Université de Lille – Nord de France

Ecole Doctorale Biologie - Santé de Lille

Comparison of YAP/TAZ and TEAD Activators on Cellular Models

Comparaison des effets de différents activateurs de YAP/TAZ et TEAD sur plusieurs modèles cellulaires

By

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*-**Sir Francis Drake***

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Publications-Poster Presentations-Conferences-Training Courses

Publications:

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- Sturbaut M, Bailly F, Coevoet M, **Sileo P**, Pugniere M, Liberelle M, Magnez R, Thuru X, Chartier-Harlin MC, Melnyk P, Gelin M, Allemand F, Guichou JF, Cotelle P. Discovery of a cryptic site at the interface 2 of TEAD - Towards a new family of YAP/TAZ-TEAD inhibitors. Eur J Med Chem. 2021.

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Conferences:

- 2nd Meeting of LiCend (14 November 2019 - 15 November 2019) Lille, FRANCE
- AD/PD™ 2022 International Conference on Alzheimer's and Parkinson's Diseases and related neurological disorders (15 March 2022-20 March 2022) Barcelona, SPAIN

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Abstract

The Hippo pathway (HP) consists in a cascade of kinases which controls phosphorylation of the co-activators YAP/TAZ. When unphosphorylated YAP and TAZ translocate into the nucleus, where they mainly bind to the TEAD transcription factors and activate genes related to cell proliferation, stemness and survival. In this way inhibition of HP's kinases could promote cell survival and proliferation. Research has focused more on trying to reduce YAP/TAZ-TEAD activity given its known involvement in cancer. However, recently new findings have highlighted the importance of increasing this activity given the involvement of their downregulation in various diseases, such as certain types of cancer or in neurodegenerative diseases. Indeed, it has recently emerged that HP is involved in several neurodegenerative diseases such as Huntington's disease (HD), Alzheimer's disease (AD) or Parkinson's disease (PD). Numerous deregulations of HP have been detected in post-mortem brains of HD patients, in murine and *in vitro* models of HD. An increased level of phosphorylated YAP was observed, which leads to a decreased localisation of YAP in the nucleus, decreased YAP-TEAD binding and consequent downregulation of their target genes. Recent data suggest that targeting the Hippo pathway might be beneficial in murine models of these disorders. Furthermore, increasing YAP/TAZ binding with TEAD and thus their activity may also have beneficial effects on regeneration and wound healing.

The aim of this project is to compare several YAP/TAZ and TEAD activators, selecting the best activator capable of increasing YAP/TAZ-TEAD activity, increasing the expression of their targets and inducing an increase in cell growth in different cell models.

Firstly, we selected the best activator(s) of TEAD transcriptional activity out of a series of different molecules acting on different HP targets using a TEAD reporter Luciferase assay. Secondly, we evaluated the selected drug, TRULI (a LATS1/2 inhibitor), for its capability to increase YAP/TAZ-TEAD target genes (*Cyr61* and *CTGF*) at both RNA and protein levels and their effects on cellular growth in HEK293T and small molecule neuronal progenitor cells (smNPCs), demonstrating that the molecule increases cell growth and the expression of target genes in both cell models. Next, we analysed cell growth, proliferation and the expression of HP target genes in fibroblasts taken from HD patients compared to fibroblasts taken from healthy individuals, since it has previously been seen that in HD fibroblasts proliferation is decreased. However, we observed no significant changes in cell growth, proliferation and expression of HP target genes.

Finally, we tested TRULI on primary fibroblast lines from healthy individuals. TRULI treatment leads to increased cell growth in two out of four of the samples analysed; moreover, there are variable effects on the expression of HP target genes analysed. Indeed, TRULI decreases the expression of *CTGF* and *Cyr61* (classical HP target genes), but increases expressions of *BIRC5*, *CENPF*, and *BUB1B,* in most of the samples. However, we noticed individual variations between the different samples in cell growth, proliferation and in the expression of HP target genes upon TRULI treatment.

Further studies are needed to verify potential off-target effect and tissue-specific effect of TRULI. However, the next step is to study the effect of TRULI in neuronal models of Huntington's disease, given the encouraging results the molecule had in a neural model such as smNPCs, even in those derived from PD patients. Furthermore, it would be interesting to test the effects of this compound in fields such as regeneration and wound healing. Moreover, future studies should also investigate the effect of new emerging modulator of Hippo pathway.

Résumé

La voie Hippo (HP) consiste en une cascade de kinases qui contrôle la phosphorylation des co-activateurs YAP/TAZ. Lorsqu'ils sont non phosphorylés, YAP et TAZ se transloquent dans le noyau, où ils se lient principalement aux facteurs de transcription TEAD et activent les gènes liés à la prolifération cellulaire, au caractère cellule-souche et à la survie. De cette manière, l'inhibition des kinases de HP pourrait favoriser la survie et la prolifération des cellules. La recherche s'est davantage concentrée sur la tentative de réduire l'activité YAP / TAZ-TEAD compte tenu de son implication connue dans les cancers. Cependant, des découvertes récentes ont mis en évidence l'importance d'augmenter cette activité compte tenu de l'implication de leur régulation à la baisse dans diverses maladies, telles que certains types de cancer ou dans les maladies neurodégénératives. En effet, il est récemment apparu que HP est impliquée dans plusieurs maladies neurodégénératives telles que la maladie de Huntington (MH), la maladie d'Alzheimer (MA) ou la maladie de Parkinson (MP). De nombreuses dérégulations de HP ont été détectées post-mortem dans des cerveaux de patients MH, dans des modèles murins et des modèles in vitro de MH. Un niveau accru de YAP phosphorylé a été observé, ce qui entraîne une diminution de la localisation de YAP dans le noyau, une diminution de la liaison YAP-TEAD et une régulation négative conséquente de leurs gènes cibles. Des données récentes suggèrent que le ciblage de la voie Hippo pourrait être bénéfique dans les modèles murins. De plus, l'augmentation de la liaison YAP/TAZ avec TEAD et donc l’augmentation de l’activité transcirptionnelle de TEAD peut également avoir des effets bénéfiques sur la régénération et la cicatrisation des plaies.

