containing	NM_009605	F: 5′-CAGGCATCCCAGGACATCC-3′
			R: 5′-CCAAGAAGACCTGCATCTCCTTT-3′
Col2a1	Collagen, type II, alpha 1	NM_001113515	F: 5′-GGTGGCTTCCACTTCAGCTAT-3′
			R: 5′-TCATTGGAGCCCTGGATGAG-3′
Col10a1	Collagen, type X, alpha 1	NM_009925	F: 5′-TTCTCCTACCACGTGCATGTG-3′
			R: 5′-AGGCCGTTTGATTCTGCATT-3′
CtsC	Cathepsin C	NM_009982	F: 5′-CCAACTGCACCTACCCTG-3′
			R: 5′-CTGAACGGTATTGATGGCCT-3′
CtsS	Cathepsin S	NM_021281	F: 5′-TGGTGACGAAGATGCCCTGAAAGA-3′
			R: 5′-TGCCATCAAGAGTCCCATAGCCAA-3′
Erg	Avian erythroblastosis virus E-26 (v-ets) oncogene related	NM_133659	F: 5′-GTGGGCGGTGAAAGAATATGG-3′
			R: 5′-CTTTGGACTGAGGGGTGAGG-3′
DN-Erg	Transgen Dominant-Negatif Erg	-	F: 5′-ACCCACAGAAGATGAACTTTG-3′
			R: 5′-GGATCCACTAGTTCTAGAGG-3′
Fabp4	Fatty acid binding protein 4, adipocyte	NM_024406	F: 5′-CAAAATGTGTGATGCCTTTGTG-3′
			R: 5′-GGCTCATGCCCTTTCATAAAC-3′
Hprt	Hypoxanthine guanine phosphoribosyl transferase	NM_013556	F: 5′-GCTGGTGAAAAGGACCTCT-3′
			R: 5′-AAGTAGATGGCCACAGGACT-3′
Matr3	Matrin3	NM_010770	F: 5′-TTACCAGCACCCAGATTTCC-3′
			R: 5′-TGGAGCAAGTCACAGTCGTC-3′
Mmp3	Matrix metallopeptidase 3	NM_010809	F: 5′-TGACCCACATATTGAAGAGC-3′
			R: 5′-ACTTGACGTTGACTGGTGTC-3′
Mmp9	Matrix metallopeptidase 9	NM_013599	F: 5′-ACTCACACGACATCTTCCAG-3′
			R: 5′-AGAAGGAGCCCTAGTTCAAG-3′
Mmp13	Matrix metallopeptidase 13	NM_008607	F: 5′-TTTATTGTTGCTGCCCATGA-3′
			R: 5′-TTTTGGGATGCTTAGGGTTG-3′
Plin	Perilipin 1	NM_001113471	F: 5′-TGCTGGATGGAGACCTC-3′
			R: 5′-ACCGGCTCCATGCTCCA-3′
Pparg	Peroxisome proliferator activated receptor gamma	NM_011146	F: 5′-GCATCAGGCTTCCACTATGGA-3′
			R: 5′-AAGGCACTTCTGAAACCGACA-3′
Runx2	Runt related transcription factor 2	NM_001145920	F: 5′-GAGGCCGCCGCACGACAACCG-3′
			R: 5′-CTCCGGCCCACAAATCTCAGA-3′
Scin	Scinderin	NM_009132	F: 5′-AACAGTGGTAGAGTCCAGATT-3′
			R: 5′-GTGATAGATGCCAGGTTCCTC-3′
Sox9	SRY-box containing gene 9	NM_011448	F: 5′-TGGCAGACCAGTACCCGCATCT-3′
			R: 5′-TCTTTCTTGTGCTGCACGCGC-3′

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gene primer set), using the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene as an endogenous control. The wt control was set to 1 and expression data are presented as bar graphs of the mean values with their SD.

Microarray Analysis

Hybridisation was carried out following the Two-Colour Microarray-Based Expression Analysis protocol (Agilent Technologies). For each sample, 1 μg of total RNA per sample was divided into two equal aliquots to enable technical replication known, as a dye-swap hybridisation, and amplified. Dye-swap hybridisations

were performed by reversing the dyes for each of the RNA samples. The reverse transcription and the labelling were conducted using the protocol recommended by Agilent (Agilent Low RNA Input Fluorescent Linear Amplification kit). Whole Mouse Genome Oligo Microarrays from Agilent Technologies were used and hybridisations were performed for 17 h at 65°C (Gene Expression Hybridization kit, Agilent Technologies). Slides were washed and scanned using Innoscan 700 (Innopsys) and raw data were processed using the Limma package (Linear Models for Microarray Data) running under the statistical language R. A normalisation protocol consisting of a within-array loess normalisation to correct for dye and spatial effects was applied on the median or mean intensities of the spots. After normalisation, identification of statistically significant deregulation was performed using a moderated Student's *t*-test with empirical Bayes shrinkage of standard errors. We selected genes whose expression was significantly upregulated (mean log-ratio >3.3x; adjusted p-value <0.005) or downregulated (mean log-ratio <-3.3x; significant at the adjusted p-value <0.005 level). The data were further analysed using MultiExperiment Viewer (version 4.5), Fatigo and Pathway-Express. The latter was used to predict molecular signalling pathways by assessing an impact factor that accounts for contribution to the proportion of differentially regulated genes in a given pathway.

Results

Morphological phenotype of adipocytes in monolayer-cultured DN-Erg chondrocytes

To investigate how modified Erg function (via dominant negative transgene expression) affects chondrocyte phenotype, we isolated chondrocytes from the ribs of wt or DN-Erg E18.5 embryos and cultured them in a conventional monolayer culture. After plating chondrocytes in culture dishes (designated as day 0), cells showed a polygonal shape until confluence (Fig. 1, day 0 to day 3). Thereafter, the morphology of DN-Erg chondrocytes changed rapidly and dramatically. Some DN-Erg cells became enlarged fibroblast-like cells and accumulated vesicles. These vesicles were strongly stained with Oil red O, suggesting they were lipid droplets. Compared with DN-Erg cells, which accumulated lipid droplets from day 9 to day 20 (Fig. 1 and Fig. S1), wt chondrocyte cultures only just begun to show weak Oil red O staining as of day 20. Using an Alcian blue stain as a chondrocyte-specific matrix stain, DN-Erg cultures showed a decrease in cartilage matrix deposition in comparison to wt cultures (Fig. S2). These morphological and cytochemical observations showed that monolayer-cultured DN-Erg chondrocytes display an adipocyte-like phenotype, suggesting that DN-Erg transgene expression favours an adipocyte-like phenotype in monolayer culture over time.

Decrease in chondrocyte marker expression and detection of adipocyte-related genes in cultured transgenic DN-Erg chondrocytes

We observed great morphological differences between DN-Erg and wt chondrocytes over the 20 day culture period. To characterize the adipocyte-like phenotype of DN-Erg chondrocytes, we paid special attention to the expression of specific chondrogenic and adipogenic markers using quantitative real-time PCR (Fig. 2). Total RNA was collected from wt and DN-Erg chondrocytes after 0, 6, 10, 15 and 20 days of culture.

We studied the expression of four major chondrocyte markers, namely *Col2a1*, *Sox9*, *Col10* and *Runx2* (Fig. 2A). As expected, in wt chondrocytes, *Col2a1* and *Sox9* showed a peak in expression during the first 6 days of culture, followed by a dramatic decrease. The expression level of these two markers was 2-fold and 4-fold lower, respectively, in DN-Erg chondrocytes compared with wt chondrocytes. Likewise, *Col10* and *Runx2* expression, which were 2- to 3-fold downregulated in DN-Erg, decreased rapidly in both chondrocyte cultures within 6 and 10 days of culture, respectively, and remained at low levels throughout the rest of the culture period. We also verified DN-Erg expression. As expected, since the transgene is under *Col2a1* promoter control, the transgene expression was expressed during the culture, and its pattern was similar to the *Col2a1* pattern (Fig. S3).

Because Oil red O staining revealed lipid vesicles in cultured DN-Erg cells, we assayed the expression of adipocyte markers, such as the *adiponectin* (*Adpn*) and *peroxisome proliferator-activated receptor gamma* (*Pparγ*) genes (Fig. 2B). In both chondrocyte cultures, we detected *Adpn* transcripts at very low levels until day 6 of culture. *Adpn* expression was upregulated at day 20 compared to day 0, when mRNA levels were 200- and 600-fold greater in wt and DN-Erg chondrocytes, respectively. Although *Pparγ* mRNA level remained constant throughout wt chondrocyte culture, it increased in DN-Erg chondrocytes after 6 days of culture.

Taken together, these results validated the observed phenotypic changes. DN-Erg chondrocytes cultured as monolayers experienced a dramatic decrease in the expression of chondrocyte-specific markers and a strong emergence of adipocyte-specific markers.

Comparative differential transcriptomic analysis of monolayer-cultured wt and DN-Erg chondrocytes

To investigate and compare all the modulated genes in monolayer-cultured DN-Erg and wt chondrocytes over time, we monitored the change in genome-wide expression patterns with a global differential approach using whole Mouse Genome Oligo Microarrays (Agilent). Using the monolayer cultures, total RNA isolated from day 0 to day 20 were hybridised to a microarray containing 44,000 probe sets representing nearly 41,000 mouse genes. Comparative microarray analyses were done using Mapix 3.2 (Innopsys). Because the number of differentially expressed genes was high, we chose a cut-off value of a 10-fold change in expression and an adjusted p-value of <0.005.

The results show that 93 transcribed genes (42 upregulated and 51 downregulated) were significantly modified 10-fold or more between day 0 and day 20 in cultured wt chondrocytes (Fig. 3A and Table S1), 585 genes (442 upregulated and 143 downregulated) were modified between day 0 and day 20 in cultured DN-Erg chondrocytes (Fig. 3A and Table S2). Finally, even with the stringent cut-off criterion (i.e. value of 10-fold or more), the number of modified genes was six times greater in DN-Erg chondrocytes than in wt chondrocytes between day 0 and day 20 of culture.