L'objectif de ce projet est de comparer plusieurs activateurs YAP/TAZ et TEAD, en sélectionnant le meilleur activateur capable d'augmenter l'activité YAP/TAZ-TEAD, d'augmenter l'expression de leurs cibles et d'induire une augmentation de la croissance cellulaire dans différents modèles cellulaires.

Dans un premier temps, nous avons sélectionné le(s) meilleur(s) activateur(s) de l'activité transcriptionnelle TEAD parmi un panel de molécules différentes agissant sur différentes cibles HP à l'aide d'un test de luciférase reporter TEAD. Deuxièmement, nous avons évalué la molécule sélectionnée, TRULI (un inhibiteur de LATS1/2), pour sa capacité à augmenter les gènes cibles YAP/TAZ-TEAD (Cyr61 et CTGF) aux niveaux ARN et protéiques et son effet sur la croissance cellulaire dans HEK293T et les cellules progénitrices neuronales à petites cellules (smNPC), démontrant que la molécule augmente la croissance cellulaire et l'expression de gènes cibles dans les deux modèles cellulaires. Nous avons ensuite analysé la croissance

cellulaire, la prolifération et l'expression des gènes cibles de HP dans des fibroblastes prélevés sur des patients MH en comparaison avec des fibroblastes prélevés sur des individus sains, car il a déjà été observé que dans les fibroblastes MH, la prolifération diminue. Cependant, nous n'avons observé aucun changement significatif dans la croissance cellulaire, la prolifération et l'expression des gènes cibles HP.

Enfin, nous avons testé TRULI sur des lignées primaires de fibroblastes d'individus sains. Le traitement TRULI entraîne une augmentation de la croissance cellulaire dans deux des quatre échantillons analysés ; de plus, il existe des effets variables sur l'expression des gènes cibles HP analysés. En effet, TRULI diminue l'expression de CTGF et Cyr61 (gènes cibles HP classiques), mais augmente les expressions de BIRC5, CENPF et BUB1B, dans la plupart des échantillons. Cependant, nous avons remarqué des variations individuelles entre les différents échantillons dans la croissance cellulaire, la prolifération et l'expression des gènes cibles HP lors du traitement TRULI.

D'autres études seront nécessaires pour vérifier l'effet potentiel hors cible et l'effet tissu-spécifique de TRULI. Cependant, la prochaine étape consistera à étudier l'effet de TRULI dans des modèles neuronaux de la maladie de Huntington, compte tenu des résultats encourageants de la molécule dans un modèle neuronal tel que les smNPC, même chez ceux prélevés sur des patients parkinsoniens. De plus, il serait intéressant de tester les effets de ce composé dans des domaines tels que la régénération et la cicatrisation.

List of Abbreviations

α-cat: α-catenin
AC: Arrhythmogenic cardiomyopathy
AD: Alzheimer's disease
AJs: Adherens junctions
ALS: Amyotrophic lateral sclerosis
AMOT: Angiomotin
AMPK: AMP-activated protein kinase
AO: Age at onset
AP-1: Activator protein 1
APC: Adenomatous polyposis coli
APC/C: Anaphase-promoting complex/cyclosome
ARID1A: AT-rich interactive domain-containing protein 1A
ASOs: Allele-specific oligonucleotides
Axl: AXL Receptor Tyrosine Kinase

BDNF: Brain-derived neurotrophic factor
BIRC5: Baculoviral inhibitor of apoptosis repeat-containing 5
BMPs: Bone morphogenic proteins
BUB1B: Mitotic Checkpoint Serine/Threonine Kinase B

CENPF: Centromere Protein F
CIP: Contact inhibition of proliferation
CK1: Casein Kinase 1
CMA: Chaperone-mediated autophagy
CNS: Central nervous system
CREB: cAMP response element-binding protein
CRISPR: Clustered regularly interspaced short palindromic repeats
CSCC: Cervical squamous cell carcinoma
CTGF: Connective tissue growth factor
CTNF: Ciliary neurotrophic factor
Cyr61: Cysteine-rich angiogenic protein 61

DA: Dopamine
DBS: Deep brain stimulation
Dlg: Discs large
DRP1: Dynamin-related protein 1
DRPLA: Dentatorubral-pallidoluysian atrophy

E-cad: E-caderin
ECM: Extracellular matrix
Ed: Echinoid
EF3: Elongation factor 3
EGF: Epidermal growth factor
eIF2α: Eukaryotic initiation factor 2α
EMT: Epithelial-mesenchymal transition
ER: Endoplasmic reticulum
ESCs: Embryonic stem cells
Ex: Expanded

F-actin: Filamentous actin
FAs: Focal adhesions

GDNF: Glial cell line-derived neurotrophic factor
GFAP: Glial fibrillary acidic protein
GPCRs: G-protein coupled receptors
GWAS: Genome-wide association study

HD: Huntington's disease
HDAC: Histone deacetylase
HEAT: Huntingtin, EF3, PP2A, yeast kinase TOR1
HP: Hippo pathway
Hpo: Hippo
HSP90: Heat shock protein 90
HSR: Heat Shock Response
HS: Heat shock
HTT: Huntingtin

ICM: Inner cell mass
IGF-1: Insulin-like growth factor-1
iPSCs: induced pluripotent stem cells

JNK: c-Jun N-terminal kinase

KDAC: Lysine deacetylase

LATS1/2: Large tumor suppressor kinase1/2
LCA: Leber congenital amaurosis
Lgl: Lethal giant larvae
LKB1: Liver kinase B1
LPA: Lysophosphatidic acid

Mats: Mob as tumor suppressor
Mer: Merlin
MMB: Myb-MuvB
MOB1: Mps one binder homolog1
MSCs: Mesenchymal stem cells
MSNs: Medium spiny neurons
MST1/2: Mammalian sterile 20–like kinase-1/2
MTs: Microtubules
mTOR: mammalian target of rapamycin
Myc: MYC Proto-Oncogene

NASH: Nonalcoholic steatohepatitis
NF2: Neurofibromin 2
NMDA: N-Methyl-D-Aspartate
NSCs: Neural stem cells
NTN1: Netrin1

PD: Parkinson's disease
PERK: PKR-like ER kinase
PGC-1α: Peroxisome proliferator-activated receptor-g coactivator-1 alpha
PKA: Protein Kinase A
Plk1: Polo-like kinase 1
PP2A: Proteinphosphatase 2A
PRD: Proline-rich domain

RCC: Renal cell carcinoma
ROCK: Rho-associated serine/threonine kinase
ROS: Reactive oxygen species
RNAi: RNA interference

S1P: Sphingosine-1-phosphate
Sav: Salvador
SAV1: Protein salvador homolog 1
SCLC: Small cell lung cancer
SCRA: Sveinsson chorioretinal atrophy
Scrib: Scribble
Sd: Scalloped
smNPCs: small molecule Neural Precursor Cells
SNc: Substantia nigra pars compacta
STAAD: Stanford type A aortic dissection
SWH: Salvador-Warts-Hippo
SWI/SNF: Switch/sucrose nonfermentable

TAF4: TATA-box binding protein associated factor 4
TALENs: Transcription activator-like effector nucleases
TAOK: TAO kinase
TAZ: WW domain–containing transcription regulator protein 1
TE: Trophectoderm
TEAD1-4: TEA domain family member1-4
TGF-β: Transforming growth factor beta
TJ: Tight junction
TOPIC: Toxic exon 1 protein
TRIAD: Transcriptional Repression-Induced Atypical cell Death

UPR: Unfolded protein response
UPS: Ubiquitin-proteasome system

VEGFs: Vascular endothelial growth factors
VGLL4: Vestigial-like protein 4

Wnt: Wingless/Integrated
Wts: Warts

YAP: Yes-associated protein
Yki: Yorkie

ZFNs: Zinc-finger nucleases

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1. Introduction

1.1 Hippo pathway

1.1.1 A Brief History of Hippo pathway

The Hippo pathway was discovered in 1995 in *Drosophila melanogaster* through genetic mosaic screens with the aim of identifying genes involved in growth regulation and overgrowth-causing mutations (Justice et al., 1995; Xu et al., 1995). The results of these studies revealed one of the key kinases of the Hippo pathway: Warts (Wts). The role of Warts was poorly understood for a decade, until other core components of the Hippo pathway were identified with analogous screening: the adaptor proteins Salvador (Sav) and Mob as tumor suppressor (Mats), and the protein kinase Hippo (Hpo) that regulates and activates Warts (S. Ma et al., 2019; Pan, 2010; S. Piccolo et al., 2014; F. X. Yu et al., 2015). The pathway was initially called Salvador-Warts-Hippo (SWH) pathway and then renamed as "Hippo pathway" after Hippo mutants which led to the uncontrolled growth of Drosophila organs and making them look like a hippopotamus. The downstream effector of the kinase cascade was identified only in 2005 and named Yorkie (Yki) in *Drosophila*. Yorkie is a transcriptional coactivator and an interactor of Warts, it is involved in cell proliferation and survival (J. Huang et al., 2005). Yki mutants or its inhibition decrease proliferation, on the other hand its overexpression causes overgrowth, showing an opposite behaviour to Wts. In *Drosophila*, Warts together with the cofactor Mats phosphorylates Yorkie preventing its translocation into the nucleus and remains inactive in the cytoplasm blocked by 14-3-3. This explains the opposite behaviour of Yorkie and Warts, in fact when the Hippo pathway is active Yorkie is inactivated and *vice versa*. Yorkie, when active and in the nucleus, activates genes related to cell proliferation and survival, however it lacks DNA-binding domains and therefore must interact with some transcription factors which in Drosophila is Scalloped (Sd), a TEA-domain transcription factor (S. Wu et al., 2008; L. Zhang et al., 2008). The Hippo pathway is highly conserved across the different species and particulararly in mammals: **MST1/2** (mammalian sterile 20–like kinase-1/2) are Hippo orthologs, **SAV1** (Protein salvador homolog 1) is Sav ortholog, **MOB1** (Mps one binder homolog1) is Mats ortholog, **LATS1/2** (Large tumor suppressor kinase) are Warts ortholog, **YAP** (Yes-associated protein) and **TAZ** (WW domain–containing transcription regulator protein 1) are Yorkie orthologs (TAZ is YAP paralog) and **TEAD1-4** (TEA domain family member) are Scalloped orthologs.

1.1.2 Components of Hippo pathway

The core of the Hippo pathway in mammals is a kinase cascade mainly represented by the serine threonine kinases MST1/2 and LATS1/2, and their regulatory adaptor proteins respectively SAV1 and MOB1A/B (Fig. 1). Activated by different signals, MST1/2 bind with SAV1 and then phosphorylate LATS1/2 and MOB1A/B (E. H. Y. Chan et al., 2005; Praskova et al., 2008). Once phosphorylated and then activated, the LATS1/2-MOB1A/B complex consequently phosphorylates YAP and its paralogue TAZ (S. Piccolo et al., 2014; Zheng & Pan, 2019). LATS1/2 can phosphorylate YAP at five different serine/threonine residues, but the most important phosphorylations in blocking YAP and avoiding its entry into the nucleus are: S127 and S381 (in TAZ, which has 4 out of 5 YAP residues, these represent S89 and S311) (B. Zhao, Li, Lei, et al., 2010). Phosphorylations on these sites lead to an accumulation of YAP/TAZ in the cytoplasm and their subsequent binding to the 14-3-3 protein which inactivates them (Basu et al., 2003). Moreover, these modifications by LATS prepare YAP for additional phosphorylation by Casein Kinase 1 (CK1) and the subsequent recognition by $SCF^{\beta\text{-}TRCP}$ E3 ubiquitin ligase which results in YAP/TAZ polyubiquitination and proteasomal degradation (C. Y. Liu et al., 2010; B. Zhao et al., 2007a; B. Zhao, Li, Tumaneng, et al., 2010). MST1/2 are not the only kinases capable of phosphorylating LATS1/2, in fact also the MAP4K family proteins (MAP4K1/2/3/4/5/6/7) and TAO kinases (TAOK1/2/3) can phosphorylate and activate LATS1/2, moreover TAOK is also able to phosphorylate and activate MST1/2 (S. Li et al., 2015; S. Ma et al., 2019; Z. Meng et al., 2015, 2016). However, it is important to emphasize that the LATS1/2-MOB1A/B complex is essential for the inhibition of YAP/TAZ via phosphorylation, while the individual deletion of MST1/2, MAPK4 family and TAOKs leads only to a partial inhibition of YAP/TAZ. Nevertheless, simultaneous deletion of MSTs, MAP4Ks and TAOKs causes a large decrease in YAP/TAZ phosphorylation (Z. Meng et al., 2015; Plouffe et al., 2016).