To classify the genes whose expression had been modified, further examination was performed on the basis of Gene Ontology (GO) annotations (Fig. 3B). We first focused on genes classified by the following functional annotations from the Gene Ontology Database: "extracellular matrix region", "metallopeptidase activity", "cartilage condensation and development", and "ossification" annotations. We then used hierarchical clustering to group together genes with similar expression patterns (Table S3). This process resulted in a selection of 190 upregulated or downregulated genes coding for collagens, proteoglycans, matrix-modifying enzymes, growth factors and transcription factors (Fig. 3B). Among these 190 genes, 9 genes whose expression is known to decrease during chondrocyte dedifferentiation were assayed and found to be dramatically downregulated in cultured DN-Erg

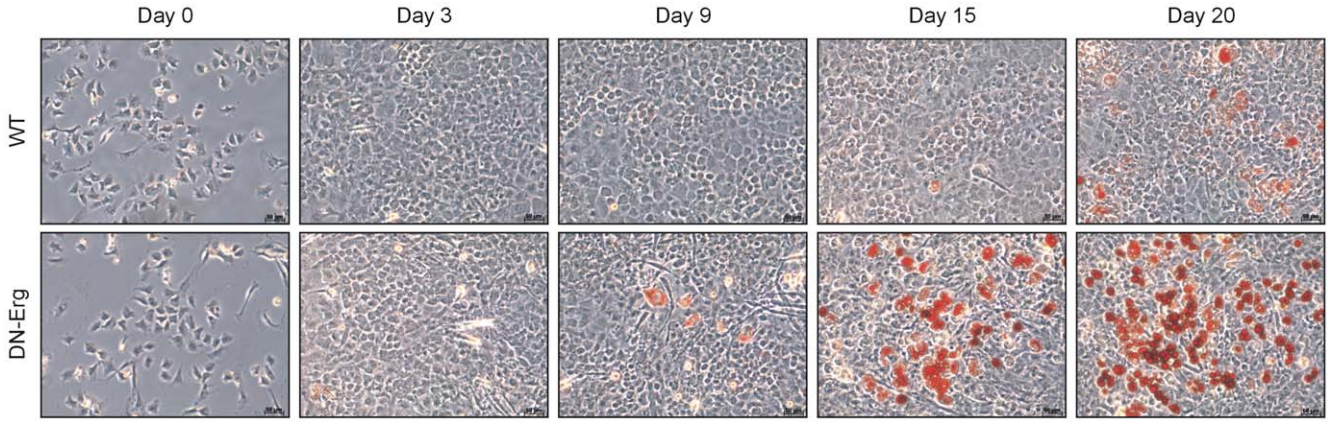

Figure 1. Morphological changes in wt and DN-Erg E18.5 chondrocytes in culture. Chondrocytes from freshly isolated from ribs of wt and DN-Erg transgenic mouse embryos (at E18.5) were cultured for up 20 days and stained with Oil red O. Phase-contrast images at days 0, 3, 9, 15 and 20 (with day 0, the day of plating) are shown (Scale bar, 50 μm).
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chondrocytes: *scinderin* (*Scin*), *hyaluronan and proteoglycan link protein1* (*Hapln1*), *matrilin 3* (*Matn3*), *SPAR like 1*, *Col11a2*, *epiphycan* (*Epyc*), *col27a1*, *fibroblast growth factor receptor* (*Fgfr3*) and the transcription factor Sox9, which showed decreased expression in the previous experiment (Fig. 2B). Likewise, the following genes were substantially upregulated: matrix metallopeptidase and aggrecanase (*Adamts5*, *Adamts2*, *Adamts15*, *Mmp 13*, *Mmp3*, *Mmp9*, and *Mmp11*).

To identify functionally related patterns from the list of genes differentially expressed between day 0 and day 20, we carried out a pathway analysis and compared the relative distribution of functions (Fig. 3C). Genes modified between day 0 and day 20 in DN-Erg chondrocytes were involved in several functional pathways. In accordance with what we observed in the cultured DN-Erg cell plate, we noticed clusters of genes involved in the 'PPAR signalling pathway' and in 'adipocyte differentiation' (from the Gene Ontology Database) (Table S4). Nine transcripts (*Adpn*, periplin (*Plin*), fatty acid binding protein 4 (*Fabp4*), lipoprotein lipase (*Lpl*), carnitine palmitoyltransferase 1a liver (*Cpt-1a*), acyl-Coenzyme A oxidase 2 branched chain (*Acox2*), angiopoietin-like 4 (*Angptl4*), CD36 antigen (*CD36*), adipose differentiation related protein (*Adfp*)) were associated with the 'PPAR signalling pathway' (Fig. S4) and were significantly upregulated in DN-Erg chondrocytes cultured for 20 days (Fig. 3D and Table S4).

Validation of changes in gene expression by real-time PCR

To confirm the observed differences in gene expression between day 0 and day 20 of cultured DN-Erg and wt cells by microarray assay, we performed a quantitative RT-PCR (RT-qPCR) analysis on 11 additional selected genes products (Table 2) in addition to *Sox9* and *Adpn* genes (Fig. 2). In all cases, the direction of change in expression was concordant between the microarray and RT-qPCR results, although absolute values of the microarray-estimated fold change and RT-qPCR-calculated fold change were different (e.g. *Matn3*). Nevertheless, RT-qPCR results were in line with microarray data and confirmed the loss of the chondrocyte phenotype and the dramatic upregulation of adipocyte markers in cultured DN-Erg chondrocytes.

Predominant adipogenesis in bone marrow of adult DN-Erg mice

Since the cultured primary chondrocytes were isolated from the ribs of 18.5 day-old wt and DN-Erg embryos, we compared the cartilaginous framework phenotype of wt and DN-Erg embryos (Fig. 4A and unpublished data). The ribs were morphologically homogenous in appearance and consisted only of chondrocytes evenly distributed in extracellular matrix (Fig. 4B).

The particular phenotype of DN-Erg transgenic mice, such as early-ageing processes, including hyperlordosis/hyperkyphosis and reduced mobility, appeared with age and was associated with an arthritis-like phenotype (unpublished data). Given these results, we were interested in features of bone marrow from tibia and femur of transgenic mice. Histological examination of limb sections stained with hematoxylin/eosin showed the distinct appearance of adipocytes occupying the marrow cavity (Fig. 5A and Fig. S5A). A clear increase in the number of adipocytes present in the bone marrow of mice was observed upon ageing (Fig. 5A, S5A). Adipocyte densities were significantly higher in DN-Erg compared to wt mice at weeks 6 and weeks 40 (Fig. 5B, 5C). Although adipocytes were readily distinguishable from other cell types present in bone marrow by their morphology (Fig. 5C), Sudan black B staining and immunocytochemistry staining of adiponectin were performed to confirm our observations (Fig. S5B and S5C).

Taken together, these histological investigations of bone marrow from adult mice femur revealed that ETS transcription factor family may contribute to the homeostatic balance in skeleton cell plasticity *in vivo*.

Discussion

Erg expression is associated with early events of cartilage formation during development [21,24,31], but its physiological role in this mechanism has been poorly understood. Several specific transcription factors (such as Nkx3.2, Sox9, etc.) have been shown to be involved in the maintenance of the chondrogenic state [32,33] or in the promotion of transdifferentiation into an adipogenic state [34]. In the present study, we showed that Erg is an important component in the maintenance of the chondrogenic phenotype *in vitro*, and in the healthy ageing of the skeleton. However, since other ETS family genes are also expressed, to a

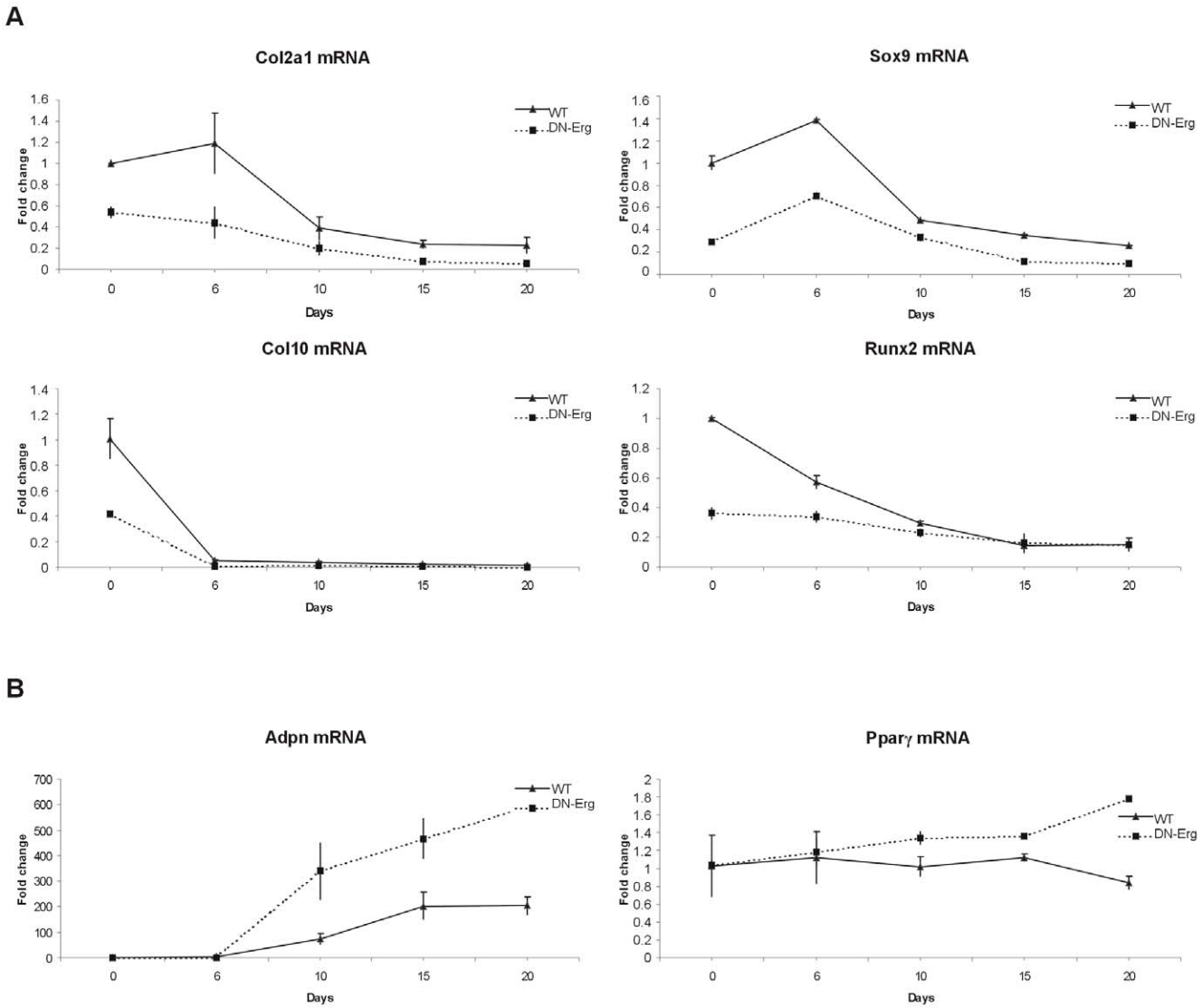

Figure 2. Expression of chondrogenic and adipogenic markers during monolayer culture of wt and DN-Erg chondrocytes. A. Expression of chondrogenic genes *Col2a1*, *Sox9*, *Col10*, and *Runx2*. Gene expression was evaluated by RT-qPCR. B. Expression of adipogenic genes *Adpn*, and *Pparγ*. Gene expression was evaluated by RT-qPCR. The reported target gene: *Hprt* transcript ratio in chondrocytes was normalised to the target gene:*Hprt* transcript ratio (set to 1) of freshly plated wt chondrocytes (day 0). Data represent the mean of at least 2 independent chondrocyte cultures from 2 distinct mice for each genotype.
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lesser extent, in cartilage, we cannot rule out that the binding of these transcription factors is also potentially disturbed.

Chondrocytes that are grown in a standard monolayer culture are known to lose their phenotype after a few passages [35]. Furthermore, studies have shown that chondrocytes of the mouse process may transdifferentiate into adipocytes under culture conditions and accumulate lipid droplets [8,34]. In our study, we observed that chondrocytes isolated from embryos of a transgenic mouse model (expressing a dominant negative form of Erg during cartilage formation and cultured *in vitro*) and a wt mouse rapidly lose their chondrogenic phenotype. In contrast, only DN-Erg chondrocytes expressed the adipogenic phenotype over time. Significant morphological differences, such as cell morphology and lipid accumulation, were observed in DN-Erg chondrocytes stained in monolayer culture. These observations were confirmed by a comparative transcriptomic analysis of cultured DN-Erg chondrocytes. A high cut-off value was needed to analyse cDNA microarray because of the high number of deregulated genes. GO analysis identified specific groups of regulated gene products associated with a loss of the chondrogenic phenotype and the appearance of adipogenic differentiation markers. Expression of several genes involved in chondrocyte differentiation and hypertrophic maturation dramatically decreased between day 0 and day 20 in DN-Erg compared with wt chondrocytes, whereas the expression of genes involved in matrix metalloproteinases (MMPs) and aggrecanases, leading to articular cartilage destruction, were upregulated. Moreover, the expression of several key features of osteoarthritis pathology [36] and pro-inflammatory mediators (e.g. cysteine proteases cathepsin S and cathepsin C [37]), were detected among the highly regulated genes during DN-Erg chondrocyte culture.