When the Hippo cascade is inhibited YAP/TAZ can translocate into the nucleus where they mainly bind to the TEAD transcription factor family, indeed YAP/TAZ are transcriptional coregulators lacking DNA-binding domains, therefore in the nucleus they need to interact with transcription factors to activate their target genes and TEADs are their main partners (Misra & Irvine, 2018). The YAP-TEAD complex regulates various aspects related to growth such as cell proliferation, cell survival, inhibition of apoptosis by activating genes such as connective tissue growth factor (*CTGF*) and

cysteine-rich angiogenic protein 61 (*Cyr61*), but also direct regulators of cell cycle progression such as CDKs, factors necessary for DNA synthesis, replication and repair involved in mitosis. Moreover, they regulate also anchorage-independent growth, epithelial-mesenchymal transition (EMT) and oncogenic transformation (X. Liu et al., 2016; Zanconato et al., 2015; L. Zhang et al., 2008; B. Zhao et al., 2008). When YAP/TAZ are not in the nucleus, TEAD has the role of a repressor, it binds to Vg domain–containing protein VGLL4 (transcription cofactor vestigial-like protein 4) and inhibits their target genes expression (T. Guo et al., 2013; Koontz et al., 2013).

It is important to underline that the regulation of the Hippo pathway is not an ON and OFF effect, but it is fluid and dynamic. Indeed, the phosphorylation status of YAP/TAZ are constantly changing and each individual signal or regulator of the Hippo pathway alone does not guarantee the complete activation or deactivation of the pathway and the related phosphorylation or dephosphorylation of YAP/TAZ, but rather the final effect is a delicate balance between the sum of several positive and negative factors.

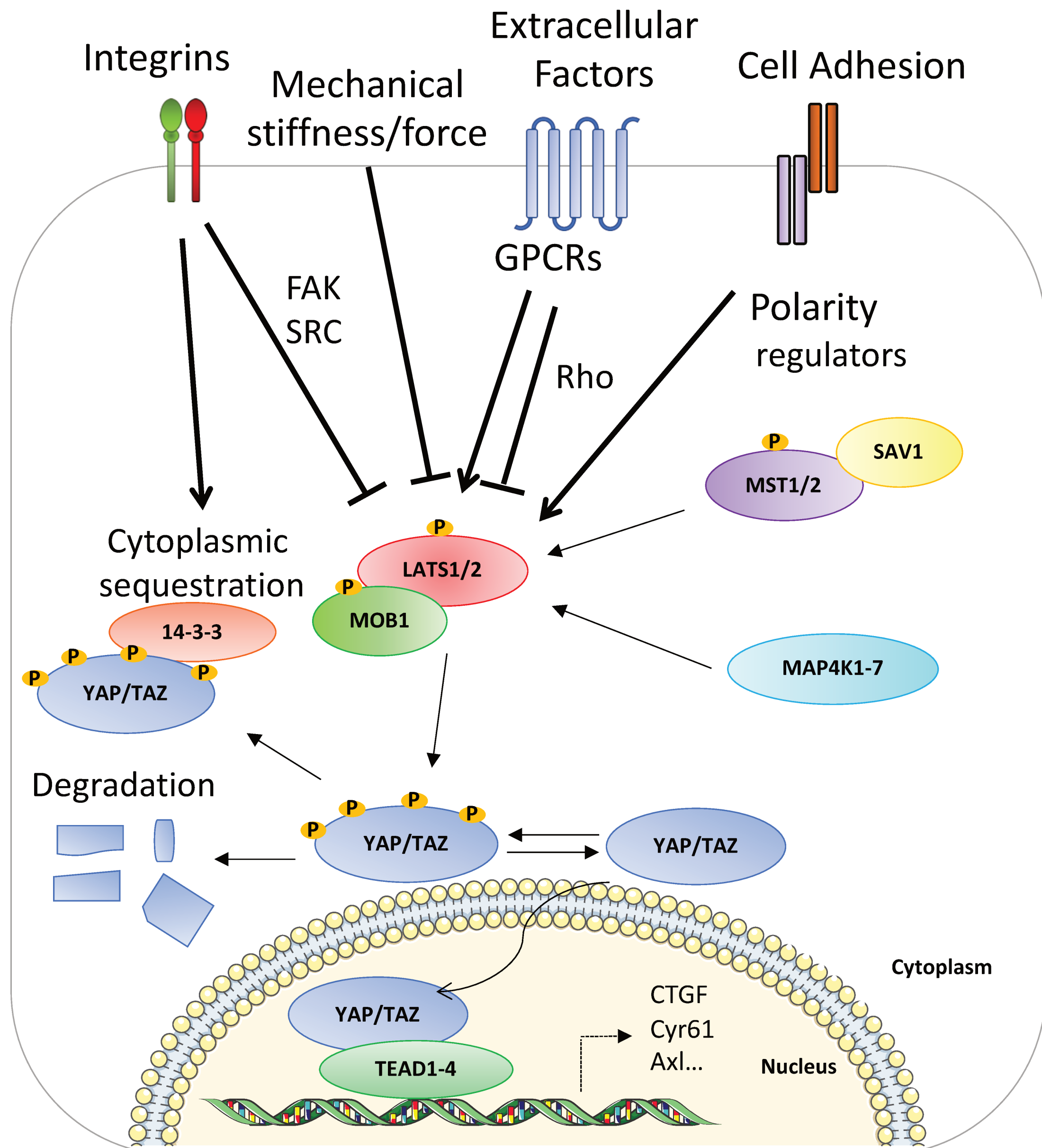


Figure 1. Graphical representation of the Hippo signalling pathway. Both the active and inactive state of HP are represented in the figure. Activation of the kinase cascade induces phosphorylation of YAP/TAZ, leading to their sequestration and degradation. Conversely, the inactive state of the pathway leads to an accumulation of YAP in the nucleus, where it interacts with TEAD1-4, activating their target genes. See text for details. Figure adapted and modified from Sileo et al., 2022.

1.1.3 Upstream Regulators of Hippo pathway

Unlike other pathways which are activated by very specific signals and ligands, the Hippo pathway is regulated by numerous upstream signals and factors such as cell polarity, cell density, adherens junction (AJs), cytoskeleton, mechanical forces, G-protein coupled receptors (GPCRs) ligands, energy and stress signals (Kwon, 2021; S.