The expression of three master genes *Sox9*, *Runx2* and *Pparγ*, which are critical for phenotype determination at early stages of mesenchymal cells in cartilage formation, osteogenesis and

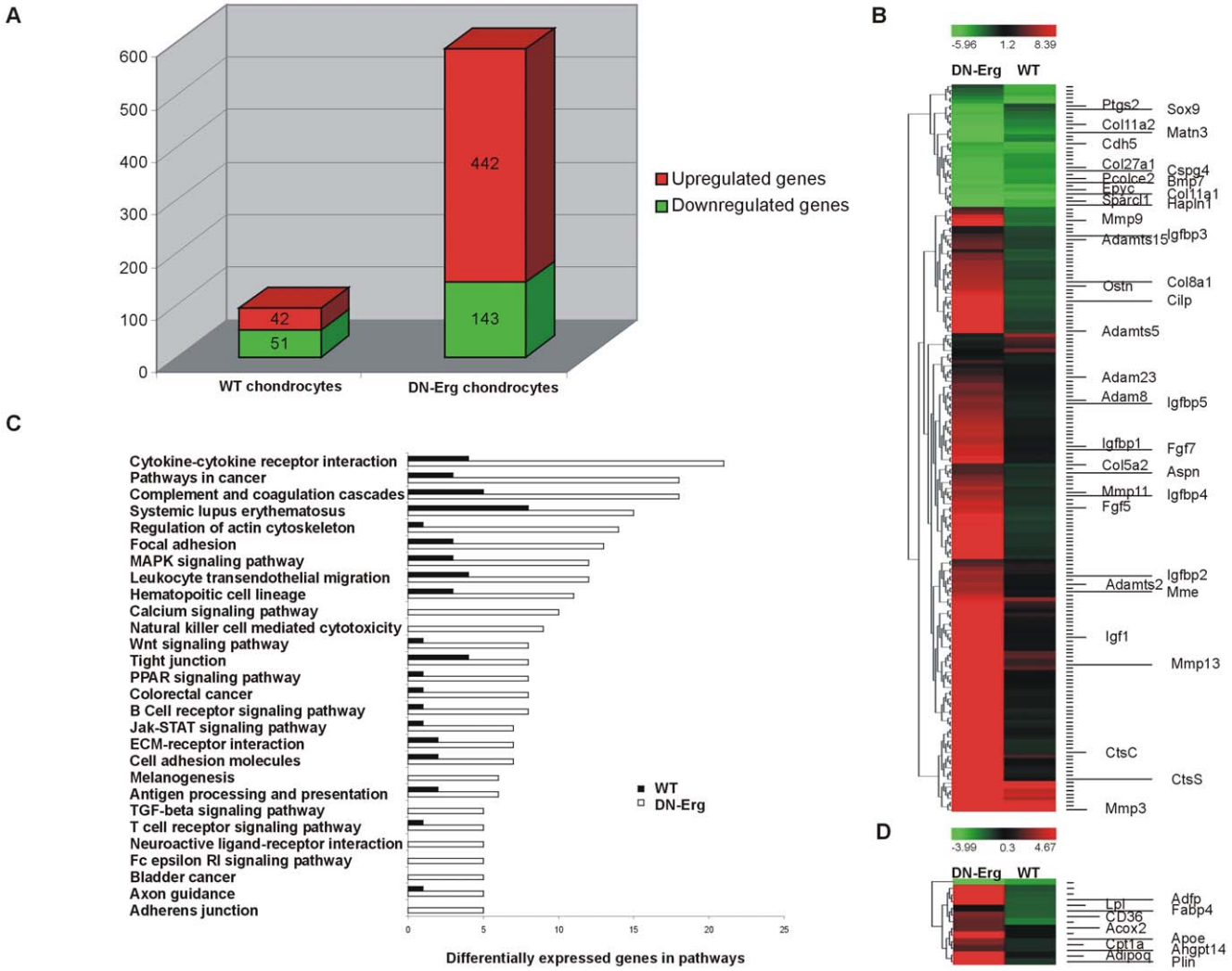

Figure 3. Microarray analysis and gene ontology analysis of signalling pathways. A. Numbers of genes with differential expression between monolayer culture day 0 and day 20 in wt and DN-Erg embryo (E18.5) chondrocytes. Probe sets were filtered according to a 10-fold change cut-off. B. Hierarchical Clustering (HCl) diagram with clusters genes corresponding to the "extracellular matrix", "metallopeptidase activity", "Cartilage condensation and development", "Ossification" annotations. C. Major signalling pathways predicted using Pathway-Express. Pathways listed are pathways with at least 5 or more genes which expression was modified during culture, as determined by Pathway Express. D. Hierarchical Clustering (HCl) tree with clusters of "Lipid metabolism process" and "Lipid transport" genes.
doi:10.1371/journal.pone.0048656.g003

adipogenesis, respectively, were affected in freshly isolated and in cultured DN-Erg chondrocytes. Knowledge on each transcription factor in the commitment and the maintenance of chondrogenic cell lineages suggests that their deregulation may contribute to the dedifferentiation of chondrocytes and the switch of chondrocytes into adipocytes. Sox9 plays an essential role in the promotion of chondrogenesis [38] and may maintain chondroblasts in an immature state. In addition, the transcription factor Runx2 is a critical enhancer of chondrocyte maturation and osteoblast differentiation. The expression of these two transcription factors was decreased by over 60% in DN-Erg chondrocytes relative to wt chondrocytes. Such low expression levels of key chondrogenesis regulators may explain the early dedifferentiation of cultured DN-Erg chondrocytes. Moreover, as for the depletion of Runx2 in chondrocytes [39], the competition of Erg with a transdominant negative protein favours the loss of the well-established chondrocyte phenotype and the emergence of adipocytes. To show the potent inhibition of the Erg transcription factor in the adipocyte differentiation process, we followed *Erg* expression in 3T3L1 preadipocytes cells (data not shown). The *Erg* gene is expressed at a very basal level in 3T3L1 cells, and its level did not change when adipocyte differentiation was induced. Moreover *Erg* overexpression did not affect adipogenenic differentiation of 3T3L1 cells (data not shown). Altogether, these results suggest that although the Erg protein may be involved in the inhibition of the transdifferentiation of chondrocytes into adipocytes, it was not associated with the adipogenic process.

The nuclear receptor Pparγ, mainly involved in the regulation of adipogenesis and in the expression of adipocyte-related differentiation marker genes, also plays an important, albeit complex, role in bone metabolism. On the one hand, Pparγ favours the differentiation of mesenchymal stem cells into adipocytes rather than osteoblasts or chondrocytes [40]; *Pparγ* overexpression has been reported to promote adipogenic differentiation in growth plate chondrocytes [34], but it has no effect on fully differentiated osteoblasts or osteoclasts [41]. On the other

Table 2. Correlation of microarray data and RT-qPCR analysis.

		Microarray		Real-Time qPCR	
		(Fold-change between day 20 vs day 0 of culture)		(Fold-change between day 20 vs day 0 of culture)	
Sequence Name	**Accession**	**WT**	**DN-ERG**	**WT**	**DN-ERG**
Cartilage Development					
Scinderin	NM_009132	−2	−71	−14	−795
Matn3	NM_010770	−7	−39	1	−2
Metalloproteinases					
ADAMTS5	NM_011782	2	49	4	56
MMP3	NM_010809	40	284	13	87
MMP9	NM_013599	−1	26	1	137
MMP13	NM_008607	11	49	10	71
Cathepsine					
CTS C	NM_009982	4	131	39	317
CTS S	NM_021281	6	288	52	1116
Adipocyte Differentiation					
Plin	NM_175640	4	64	3	16
FABP4	NM_024406	3	16	146	284
Adipoq	NM_009605	9	84	200	467

Comparison of fold change in the expression of 11 genes implicated in chondrogenesis or adipogenesis as determined by microarray analysis and RT-qPCR.
Fold change between day 0 and day 20 observed by microarray-analysis and RT-qPCR for wt and DN-Erg chondrocytes are shown. Expression levels of target genes obtained by RT-qPCR were normalised to *Hprt*. Upregulation is indicated by positive values and downregulation is indicated by negative values.
doi:10.1371/journal.pone.0048656.t002

hand, *in vivo* Pparγ has been shown to be expressed and activated in articular chondrocytes. It is required for endochondral ossification and cartilage development, and has chondroprotective properties against osteoarthritis [42,43,44,45]. Several studies have shown that Pparγ is expressed in hypertrophic chondrocytes. Even if its role remains incompletely resolved, the expression of this transcriptional factor is involved in lipid and energy metabolism [46]. In the present study, *Pparγ* was expressed in wild-type and DN-Erg chondrocytes, and no difference in the level of *Pparγ* mRNA expression was found in freshly isolated chondrocytes. However, the expression of *Pparγ* and its target genes were progressively increased in DN-Erg chondrocytes during culture, which may affect chondrocyte features and accelerate the differentiation pathway to adipocytes.

The present results obtained with primary chondrocytes prompted us to compare skeletal features of transgenic mice with wt mice. It is well known that, with age, bone marrow becomes enriched into adipocytes [47,48]. Moreover, age-related diseases such as osteoporosis and osteopaenia are described as accompanied by a pronounced accumulation of adipocytes in bone marrow