Ma et al., 2019; S. Piccolo et al., 2014; Zheng & Pan, 2019) (Fig. 1). Although the kinase cascade is well known in both *Drosophila* and mammals, these upstream signals are still a source of in-depth investigation by researchers as the regulation of the Hippo pathway is not yet as well characterized as the core kinase cascade.

- *Cell Polarity*

  The first upstream regulators of Hippo pathway discovered in *Drosophila* were the tumor suppressors Merlin (Mer, NF2 in mammals) and Expanded (Ex) (Hamaratoglu et al., 2005). Mer and Ex along with two more tumor suppressors, Kibra and Pez, interact each other and function cooperatively in the apical domain of epithelial cells to regulate Hippo signaling in different developmental contexts (Baumgartner et al., 2010; Genevet et al., 2010; Poernbacher et al., 2012; J. Yu et al., 2010). NF2 can rather directly bind to and recruit LATS1/2 to the plasma membrane for activation by the MST-SAV complex (Yin et al., 2013). In the epithelial cells, the apical and basal domains are defined by different polarity protein complexes and are separated from each other by cell-cell junctions. These are represented by two apical complexes, the Crb complex and the aPKC-PAR complex, as well as the basal-laterally localized SCRIB group proteins including Scribble (Scrib), Lethal (2) giant larvae (Lgl), and Discs large (Dlg). In *Drosophila*, the apical domain determinant Crb directly recruits Ex to the apical domain to promote Hippo signaling (C. L. Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010). For instance, Scrib interacts with the major Hippo kinases MST1/2 and LATS1/2, thus activating LATS1/2 (Skouloudaki et al., 2009; Yang et al., 2015). Moreover, Crb and tight junction (TJ) protein PTPN14 are able to directly interact and inhibit YAP/TAZ (J. M. Huang et al., 2013; X. Liu et al., 2013; Szymaniak et al., 2015; W. Wang et al., 2012). In mammals an ortholog of Ex has not yet been found or does not exist, but the participation of Crb in Hippo pathway seems to be conserved, indeed CRB3 acts with Angiomotin (AMOT)-like proteins to activate Hippo signaling (Varelas et al., 2010). Noteworthily, members of the AMOT family of TJ proteins regulate YAP/TAZ in different ways: they can promote YAP/TAZ phosphorylation by binding and activating LATS2 (Paramasivam et al., 2011), or they can directly interact with YAP/TAZ bypassing the kinase cascade (S. W. Chan et al., 2011; W. Wang et al., 2011; B. Zhao et al., 2011). Moreover, AMOTs emerged as direct substrates of LATS1/2 and their phosphorylation by these kinases promotes

stabilization and abundance of AMOTs, thus limiting YAP/TAZ activity (Adler et al., 2013).

- *Adherens Junctions*

Cell-cell junction proteins, like AJs and TJs elements, are known to be regulators of Hippo pathway. For instance, E-caderin (E-cad) cooperating with NF2, KIBRA and LATS, is able to regulate contact inhibition of proliferation (N. G. Kim et al., 2011a). Moreover, α-catenin (α-cat), which is another AJ component, limits YAP activity both directly and indirectly. Indeed, α-cat can bind directly to YAP (Schlegelmilch et al., 2011a) or is able to inhibit integrin-mediated activation of SRC tyrosine kinase (P. Li et al., 2016). On the contrary, it was shown in *Drosophila* that E-cad and α-cat seem to promote Yorkie activity (Rauskolb et al., 2014; C. C. Yang et al., 2015). Otherwise, another AJ component Echinoid (Ed), has been observed to decrease Yki activity by furthering Sav membrane association and stability (Yue et al., 2012).

Besides AJs and TJs mentioned above, also focal adhesions (FAs) have been shown to be implicated in the regulation of Hippo signaling through several mechanisms. For instance, some of these mechanisms include: ILK-mediated suppression of NF2 (Serrano et al., 2013), FAK-Src-PI3K-mediated inhibition of LATS1/2 (N. G. Kim & Gumbiner, 2015), Gα13-mediated suppression of RhoA (L. Wang et al., 2016), and mechanical forces (Elosegui-Artola et al., 2017).

Furthermore, integrins have an important role in mediating signaling from the extracellular matrix (ECM) and they are implicated in YAP activity. Indeed, in multilayered tissues like the skin, integrin signaling contributes to YAP activation in basal layers, despite the inhibition of YAP in apical cell layers (Elbediwy et al., 2016).

- *G-Protein Coupled Receptors and Soluble Factors*

GPCRs are the largest family of membrane receptors in mammals and are involved in the regulation of several biological processes. In the last years several diffusible hormones and soluble factors were discovered to regulate YAP/TAZ. The first hormones discovered to activate YAP/TAZ were lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P). These hormones potently activate YAP/TAZ through their cognate GPCRs by inhibiting LATS (Miller et al., 2012; F. X. Yu et

al., 2012b). Interestingly, different GPCR family members can have positive or negative control on YAP/TAZ activity, depending on which coupled heterotrimeric G protein are induced by the different ligands. Indeed, ligands acting through $G\alpha_{12/13}$, $G\alpha_{i/o}$, or $G\alpha_{q/11}$, such as LPA, thrombin, angiotensin II and estrogen, activate YAP/TAZ promoting their nuclear translocation and transcriptional activity; on the other hand, ligands acting on $G\alpha_s$, (including β2 adrenergic receptors) like epinephrine and glucagon, inhibit YAP/TAZ activity (F. X. Yu et al., 2012b; Zhou et al., 2015).

All the mechanistic details of the regulation of YAP/TAZ by GPCRs are not yet completely understood, however it is clear that Rho GTPase is involved as downstream effector and also F-actin cytoskeleton is implicated in the Hippo signaling regulation (Miller et al., 2012; F. X. Yu et al., 2012b). In addition, activation of Protein Kinase A (PKA) upon cAMP production was demonstrated to inhibit YAP/TAZ activity by promoting LATS1/2 after $G\alpha_s$ activation (M. Kim et al., 2013; F. X. Yu et al., 2013).

However, there are several soluble factors that act independently from GPCRs and they are also involved in the regulation of cell growth, proliferation and tissue homeostasis by acting on the Hippo pathway. Some of these factors are: VEGFs (vascular endothelial growth factors), TGF-β (transforming growth factor beta), Wnt (Wingless/Integrated), insulin, EGF (epidermal growth factor), and BMPs (bone morphogenic proteins) (Azad et al., 2018; Miranda et al., 2017; Sorrentino et al., 2017; X. Wang et al., 2017; F. X. Yu et al., 2015).

- *Cell Morphology, Mechanical Cues and Cytoskeleton*

  Cells are sensitive of the physical aspects of their microenvironment such as cell-cell contact, changes in cell shape or the elasticity of the extracellular matrix and they are able to translate these inputs in biochemical signals (S. Piccolo et al., 2014). All the mechanisms that allow cells to perceive and consequently respond to mechanical cues are known as mechanotransduction, and they regulate cell survival, proliferation, migration and differentiation (Hoffman et al., 2011).

  Recent discoveries shown that YAP/TAZ have a pivotal role in mechanotransduction, regulating gene expression upon mechanical cues, changing in this way the cellular behavior (Halder et al., 2012; Low et al., 2014). As a matter of fact, when the ECM is stiff, remodelling of the cytoskeleton takes place in the

cell inactivating the Hippo signaling pathway thus allowing YAP/TAZ to enter the nucleus. Conversely, when the ECM is soft, Hippo signaling is active and this leads to the phosphorylation of YAP/TAZ, thus preventing their entry into the nucleus (Dupont et al., 2011). Moreover, cells grown on stiff ECM or spread across a large surface are flat (YAP/TAZ are nuclear), while cells grown on soft ECM or on a small surface are round (YAP/TAZ are cytoplasmic) (Dupont et al., 2011; Wada et al., 2011). The responsible for the transmission of stiffness signals seems to be RhoA GTPase, regulating YAP/TAZ translocation, promoting actin polymerization and stress fiber formation. Indeed, inhibiting RhoA, Rho-associated serine/threonine kinase (ROCK) or myosin type II with small molecules leads to YAP/TAZ cytoplasmic localization and their consequent inactivation (Dupont et al., 2011; Wada et al., 2011; B. Zhao et al., 2012). The role of Hippo signaling cascade in this process is controversial, in fact there are studies in which the YAP regulation by Rho is Hippo pathway independent, while there are recent studies in which YAP regulation is mediated by the actin cytoskeleton and Hippo signaling, especially YAP phosphorylation by LATS1/2 is involved (Codelia et al., 2014; Wada et al., 2011; B. Zhao et al., 2012).

Moreover, YAP/TAZ are able to bind with the switch/sucrose nonfermentable (SWI/SNF) complex through AT-rich interactive domain-containing protein 1A (ARID1A) in soft matrix conditions. On the contrary, on stiff ECM, nuclear filamentous actin (F-actin) rises, increasing their interaction with ARID1A-SWI/SNF. Therefore, YAP/TAZ can dissociate from the complex and interact with TEAD (Boopathy & Hong, 2019; Chang et al., 2018; H. J. Lee et al., 2017). Indeed, the levels of F-actin have an important role in the mechanical regulation of YAP activity (Mana-Capelli et al., 2014; B. Zhao et al., 2012).

Contact inhibition of proliferation (CIP) is a biological paradigm in which cultured cells stop dividing when they reach confluency and YAP/TAZ are involved in this phenomenon (B. Zhao et al., 2007b). Indeed, at low density YAP/TAZ are active in the nucleus, while at high density they are cytoplasmic, in fact at high cell density LATS1/2 kinase activity is enhanced leading to an increased YAP phosphorylation during CIP (Fig. 2). Nevertheless, how cell confluency activates LATS1/2 is not yet totally understood. One mechanism seems to be the AMOT complex at the TJs, which mediates cell-cell contact signals to regulate Hippo signaling (Y. Li et al., 2015). Another potential mechanism is the *trans*-dimerization of E-cadherin at AJs,

which stimulates the Hippo kinase cascade MST1/2-LATS1/2 through α/β-catenin, Kibra and Merlin (N. G. Kim et al., 2011b). The cytoplasmic retention of YAP/TAZ at high density implicates also F-actin polymerization and stress fiber formation (Aragona et al., 2013). Aragona and colleagues shown that cell-cell contact regulates YAP activity also altering cell geometry; indeed, low density plated cells have a flat and spread-out morphology, while high density cells are round and compact (Aragona et al., 2013). The geometrical changes described inhibit RhoA activity, reduce stress fiber formation, inactivating YAP (Aragona et al., 2013; Dupont et al., 2011; Wada et al., 2011). Nonetheless, this regulation needs further studies to be totally understood.