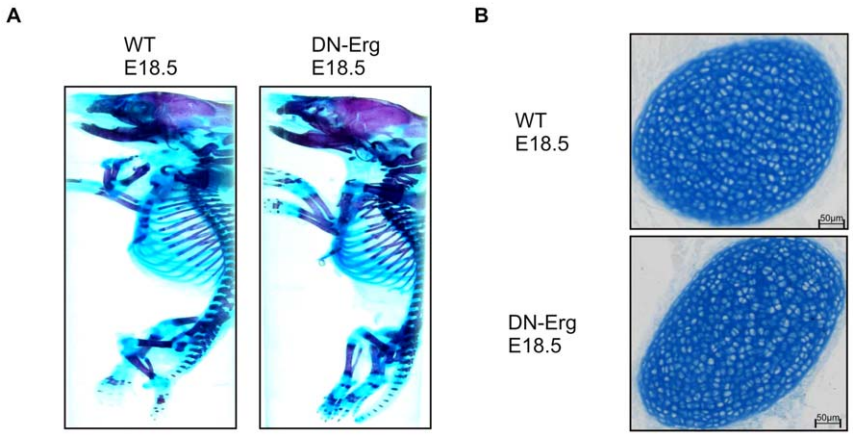

Figure 4. Histological examination of 18.5 day-old embryos. A. Skeletal and cartilage preparations of wt (left) and DN-Erg (right) mice at E18.5. Cartilage stained with Alcian blue, bone with Alizarin red. DN-Erg E18.5 embryos did not show any overt abnormalities in cartilaginous or skeletal development. B. Distribution of chondrocytes on sections of rib. Paraffin-embedded sagittal sections of wt (left) and DN-Erg (right) newborn mice were stained with Alcian blue. Bars = 50 µm.
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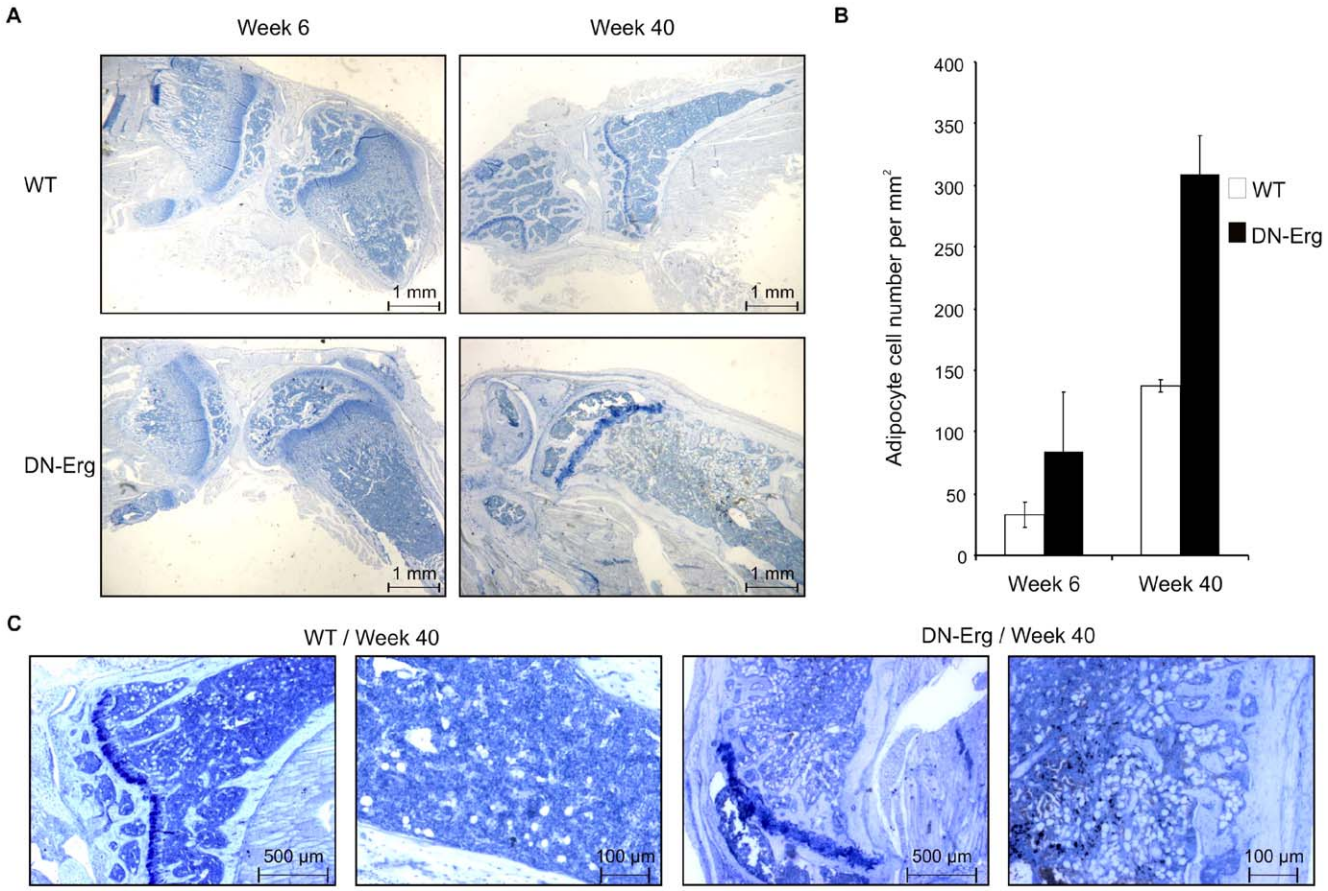

Figure 5. Cytological analysis and adipocyte quantification of femoral bone marrow of wt and DN-Erg mice. A. Sections of femorotibial joint of week 6- and week 40- wt or DN-Erg mouse. Bars = 1 mm. B. Quantification of adipocytes in bone marrow of DN-Erg and wt femoro-tibial section. The result was expressed as the mean of total adipocyte number per square millimeter of marrow tissue area in the analysed fields of at least three different mouse. C. Cytological examination of bone marrow in a femur section of 40 week-old wt and DN-Erg mice. Left, Magnification: ×2.5. Right: higher magnification view (×10).
doi:10.1371/journal.pone.0048656.g005

[49,50,51,52]. In contrast with adult wt mice, DN-Erg mice showed predominantly fatty marrow. The function and origin of fatty marrow are still largely unknown [53]. However, these little-studied cells play a more active role than just passively filling the bone cavity [54]. Given the abundant variety of adipokines secreted by adipocytes, the increase in adipocytes in the bone marrow helps to inhibit functions of other bone cells, including osteoblasts and hematopoietic stem cells [55,56], and structural changes in the surrounding cartilage matrix, thereby disturbing bone regeneration [49,57,58,59]. All these results raise the question of the origin of adipocytes in bone marrow. The bone marrow formation occurs post-natally via the invasion of capillaries through the cortical bone shaft. Downregulation of Sox9 in the hypertrophic zone of the growth plate is required for cartilage-bone transition and bone marrow formation [60]. In this context, our results suggest that DN-Erg transgene expression during chondrogenesis may affect skeletal plasticity and bone quality in adults.

In summary, the present study strongly suggests that the Erg transcription factor is involved in chondrogenesis and plays an important role in the maintenance of the chondrocyte pathway. The potential role of the Ets protein in skeletal cell plasticity was further highlighted by *in vivo* observations of accelerated formation of fatty marrow in adult DN-Erg mice. Finally, factors affecting cartilage formation may have a high incidence on the occurrence of ageing-related diseases.

Supporting Information

Figure S1 Chondrogenic phenotype was assessed by Alcian blue staining. Chondrocytes were cultured for 15 days, then stained with Alcian blue and observed under a phase-contrast microscope at ×10 magnification.
(TIF)

Figure S2 Oil red O staining of chondrocytes isolated from WT and DN-Erg E18.5 mice and cultured for 20 days. Low-magnification; inset: ×10 magnification.
(TIF)

Figure S3 PCR Analysis of mRNA levels in DN-Erg mice after 0, 6, 10, 15 and 20 days of culture. The reported target gene (i.e. DN-Erg):*Hprt* transcript ratio at each time was determined par qPCR and was normalised to DN-Erg:*Hprt* transcript ratio (set to 1) on day 0. ND = not determined.
(TIF)

Figure S4 PPAR signalling pathway based on Pathway-Express. In the network diagrams, the red boxes indicate genes whose mRNA levels changed during the 20 days of culture.
(TIF)

Figure S5 Histological analysis of bone marrow and Adiponectin detection in femur sections of DN-Erg. A. Cytological examination of bone marrow in femur sections of 6, 24 and 40 week-old wt and DN-Erg mice. Scale bar: 100 µm. B. Lipid staining with Sudan black B (Merck) was used to evaluate lipid droplets within *adipocytes* according to Soldani et al. [61] on section from femur of 40 week-old DN-Erg mice. Scale bar: 50 µm. C. Adiponectin immunocytochemical staining in bone marrow of 40 week-old DN-Erg mice femur. Scale bar: 50 µm.
(TIF)

Table S1 List of genes with a 10-fold or more change in expression between day 0 and day 20 of culture in wt chondrocytes in microarray-derived data.
(XLS)

Table S2 List of genes with a 10-fold or more change in expression between day 0 and day 20 of cultured in DN-Erg chondrocytes in microarray-derived data.
(XLS)

Table S3 List of genes classified by the ''extracellular matrix region'', ''metallopeptidase activity'', ''cartilage condensation and development'', ''ossification'' annotations in the Gene Ontology Database.
(XLS)

Table S4 List of genes classified by the ''PPAR signalling'' and ''adipocyte differentiation'' annotations in the Gene Ontology Database.
(XLS)

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Author Contributions

Conceived and designed the experiments: SF DH MDC. Performed the experiments: SF TT LH NT AF PD. Analyzed the data: SF TT LH MHE DH FMG MDC. Contributed reagents/materials/analysis tools: SF TT LH NT AF MHE ML DH MDC. Wrote the paper: SF MDC.

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Signaling and Regulation

Molecular Cancer Research

Abnormal Expression of the ERG Transcription Factor in Prostate Cancer Cells Activates Osteopontin

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Abstract

Osteopontin (OPN) is an extracellular matrix glycophosphoprotein that plays a key role in the metastasis of a wide variety of cancers. The high level of OPN expression in prostate cells is associated with malignancy and reduced survival of the patient. Recent studies on prostate cancer (PCa) tissue have revealed recurrent genomic rearrangements involving the fusion of the 5′ untranslated region of a prostate-specific androgen-responsive gene with a gene coding for transcription factors from the ETS family. The most frequently identified fusion gene is *TMPRSS2:ERG*, which causes ERG protein overexpression in PCa cells. ERG is a transcription factor linked to skeletogenesis. This study was designed to test whether ERG and the product of the *TMPRSS2:ERG* fusion gene modulate *OPN* gene expression in PCa cells. To characterize ERG and TMPRSS2:ERG transcriptional activity of *OPN*, we focused on ETS binding sites (EBS) localized in conserved regions of the promoter. Using *in vitro* and *in vivo* molecular assays, we showed that ERG increases *OPN* expression and binds to an EBS (nt −115 to −118) in the *OPN* promoter. Moreover, stable transfection of prostate tumor cell lines by TMPRSS2:ERG upregulates endogenous OPN expression. Finally, in human prostate tumor samples, detection of the *TMPRSS2:ERG* fusion gene was significantly associated with OPN overexpression. Taken together, these data suggest that *OPN* is an ERG-target gene in PCa where the abnormal expression of the transcription factor ERG, due to the TMPRSS2:ERG fusion, disturbs the expression of genes that play an important role in PCa cells and associated metastases. *Mol Cancer Res; 9(7); 914–24. ©2011 AACR.*

Introduction

Prostate cancer (PCa) is the most commonly diagnosed nonskin cancer in men and continues to be one of leading causes of male cancer-related death among the elderly. PCa has high metastatic capacity and extensively metastasizes to bone, lymph nodes, and visceral organs, such as the liver and lungs. Research over the last few decades on metastasis has revealed detailed steps of this mechanistic cascade (1): some primary tumor cells detach and escape from the original tumor sites, intravasate into circulation, extravasate out into the secondary tissues in which they begin to proliferate (2). Chemotaxis plays an important role in the metastasis of cancer cells, directing the motility of metastatic cells through gradients of growth factors or chemoattractants.

Osteopontin (OPN), also called secreted phosphoprotein 1 (SPP1), is a glycophosphoprotein cytokine that is expressed by numerous cells and secreted into body fluids. This extracellular matrix protein is an active player in many physiological and pathological processes including bone mineralization, defense against infectious agents, blood vessel formation, arteriosclerosis, disruption of the growth of calcium oxalate crystals and nitric oxide production, and tumorigenesis (3, 4). Substantial evidence associates *OPN* expression with tumor invasion and metastasis (5) in a number of cancers such as breast (6), prostate (7), lung (8), stomach (9) colon (10), brain (11), and other cancers (12).

Transcriptional regulation of *OPN* is complex and involves multiple signal transductions (13). Identifying transcriptional regulators that contribute to the modulation of *OPN* expression is of interest for therapy that aims to control the *OPN*-mediated metastatic phenotype. Several known cis-acting transcription factors have been described and most of them have been localized to a conserved region at 250 bp upstream of the proximal promoter. Potential binding sites for transcriptional regulators, such as AP-1, Myc, Oct-1, USF, v-Src, TGF-b/BMPs/Smad/Hox, Wnt/β-catenin/APC/GSK-3b/Tcf-4, Ras/RRF, TF53, Runx2, and ETS family members, have been identified (14–21).