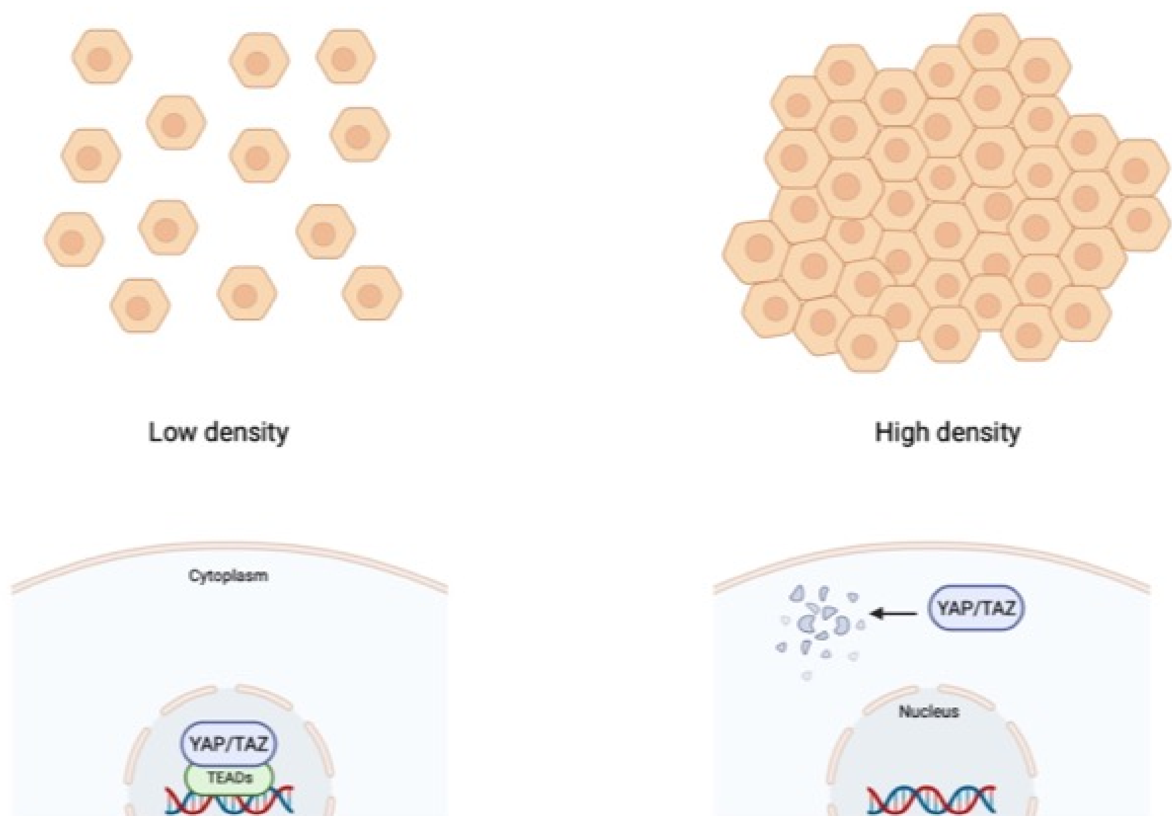


Figure 2. Graphical representation of contact inhibition of proliferation. At low density YAP/TAZ are active in the nucleus, where they interact with TEAD. At high density YAP/TAZ are excluded from the nucleus. Image created with BioRender.com.

- *Stress Signals*

The Hippo signaling pathway is sensitive to internal and environmental stresses such as energy deficit (deRan et al., 2014; J. S. Mo et al., 2015; W. Wang et al., 2015), hypoxia (B. Ma et al., 2015), osmotic stress (Hong et al., 2017; K. C. Lin et al., 2017), endoplasmic reticulum stress (H. Wu et al., 2015), oxidative stress (Lehtinen et al., 2006), cytokinesis failure (Ganem et al., 2014), and pathogens (Geng et al., 2015; B. Liu et al., 2016; F. Meng et al., 2016; S. Wang et al., 2017).

Recent data demonstrate that the energy status of the cells regulates YAP transcriptional activity in both Hippo-dependent and Hippo-independent manners. A central role in this phenomenon is played by AMP-activated protein kinase (AMPK), a Ser/Thr kinase which acts on multiple downstream substrates to restore energy homeostasis upon glucose deprivation, inhibition of glycolysis and higher AMP:ATP ratio (Hardie et al., 2012). Once activated, AMPK phosphorylate AMOTL1 leading to LATS1/2 activation. Moreover, AMPK can also directly phosphorylate YAP at multiple residues including Ser94, which is essential for YAP-TEAD interaction, thus preventing their interaction (deRan et al., 2014; J. S. Mo et al., 2015; W. Wang et al., 2015).

Besides glucose metabolism, Hippo signaling can be enhanced (leading to YAP/TAZ cytoplasmic localization and decreased transcriptional activity) by the inhibition of the mevalonate pathway, which has a crucial role for cholesterol biosynthesis and other essential bioactive molecules (Sorrentino et al., 2014; Z. Wang et al., 2014).

Hypoxia is another stress signal that inhibits the Hippo pathway, allowing YAP/TAZ activation; in fact, it activates an E3 ubiquitin ligase SIAH2, which binds to and destabilizes LATS2 (B. Ma et al., 2015).

Osmotic pressure has been observed to regulate YAP. The stress caused by osmotic pressure leads to the phosphorylation on YAP by NLK kinase, hindering the bond to 14-3-3 and the consequent YAP nuclear accumulation, which allows the expression of its target genes (Hong et al., 2017; Moon et al., 2017). Moreover, osmotic stress plays a role in YAP-TEAD interaction even in a Hippo-independent manner, indeed it activates p38, which is able to directly bind to TEAD inducing TEAD cytoplasmic translocation and disrupting YAP-TEAD-dependent transcription (K. C. Lin et al., 2017). In addition, hyper-osmotic stress strongly activates LATS, even though the molecular mechanisms are still unknown.

Accumulation of misfolded/unfolded protein in the endoplasmic reticulum (ER) leads the activation of two conserved stress response pathways: Heat Shock Response (HSR) and Unfolded protein response (UPR) that operate in the cytoplasm and endoplasmic reticulum respectively. It has been shown that both pathways activate the Hippo pathway. Initially, UPR can activate YAP by a PERK (PKR-like ER kinase)–eIF2α (eukaryotic initiation factor 2α) axis, conversely prolonged ER stress inhibits YAP and promotes apoptosis (H. Wu et al., 2015). It has been demonstrated that heat shock (HS) inhibits LATS kinase by increase heat shock protein 90 (Hsp90)-LATS interaction. Additionally, HS induces ubiquitination and degradation of LATS, which leads to a YAP dephosphorylation and activation (M et al., 2021). Thus, the stress response pathways modulate the Hippo pathway cascade inducing YAP activation under stress. Finally, pathogens may also represent a stress signal that activates the Hippo pathway. In fact, bacterial infection activates Hippo signaling cascade in both *Drosophila* and mammals (Geng et al., 2015; B. Liu et al., 2016). Moreover, the kinases MST1/2 and LATS1/2 and the effector YAP/TAZ are involved in anti-viral immune response in mammalian cells (F. Meng et al., 2016; S. Wang et al., 2017).

1.1.4 Functions of Hippo pathway

Concentrating on the target genes, the first studies shown the involvement of the Hippo pathway in cell proliferation and cell survival (Zheng & Pan, 2019). Crosstalk with others signaling pathways such as Wnt, Notch, EGFR, TGFβ and Jak-STAT, contribute to the regulation of tissue growth, proliferation and survival (Azzolin et al., 2014a; Hansen et al., 2015; S. Piccolo et al., 2014; Sileo et al., 2022). Latest data from chromatin immunoprecipitation analyses and genome-wide expression profile in human epithelial cells demonstrated that genes related to proliferation and cell death are enriched among all HP target genes (Zanconato et al., 2015; Zheng & Pan, 2019). These studies explore also new targets of Hippo signaling involved in other processes as cell migration, ECM and cytoskeleton organization. Moreover, discoveries of the last years extended Hippo pathway functions to processes like organ size control, cell fate decision or promoting stemness and regeneration (Fig. 3).