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In the past few years, the *ETS*-related gene (*ERG* gene), a member of the ETS transcriptional factor family, has been shown to be highly overexpressed in most PCa (60%–80% according to the studies; refs 22, 23). Thus, its presence in prostate cells perturbs normal gene expression. ERG overexpression (and to a lesser extent the overexpression of some other ETS family members) is the result of chromosomal translocations between the androgen-regulated *Trans-membrane protease serine 2* (*TMPRSS2*) gene promoter and the coding sequence of the *ERG* gene (24). The *TMPRSS2:ERG* fusion is associated with aggressiveness and recurrence of PCa (25), metastasis (26) and PCa-specific death (27, 28).

Two members of the ETS family, PEA3 and Ets-1, have been shown to be partially responsible for the overexpression of *OPN* in human breast cancer and in murine colorectal cancer cells, respectively (29, 30). In this study, we investigated whether *ERG* overexpression stimulates *OPN* expression in PCa cells. *OPN* promoter–luciferase deletion constructs in transient transfection experiments showed *OPN* transactivation by the ERG factor. After localizing ETS binding sites (EBS) in sequences conserved across several species, we characterized the *OPN* promoter–ERG transcription factor complex using an electromobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). Because of the expression of the *TMPRSS:ERG* fusion gene in prostate tumor cells, the overexpression of ERG in PCa cells increased OPN expression. The coincident expression and colocalization of TMPRSS2:ERG and OPN in primary prostate tumor tissue samples suggest that ERG may be implicated in the dysregulation of OPN in PCa.

Materials and Methods

Patient information and tissue selection

All PCa patients included in this study had undergone radical prostatectomy in the Lille University Hospitals. Clinical data and patient consent were provided by the referring physician. Immunohistochemical studies were conducted on formalin-fixed and paraffin-embedded prostate specimens. Frozen tissues used for RT-PCR involved primary tumors from 35 male patients (mean age: 63 years, with Gleason score >5) and were obtained from the urological collection of the local tumor tissue bank (*Tumorothèque CRRC/Canceropole Nord-Ouest*, Lille, France) after approval by the internal review board.

Plasmid constructs

We used the expression plasmids for ERG (pSG5-ERGp55 and pcDNA3.1-ERG), described in reference (31). The TMPRSS2 (exon 1):ERG (exon 4) fusion transcript was amplified and subcloned into pcDNA3.1[(+) Invitrogen] using patient cDNA samples and primers: TMPRSS2 forward primer 5′-CGCGAGCTAAGCAGGAGGC-3′ and ERG reverse primer 5′-CCTCCGCCAGGTCTTTAGTA-3′. *OPN* promoter luciferase reporters were constructed by PCR with the following primers: *OPN*-116/+77 (−116 to +77), *OPN*-136/+77 (−136 to +77), *OPN*-341/+77 (−341 to +77), and *OPN*-1441/+77 (−1441 to +77). The fragments were subsequently cloned into a pGL3-basic luciferase reporter plasmid. Consensus ERG binding site mutations corresponding to nt −121 to −114 (5′-GGAGGAAG-3′ to 5′-GGTAAAAG-3′) and to nt −112 to −106 (5′-GTAGGAG-3′ to 5′-GTCGGAG-3′) were constructed using the *OPN*-136/+77 luciferase wild-type fragment as the template. Oligonucleotides encoding for small hairpin RNAs (shRNA) against ERG mRNA [targeted sequence described in (32)] was synthesized with appropriate loop and cohesive ends sequences according to the plasmid provider instructions and was cloned into pSilencer 2.1-U6 (Ambion Inc). All constructs were verified by sequencing (Genoscreen) and restriction enzyme digestion.

Cell Culture

PC3c (a PC3 subclone provided by E.B.) and HeLa cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum, 1% gentamicin and 1% glutamin (Invitrogen). Cultures were maintained at 37°C under 5% CO_2.

Transient transfection and activity assay

One day prior to transfection, cells were plated in 12-well plates to be 50% to 60% confluent on the day of transfection. Each well was transfected using polyethylenimine (PEI, Eurogentec) according to the manufacturer's instructions, with a DNA mixture including 500 ng of the firefly luciferase reporter gene containing the *OPN* promoter, 25 ng of a control plasmid tk-luciferase (tk-renilla; Promega), and the indicated concentration of pSG5-ERG. In the RNA silencing analysis, 300 ng of pSilencer ERG or pSilencer control were cotransfected. The total amount of transfected DNA was adjusted to 1 μg per well. After 48 hours of transfection, cells were harvested and luciferase activities were assayed using the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (Berthold Biolumat centro LB960). Firefly luciferase values were normalized to those of the control renilla luciferase values. All experiments were repeated twice. Results are presented as the mean ± SEM.

Generation of PC3c cell subclones

PC3c cells stably expressing ERG or TMPRSS2:ERG were obtained by transfecting them with pcDNA3-ERG or pcDNA3-TMPRSS2:ERG plasmids. Resistant clones were selected based on antibiotic resistance over 15–21 days in DMEM containing 300 μg/mL G418. Selected colonies were isolated, allowed to proliferate and characterized for ERG and TMPRSS2:ERG expression using RT-PCR and Western blot analysis.

EMSA and supershift assays

The assays were conducted as described in reference (33) using purified ERG protein [expressed and purified using the T7-Impact System (New England Biolabs) as described

previously (34)] and ERG transfected-HeLa nuclear extracts. An oligonucleotide (Eurogentec) probe nt −123 to −101 (5′-CCAGAGGAGGAAGTGTAGGAGCAG-GT) was prepared by end-labeling double-stranded oligonucleotides with [^{32}P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase (Promega) followed by G-50 column purification (GE-Healthcare). Radiolabeled oligonucleotide probes (100,000 cpm) were incubated with protein or nuclear extracts in a total volume of 20 μL for 30 minutes at 20°C. Binding buffer consisted of 100 ng/mL of poly(dI-dC)-poly(dI-dC), 20 mmol/L Tris-HCl (pH 7.9), 50 mmol/L KCl, 1 mmol/L MgCl2, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.02% NP-40 and 10% glycerol. DNA-protein complexes were resolved by electrophoresis for 1 hour at 200 V on a 5% nondenaturing polyacrylamide gel. The gels were dried, and signals were detected by exposure to autoradiography film. Nuclear extract was used as the negative control. In competitive binding assays, unlabeled oligonucleotides were added in 100 M excess. Oligonucleotides with mutated EBS were designed as follows: mutant 1 5′-CCAGAGG-TAAAAGTGTAGGAGCAGGT, mutant 2 5′-CCAGAG-GAGGAAGTGAAAAAGCAGGT and mutant 1+2 5′-CCAGAGGTAAAAGTGAAAAAGCAGGT. Supershift assays were conducted by adding 1 μL of polyclonal antibodies directed against ERG (sc-353 and sc-354, Santa Cruz Biotechnology).

Chromatin immunoprecipitation

ChIP assays were conducted as previously described (35) by using anti-ERG (sc-353, Santa Cruz Biotechnology). Briefly, formaldehyde cross-linked chromatin was sonicated and immunoprecipitated with either IgG (control) or the indicated Ab. ChIP analyses were conducted in at least triplicate using distinct DNA preparations. Immunoprecipitated DNA was purified and quantified by PCR. The targeted *OPN* promoter sequence (−269; −75) was amplified using the following primers: 5′-CATGGGATCCC-TAAGTGCTC-3′, 5′-TGAGGTTTTCTGCCACTG-CCC-3′. The target irrelevant sequence was amplified using primers: 5′-TGAGAGCAATGAGCATTCCGATG-3′ and 5′-CAGGGAGTTTCCATGAAGCCAC-3′.

RNA preparation and RT-PCR

Total RNA was extracted using the Nucleospin RNA II kit according to the manufacturer's protocol (Macherey-Nagel). One microgram of total RNA was reverse-transcribed using Superscript II RT (Invitrogen), random hexamers (Roche), and dNTPs at 42°C for 1 hour. OPN fragments were then amplified using the High Fidelity PCR Master kit (Roche) and the appropriate oligonucleotides (A: 5′-TGAGAGCAATGAGCATTCCGATG-3′, B: 5′-CAGGGAGTTTCCATGAAGCCAC-3′) using the following cycling parameters: initial denaturation at 94°C (5 minutes), then 30 cycles of 94°C (30 seconds), annealing at 60°C (30 seconds) and extension at 72°C (30 seconds), followed by a final extension at 72°C (7 minutes). The TMPRSS2:ERG fusion transcripts were amplified with the primers: TMPRSS2 forward primer 5′-CGCGAGCTAAG-CAGGAGGC-3′ and ERG reverse primer 5′-GTAGGCA-CACTCAAACAACGACTGG-3′ described in (24). As an internal control, hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) fragments were also amplified under the same conditions (forward primer: 5′-GCTGG-TGAAAAGGACCTCT-3′ and reverse primer: 5′-AAGTA-GATGGCCACAGGACT-3′). PCR products were analyzed by electrophoresis in a 2% agarose gel.

Cellular extract preparation and Western blot analysis

Cellular extract preparation and Western blot analysis were carried out as previously described (35, 36). Immunodetections were carried out using a polyclonal anti-ERG antibody (sc-353, Santa Cruz Biotechnology), a monoclonal anti-OPN antibody (sc-21742, Santa Cruz Biotechnology) and an anti-actin monoclonal antibody (Sigma).

Immunohistochemistry

Immunohistochemistry was carried out on an automated immunostainer (Benchmark XT) with the XT ultraview diaminobenzidine kit. The primary antibodies were directed against ERG (sc-353, Santa Cruz Biotechnology, dilution 1/50) or OPN (sc-21742, Santa Cruz Biotechnology, dilution 1/100). Antigen retrieval was conducted in Tris-EDTA buffer (pH 9) for 30 minutes at 95°C for ERG and in citrate buffer (pH 6) for 60 minutes at 95°C. Negative controls were realized by omitting primary antibodies. Counterstaining was done with hematoxylin and bluing reagent.

Statistical analysis

Statistical analysis was conducted using GraphPad Quick calcs (GraphPad Software). To analyze gene expression and the correlation between the expression of *TMPRSS2:ERG* and *OPN*, we used a 2 × 2 correlation table and Fisher's exact test, and Spearman's correlation coefficient. We considered results statistically significant at $P < 0.05$.

Results

Identification of ERG binding sites in OPN promoter sequences conserved across species and transactivation of the OPN promoter by ERG proteins

To investigate the possibility of ERG-mediated OPN transactivation, we undertook a comparative genomic bioinformatics analysis to identify regions conserved between species and to define EBS. Using the ECR browser, the 5′ flanking region of the human OPN start site was compared with mouse, dog, and rhesus macaque genomes (Supplementary Fig. S1). This analysis shows that the OPN proximal promoter is well conserved in all species analyzed, and 2 domains were defined: one from nt −289 to +6 bp and the other from nt −1100 to −920 kb. These 2 conserved domains were scanned using Transfac to find potential EBS composed of the 5′ consensus sequence -GGA/W-3′ (W:A or T). We found only 1 putative

conserved EBS upstream of the OPN gene at nt −115 to −118 (EBS1). Another potential inverted EBS was identified at nt −108 to −111 (EBS2), but was found only in the mouse and rhesus macaque genomes. The computer search for transcription factor binding sites (Transfac analysis) also disclosed Runx2, SBF-1, C/EBP, and AP-1 sites, which have already been reported (30).

To test ERG transactivation, a series of sequential 5′-deletion mutant constructs were prepared from the region of the promoter sequence that confers efficient transcription of the *OPN* gene (37). The 5′-deletion sequences were cloned into the pGL3 reporter plasmid (Fig. 1A) and assayed for ERG responsiveness via luciferase activity in transient transfected HeLa cells (Fig. 1B). Moreover, since ETS-1 factors have been shown to regulate *OPN* promoter (30), we conducted luciferase assays using the same constructs (Supplementary Figs. S2A and B). As expected, ETS-1 regulate the *OPN* promoter. Since ERG protein overexpression has been associated with more than 50% of PCas, we conducted transfection assays in a biologically relevant cell type for studying *OPN* in PCa: PC3c cells that derived from the human prostate carcinoma PC3. Similar results were obtained compared with HeLa cell experiments (Fig. 1C). As shown in Figure 1B (and Supplementary Fig. S2A, assay using the plasmid pcDNA3-ETS-1), the deletion of nt −136 to −116 bp resulted in a dramatic loss of *OPN* promoter activity after ERG (or ETS-1) overexpression, whereas OPN expression was not affected after the deletions including nucleotides downstream from −136 bp. Luciferase activity increased in a dose–dependent manner with the plasmid pSG5-ERG (Fig. 1D) or with the plasmid pcDNA3-ETS-1 (Supplementary Fig. S2B). As expected, OPN expression was dramatically decreased after shRNA-ERG cotransfections compared with the shRNA control. These results showed that ERG transactivates the *OPN* promoter and this region, nt −136 to −116 bp, contains one or several essential cis-regulatory sites for ERG.

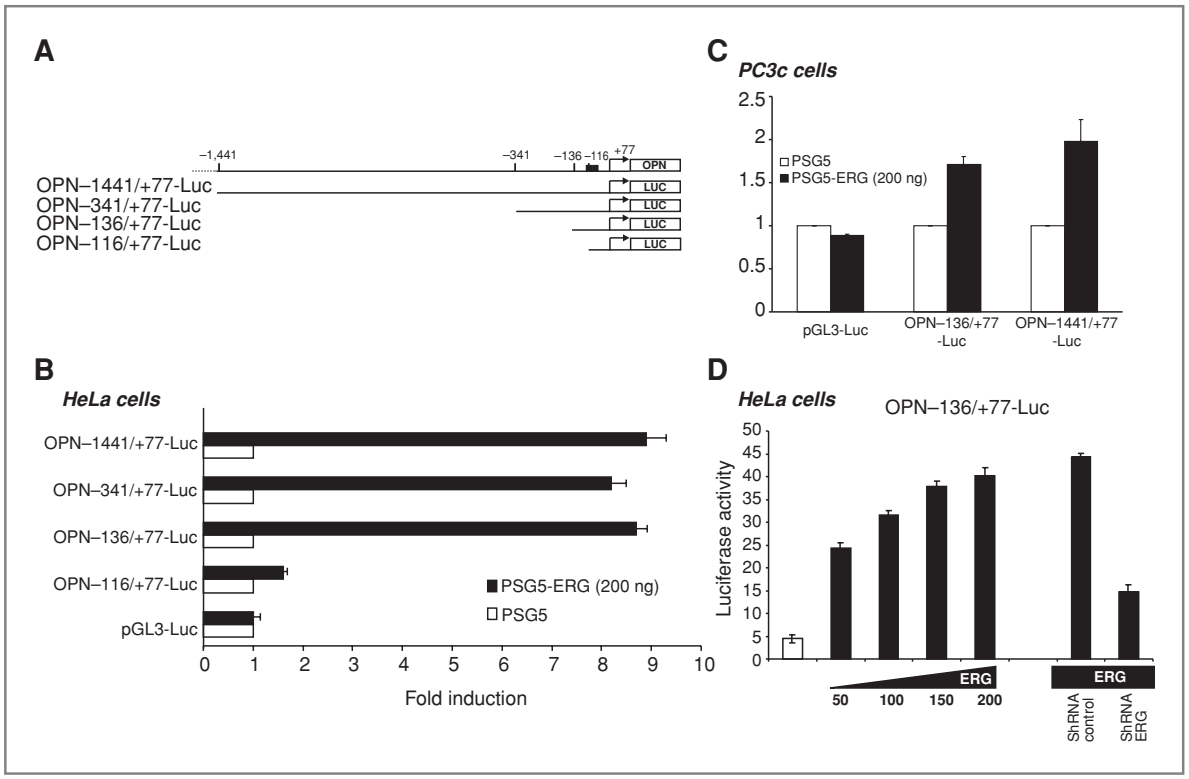

Figure 1. ERG transactivates the *OPN* promoter as determined by transient transfection with *OPN* promoter-luciferase constructs. A, 5′-deletion series of the *OPN* promoter constructs. The names of the deletion constructs indicate the location of the *OPN* promoter that was cloned in the upstream of the pGL3 luciferase gene (*Luc*). Boxes indicate EBS. B, 5′-deletion analysis of the *OPN* promoter with ERG overexpression. HeLa cells were cotransfected with the *OPN* promoter–luciferase constructs (500 ng) with or without 200 ng of pSG5-ERG, as indicated. C, luciferase activity from −136/+77 and −1441/+77 OPN promoter constructs (500 ng) with or without 200 ng of pSG5-ERG transfected in PC3c cells. D, luciferase activity of *OPN* −136/+77 luciferase constructs (500 ng) in the presence of increasing quantities of ERG expression plasmids (0 ng, 50 ng, 100 ng, 150 ng, and 200 ng), with or without shRNA targeting ERG (300 ng). In B and C, empty pSG5 vector was added to adjust the total amount of DNA to 1 μg in all transfections. Activities were assayed after 48 hours of transfection. The values obtained were normalized to the cotransfection of renilla luciferase (control). Data bars represent the mean of triplicate (or more) experiments, the error bars represent standard errors.

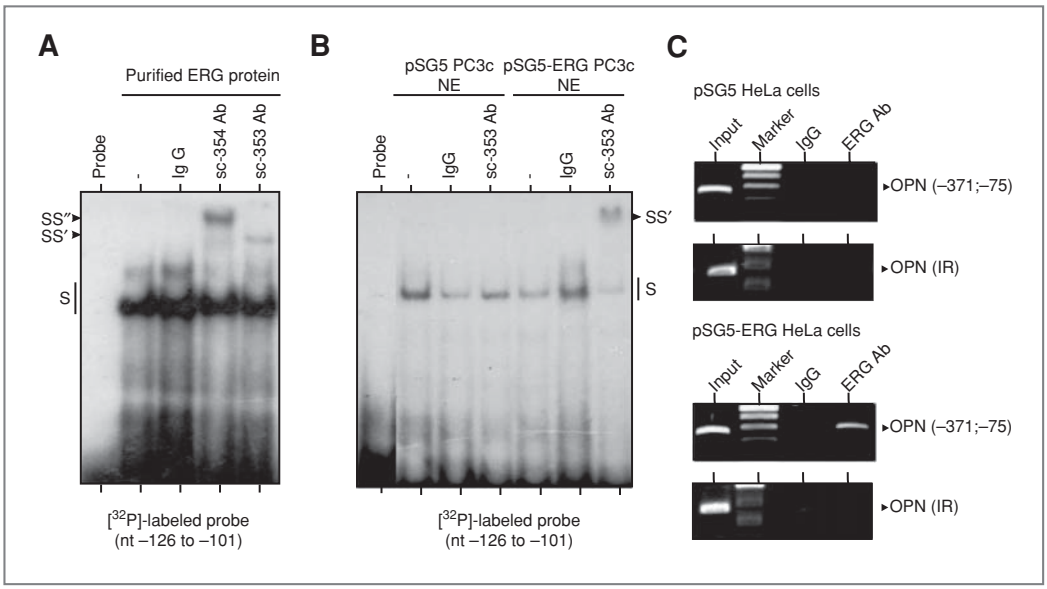

Figure 2. ERG interaction with the *OPN* promoter (A and B). EMSA validation of ERG-DNA binding to the *OPN* promoter's EBS with purified ERG protein (A) and nuclear extracts (NE) from pSG5 or pSG5-ERG transfected PC3c cells (B). EMSA were conducted with purified ERG protein or nuclear extracts from transfected PC3c incubated with ^{32}P-labeled probe (nt −126 to −101) in the presence of antibodies (Ab) directed against ERG (sc-353 Ab) or an IgG control Ab (IgG). The reaction was resolved on a nondenaturing polyacrylamide gel. The DNA-protein complexes resulting from sequence-specific transcription factor binding are designated with an S, supershift complexes are indicated by short arrowheads denoted SS′ and SS". C, ChIP analysis of ERG binding. ChIP analyses were conducted as described in Materials and Methods with pSG5 or pSG5-ERG transfected HeLa cells using 10 μg of anti-ERG Ab or the corresponding IgG isotype as a control. The input fraction corresponds to 5% of the chromatin solution before immunoprecipitation. Immunoprecipitated *OPN* promoter region (−371; −75) and an IR were analyzed on agarose gels by semi-quantitative *OPN*-specific PCR primers. Input chromatin (Input) used as PCR control and IgG are shown.

Interaction of ERG with the OPN promoter

To investigate whether these EBS were direct ERG targets, we conducted an EMSA using a ^{32}P-labeled WT nt −126 to −101 oligonucleotide substrate and purified ERG protein (Fig. 2A). Banding patterns revealed ERG affinity for these binding sites (designated as S in Fig. 2A) compared with the probe alone. In the presence of ERG antibodies (sc-353 Ab and sc-354 Ab), there were fewer S complexes and specific antibody-supershift bands SS′ and SS" were formed. No supershift was observed using the IgG isotype. Similar banding patterns were obtained when EMSA assays were conducted in the presence of nuclear extracts from HeLa cells (Supplementary Fig. S3) and PC3c (Fig. 2B) transfected with pSG5 or with pSG5-ERG. A supershift SS′ was only detected with ERG-specific antibody in pSG5-ERG transfected cells.

In accordance with these results, the shift assay revealed ERG binding on the nt −126 to −101 sequence of the *OPN* promoter. ChIP assays were then conducted with transfected HeLa cells to confirm that the EBS identified by EMSA in this portion of the *OPN* promoter is able to recruit ERG *in vivo* (Fig. 2C). As expected, ERG proteins were tethered to the endogenous *OPN* promoter. No precipitation was detected with ERG antibodies in an irrelevant region (IR), validating the specificity of binding ERG to EBS. Together, these experiments clearly showed that ERG binds to the *OPN* promoter.

Identification of a functional cis-regulatory element

To further characterize which EBS are involved in ERG binding within the WT nt −126 to −101 oligonucleotide region, we used site-directed mutant probes and constructs (Fig. 3A) and analyzed them using EMSA (Fig. 3B and 3C) and luciferase-promoter assays (Fig. 3D). A gel-shift complex (S) was present with the wt probe itself and the mutant 2 probe, but not with the mutant 1 or mutant 1+2 probes (Fig. 3B). In contrast, this complex was absent in 100-fold excess of unlabeled wt and mutant 2 probes, but persisted with mutant 1 probe (Fig. 3C). Similar patterns were obtained with nuclear extracts of transfected HeLa cells (data not shown).

Next, to assess the functionality of the EBS, we investigated the effect of ERG overexpression using a luciferase reporter construct driven by mutated EBS. The *OPN* promoter fragment −136/+77, with ERG-responsive transcriptional activity similar to the intact 1518 bp promoter (Fig. 1B), was used as a template to introduce site-directed mutations in predicted EBS1 and EBS2 sequences, named *OPN* −136/+77 mutant1 and *OPN* −136/+77 mutant2, respectively, and transfected into HeLa cells (Fig. 3D). A mutation in nt −119 to −115 from GG**AGGAA**G to

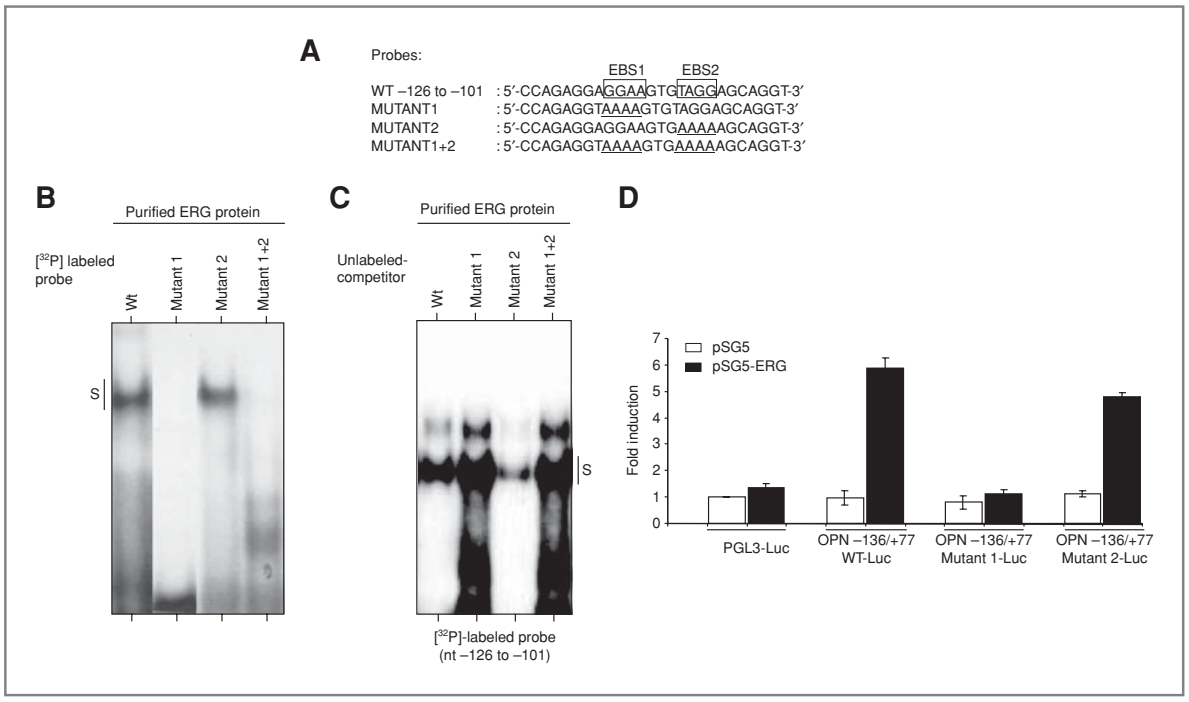

Figure 3. Identification of functional EBS *in vitro* and *in vivo*. A, sequences of the wild-type and mutant probes used in EMSA experiments. B and C, EMSA conducted with purified ERG protein. Doubled-stranded oligonucleotides were radiolabeled and incubated without (B) or with (C) a 100-fold excess of the unlabeled-competitor. D, luciferase activity from HeLa cells transfected by *OPN* (−136/+77) promoter constructs containing EBS mutations subcloned into pGL3 vector and normalized to renilla-luciferase activity. The values are expressed as the mean ± SD. of 3 triplicate experiments.

GG**TAAAA**G resulted in the suppression of ERG transactivation. In contrast, luciferase activity was not affected by mutations in nt −112 to −106 from G**TAGG**AG to G**TCGG**AG. Thus, these experiments suggest that ERG may play a direct role in *OPN* promoter activation through a very specific EBS located at nt −118 to −115.

Upregulation of endogenous OPN expression in prostate cancer cells with stable expression of ERG and TMPRSS2:ERG

A high-incidence recurrent fusion of TMPRSS2 with ERG has recently been highlighted in PCa. This fusion results in aberrant androgen-regulated ERG in prostate cells. We therefore examined endogenous OPN expression in a prostate cell line, PC3c, that we stably transfected with ERG and TMPRSS2:ERG (Fig. 4). Efficiencies of ERG and TMPRSS2:ERG overexpression were characterized in several subclones and compared with nontransfected cells and empty pcDNA3 vector-transfected cells by RT-PCR (Fig. 4A) and Western blotting (Fig. 4B). The molecular weight of ERG was expected to be 55 kD, whereas TMPRSS2:ERG was expected to be 49 kD, consistent with the deletion of 32 amino acids from the N-terminus (Fig. 4B). Results obtained with representative subclones were similar for several subclones (data not shown). Expression analysis showed that, compared with the nontransfected PC3c and empty pcDNA3 vector-transfected PC3c, OPN expression was greater in clones that overexpress ERG following transfection of ERG and TMPRSS2:ERG. Interestingly, ChIP assays revealed that ERG transcriptional factors were associated with the *OPN* promoter in clones of PC3c cells expressing ERG and TMPRSS2:ERG in which *OPN* was upregulated but not in empty pcDNA3 clones (Fig. 4C). Together, these data suggest that ERG and *TMPRSS2:ERG* gene fusion products bind to the cis-regulatory domain of the endogenous *OPN* promoter in PC3c cells and transactivate this gene.

TMPRSS2:ERG status and osteopontin/TMPRSS2:ERG expression in human primary prostate cancers

Samples of human primary PCas were assessed for *OPN* and *TMPRSS2:ERG* transcript expression using RT-PCR on total isolated RNA (Table 1): *TMPRSS2:ERG* fusions were detected in 71.4% (25/35) of the prostate tumors. This frequency of TMPRSS2:ERG fusions is in the range reported from studies conducted in Western countries (38, 39). Among these TMPRSS2:ERG+ tumors, 96% were also positive for *OPN* expression. Using a 2-tailed, cross-tabulated Fisher's exact test showed a significant association ($P < 0.01$) between TMPRSS2:ERG and OPN expression (Table 2).

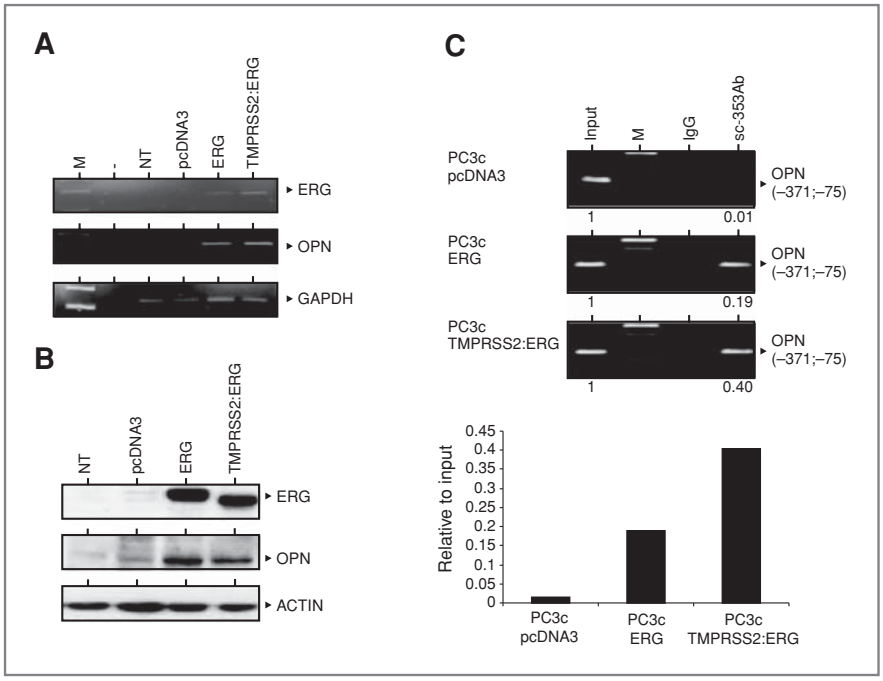

Figure 4. OPN expression in stable ERG-expression PCa PC3c cells. A, RT-PCR analysis of *ERG* and *OPN* transcript expression in stably transfected PC3c cells. M, DNA marker; –, negative control (no cDNA); NT, non transfected PC3c cells. B, representative Western blot analysis of whole cell lysates for ERG and OPN expression in stably transfected PC3c cells. Actin was also revealed to control protein loading. C, ChIP analysis of ERG binding. ChIPs were conducted in stably pcDNA3-, P55 ERG-, and TMPRSS2:ERG-transfected clones of PC3c cells. PCR product band intensities relative to input in each stably transfected PC3c cells were determined with quantitative PCR analysis [(specific antibody ChIP − control Ig ChIP)/input DNA]. Values were indicated underneath each gel results and summarized in a schematic diagram.

Next, we examined the expression of ERG and OPN from human prostate tumors (Fig. 5A). Using immunohistochemistry, we observed the coexpression of ERG and OPN in areas of prostatic adenocarcinoma. Staining against ERG was nuclear with a moderate to strong signal in adenocarcinomatous glands as shown in Figure 5. OPN was detected in the cytoplasm of adenocarcinomatous glands. As a control, in normal prostatic glands, we did not observe ERG expression except in endothelial cells of vessels (Supplementary Fig. S4), whereas, as previously described (40), none or weakly stained OPN expression was observed in the prostatic stroma. Moreover, no increase of the OPN expression level was observed in higher Gleason Score samples (Supplementary Fig. S4 and data not shown; ref 40).

Among 16 samples analyzed immunohistochemically, there were 12 (75%) cases with OPN-positive cells and 9 (56%) cases with TMPRSS2:ERG fusion positive cells (Table 3). Spearman's correlation coefficient was used to evaluate the correlation between protein expression levels (referred as % of cells stained) of ERG and OPN in IHC (Fig. 5B). Our data showed that there was a significantly positive correlation between expression levels of ERG and OPN ($r = 0.531$; $P < 0.05$).

Discussion

OPN is a SPP that has been found to facilitate anchorage of normal bone or cancer cells to mineralize tissue surfaces (41). This protein can be considered as one of the critical determinants in bone formation and tumor progression. The malignant and metastatic phenotype of human cancers has been correlated with elevated OPN expression, both at mRNA and protein levels (7). Uncovering transcriptional regulators of the *OPN* promoter has been stimulated by research seeking potential molecular targets that could modulate the metastatic phenotype and thereby constitute an effective therapeutic treatment. Several signaling pathways/transcription factors have already been proposed [for review, see ref. (42)]. In this study, we showed that ERG, a transcription factor associated with precartilaginous condensation and chondrogenesis preceding bone formation (43, 44), binds to the *OPN* promoter and transactivates this gene. Through EMSA experiments, a functional and conserved cis-regulated enhancer site was localized at nt −118 to −115. A mutation in this consensus binding site abolished ERG transactivation. In agreement with EMSA, ChIP assays revealed that ERG directly binds to the *OPN* promoter in cells. These results suggest that ERG is a critical transactivator of the *OPN* promoter. However, as noted (Supplementary Fig. S1), many transcription factors have DNA binding sites adjacent to the EBS sequence in the *OPN* promoter. Transcriptional activity of the ERG factor, as in other ETS members, is modulated by the cooperation with other key transcription factors such as AP-1, CBP-P300, Runx2, NFkB, Gata1, etc. (45–47). The combinatorial control of *OPN* regulation by the ERG factor and its partners is a fertile ground for ongoing and future investigations on ERG expression in the cell.

Recently, *ERG* has been discovered to be highly overexpressed in the majority of human prostate tumors, due to

Table 1. *TMPRSS2:ERG* and *OPN* RNA expression in human primary prostate tumor of patients

Patient number	Age	Gleason score	mRNA	
			TMPRSS2:ERG	OPN
1	67	7	+	+
2	71	7	+	+
3	63	7	–	+
4	60	6	+	+
5	61	6	+	+
6	72	7	+	+
7	78	9	–	–
8	67	7	+	+
9	62	7	+	+
10	64	9	+	–
11	56	7	+	+
12	67	8	+	+
13	75	7	+	+
14	71	7	+	+
15	49	7	–	+
16	61	7	+	+
17	63	7	+	+
18	54	7	+	+
19	58	7	+	+
20	70	7	–	+
21	70	7	–	–
22	67	6	+	+
23	51	7	+	+
24	68	7	–	–
25	54	7	+	+
26	64	6	+	+
27	70	7	+	+
28	55	7	–	+
29	67	7	+	+
30	59	7	+	+
31	55	9	–	–
32	72	9	+	+
33	73	10	–	+
34	82	8	+	+
35	58	9	–	–

NOTE: Results from *TMPRSS2:ERG* and *OPN* expression in 35 samples of patients who had given their consent (mean age, 63 years, and Gleason score >7) diagnosed with PCa. Abbreviation: ND, not determined.

the fusion of *TMPRSS2:ERG* through chromosomal rearrangements. The consequences of the *TMPRSS2:ERG* gene fusion in prostate tumorigenesis initiation remains controversial (48) and studies on its prognostic value have produced contradictory results (28, 49–51). Furthermore, PCa is characterized by its high propensity—occurring in more than 80% of patients—to spread to skeletal tissue. However, the mechanisms underlying this preferential homing of prostate cells to bone tissues are only beginning to be understood. The high level of OPN expression is closely linked to the degree of malignancy and with the reduced survival of patients (40). OPN has been shown, along with other skeletal and matrix-associated proteins, to be expressed in cancer cells as major soluble factors stimulating the migration, survival, anchorage-independent growth and invasion of PCa cells (52, 53). In this context, we expected that the abnormal expression of ERG protein in prostate tumor cells *via* the *TMPRSS2:ERG* rearrangement would induce ERG overexpression and thereby affect the expression of ERG-target genes. Among these deregulated genes, we hypothesized that the *OPN* gene would increase through ERG overexpression. In this study, we show that, in PC3c cells stably transfected by ERG or TMPRSS2 (exon 1):ERG (exon 4; the most frequent fusion in PCa), *OPN* transcription is enhanced and that ERG binds to the *OPN* promoter in PC3c DNA. Proteins encoded by this *TMPRSS2 (exon 1):ERG (exon 4)* fusion transcript are truncated, lacking 32 amino acids from the N-terminus. Despite this truncation in the N-terminal region, whose functional role remains unknown (54), both ERG and TMPRSS2 (exon1):ERG (exon4) similarly regulate *OPN* expression. However, in normal prostate cells, endogenous ETS factors may regulate a set of genes. Therefore, in PCa cells, which exhibit the TMPRSS2:ERG fusion, the ERG proteins are strongly overexpressed causing a perturbation of transcriptional gene regulation. These changes could affect newly genes or ETS targets already regulated by endogenous ETS factors. For example, since ETS-1 is able to directly activate the *OPN* promoter, a competition could exist between ETS-1 and the overexpressed ERG factors for the *OPN* promoter binding and activation.

To test the biological relevance of our findings suggesting that TMPRSS2:ERG may increase *OPN* expression levels in prostate tumors, coexpression of *OPN* and *ERG* were assessed using RT-PCR and immunohistochemical analyses in specimens from patients diagnosed with PCa (mainly biopsies from prostatectomy with Gleason scores evaluated at 7). Despite the biological heterogeneity that characterizes PCa specimens, which can complicate molecular studies, a significant association was observed between *TMPRSS2 (exon 1):ERG (exon 4)* fusion and *OPN* expression. Moreover, tumor specimens showed

Table 2. Patients diagnosed with PCa were analyzed in a 2 × 2 table using Fisher's exact test

	TMPRSS2:ERG –	TMPRSS2:ERG +	Total
OPN –	5 (14.3 %)	1 (2.8%)	6 (17.1%)
OPN +	5 (14.3 %)	24 (68.6 %)	29 (82.9%)
Total	10 (28.6%)	25 (71.4%)	35 (100%)

NOTE: $P = 0.0040$. OPN expression vs. TMPRSS2-ERG expression. Fisher's exact test: The 2-tailed P value < 0.01.

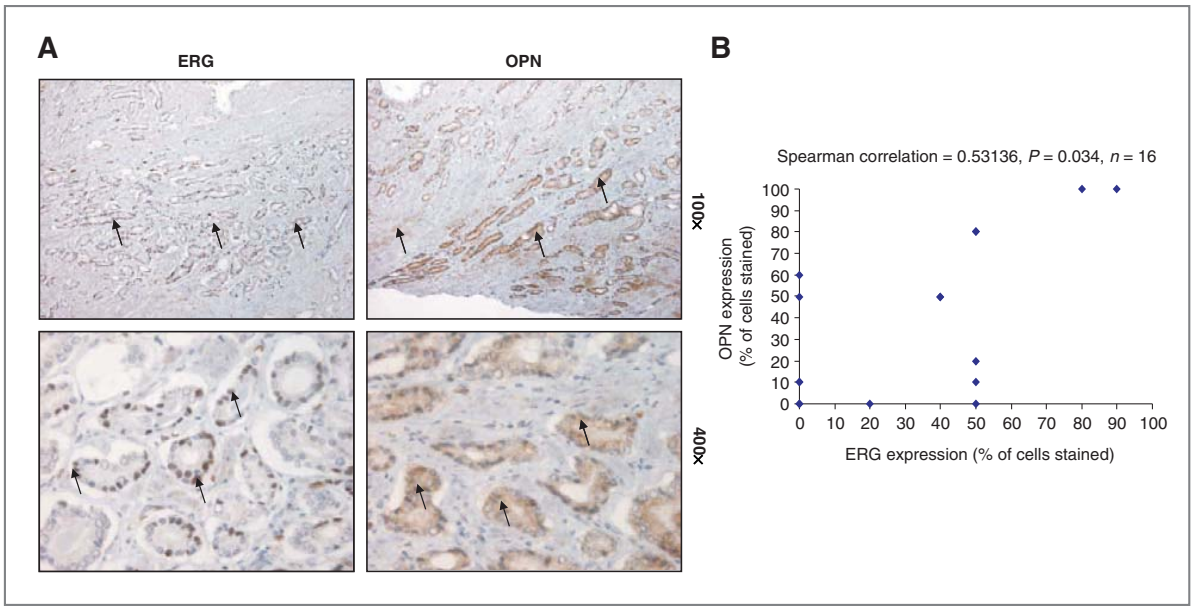

Figure 5. Immunohistochemical staining for ERG and OPN in human PCa tissues. A, OPN and ERG immunohistochemistry of consecutive PCa tissue sections. The sections were counterstained with hematoxylin (blue). Arrows indicate positive staining (brown). Original magnification, ×100 in top panels and × 400 in bottom panels. B, correlation between expression levels of ERG and OPN protein in 16 samples of PCa tissue sections (Table 3). Spearman's correlation coefficient was used to evaluate the correlation between mRNA and protein expression levels of OPN in IHC. Our data showed that there was a significantly positive correlation between expression levels of OPN mRNA and protein in HCC ($r = 0.531$; $P < 0.05$).

Table 3. ERG and OPN protein expression in human primary prostate tumor of patients

Patient number	Gleason score	ERG expression (% of cells stained)	OPN expression (% of cells stained)
1	5	0	10
2	5	50	20
3	5	40	50
4	6	0	0
5	6	0	60
6	6	40	50
7	6	20	0
8	7	0	50
9	7	0	10
10	7	50	80
11	7	80	100
12	7	90	100
13	7	50	80
14	8	0	0
15	9	50	0
16	9	50	10

NOTE: Expression status of ERG and OPN in 16 random samples of PCa tissue sections using immunohistochemical experiments. The OPN signal was cytoplasmic with a weak to moderate intensity, whereas ERG signal was nuclear with a moderate to strong intensity. The results were expressed as the percentage of cells showing staining.

similar immunostaining localization for ERG transcription and OPN. In this study, we only focused on the involvement of the most common fusion variant TMPRSS2 (exon1):ERG (exon 4) reported in *OPN* upregulation. However, this does not exclude the possibility that other fusion variants between androgen-dependent gene promoters and ETS family members are present in prostate tumor samples where TMPRSS2 (exon1):ERG (exon4) has not been detected.

OPN has multi-functional properties that promote cell survival, cell adhesion, and cell migration (42, 55). By binding to αvβ3 integrins, OPN protects cells from apoptosis through the activation and phosphorylation of the PI3-K/AKT pathway (56, 57). OPN activates various matrix-degrading proteases, such as matrix metalloproteinases (MMP) and the urokinase plasminogen activator (PLAU) that contribute to malignancies (58, 59). MMP3, MMP9, a disintegrin and metalloproteinase 19 (ADAM19) and PLAU were recently reported to be increased in benign prostate cell lines RWPE and PrEC (primary benign prostate epithelial cells) transfected with overexpressed ERG factors (60). They were identified as direct targets of ETS transcription factors. Based on our results, we can also suggest that the activation of the *OPN* by TMPRSS2:ERG mediates the matrix metalloproteinase pathway and thereby PCa progression.

In summary, the results presented here show that *OPN* is a target gene of ERG. The expression of the extracellular matrix protein OPN, which plays a crucial role in tissue remodeling, inflammation, tumor growth, angiogenesis and metastasis, is

associated with TMPRSS2:ERG expression in prostate tumors cells and thereby participates in metastasis progression and aggressiveness. Small molecules that can prevent the interaction of TMPRSS2:ERG with the promoter of ERG-target genes in PCa cells may be an interesting avenue of research for developing metastasis prevention strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Résumé

La régulation transcriptionnelle de l'expression des gènes est un processus finement orchestré. Les acteurs centraux de cette régulation sont les facteurs de transcription et leurs cofacteurs. De ce fait, ce sont des cibles de choix pour développer des stratégies thérapeutiques.

L'étude des mécanismes impliqués dans la régulation de la transcription constitue le fil conducteur de mon activité de recherche. Mon parcours scientifique a débuté avec l'étude du mécanisme de la régulation de la transcription en réponse aux rétinoïdes et le recrutement des cofacteurs du récepteur nucléaire à l'acide rétinoïque. J'ai ensuite cherché à identifier les facteurs de transcription impliqués dans la régulation de l'expression de la molécule immuno-régulatrice HLA-G. Puis j'ai appris à connaître le facteur de transcription ERG et son implication dans la chondrogenèse et dans les métastases osseuses du cancer de la prostate.

La découverte des gènes de fusion TMPRSS2 :ETS dans la majorité des cancers de la prostate a représenté une révolution dans la connaissance biologique des tumeurs prostatiques. En effet, les produits de ces gènes de fusion dans les cellules prostatiques sont des protéines ETS exprimées de façon massive et ectopique. La surexpression de la protéine ERG concourt à la perturbation des réseaux transcriptionnels associés à l'agressivité et à la progression des cancers de la prostate. Connaissant l'importance des complexes protéiques dans les mécanismes de régulation, le projet que j'envisage a pour objectif d'identifier l'interactome de la protéine issue du gène de fusion *TMPRSS2 :ERG*. En effet, la compréhension des réseaux de régulation transcriptionnelle impliquant ERG et ses partenaires protéiques devraient permettre à terme de développer de nouvelles perspectives thérapeutiques ciblées.