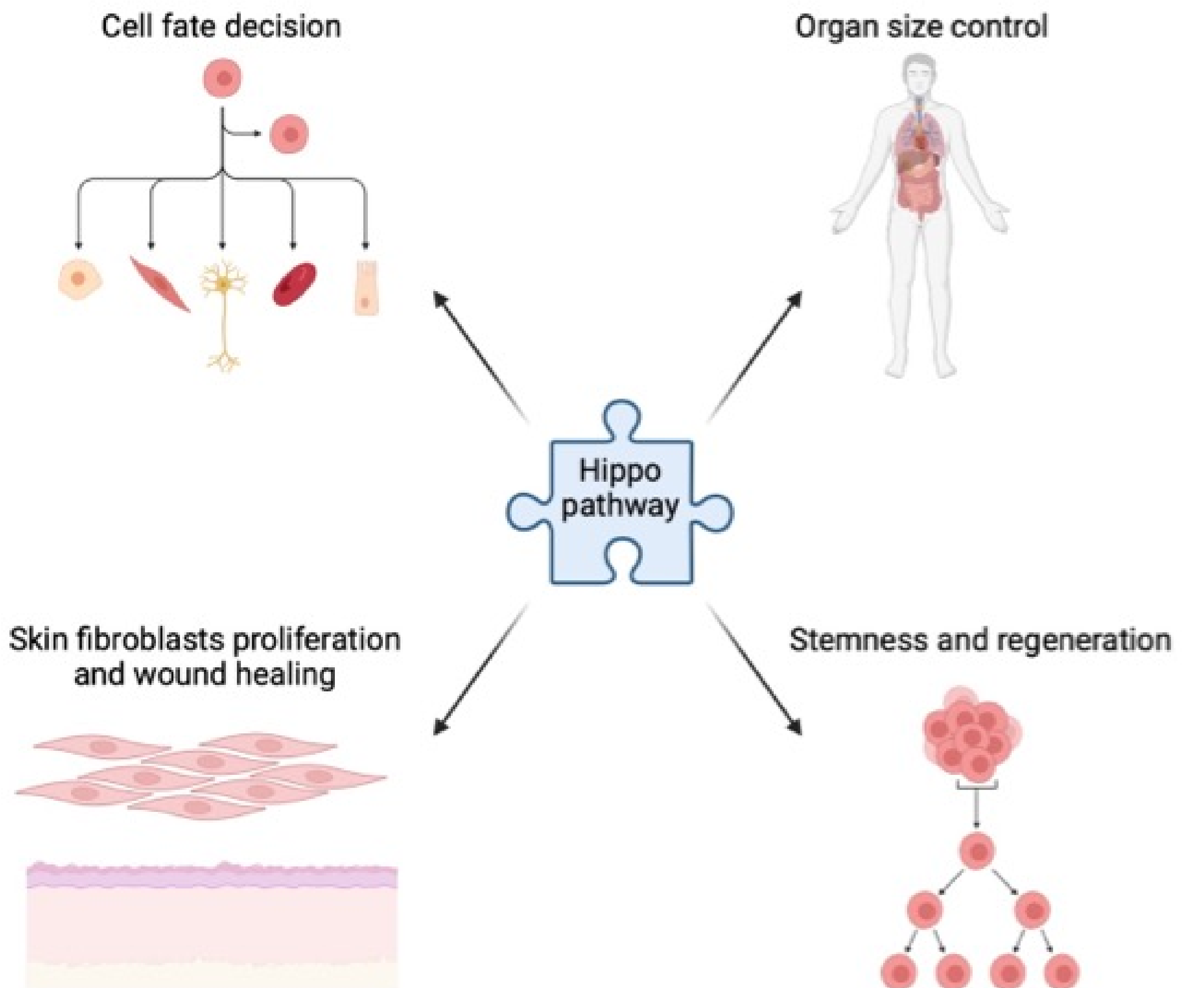


Figure 3. The Hippo pathway is involved not only in cell proliferation and survival, but also plays a role in fundamental processes such as cell fate decision, organ size regulation, stemness, regeneration and wound healing. Image created with BioRender.com.

- *Organ Size Control*

  Hippo signaling is one of the master regulators of organ growth, indeed the loss of functions of some of its components or hyperactivated YAP/TAZ in mice lead to overgrowth of many organs as liver or heart (Patel et al., 2017; Xiao et al., 2016). Moreover, mutations in HP players induce overgrowth but also undergrowth in some tissues (F. X. Yu et al., 2015). However, the organs size control by the Hippo pathway is not universal. In fact, the upregulation of YAP/TAZ or deletion in the signaling cascade cause the overgrowth of some organs such as the liver, heart, stomach and spleen (Cotton et al., 2017; Patel et al., 2017; H. Song et al., 2010; Xiao et al., 2016); in contrast in others organs such as lung, limb, intestine and mammary

gland it does not increase organ size, rather in some organs it even decreases the size, such as the pancreas and kidneys. (Cai et al., 2010; Q. Chen et al., 2014; Dong et al., 2007a; T. Gao et al., 2013; George et al., 2012; McNeill & Reginensi, 2017; Reginensi et al., 2013; H. Song et al., 2010). Hence, the role of Hippo signaling on organ size control is context dependent and influenced by other factors such as the cellular composition, the growth rate and hormonal signals.

- *Cell Fate Decision*

  In addition to controlling organ size, the Hippo pathway also plays a crucial role in cell differentiation and in cell fate decisions.

  During mouse embryogenesis, in outer blastocyst cells Hippo signaling is inactive, hence YAP is nuclear and actives TEAD4-dependent genes to specify the trophectoderm (TE) lineage. Conversely, in inner cells HP is active, YAP is cytoplasmic and this promotes the formation of the inner cell mass (ICM) (Nishioka et al., 2009; Sasaki, 2015).

  Hippo pathway is involved in differentiation path of mesenchymal stem cells (MSCs). Indeed, cultured MSCs can be differentiated controlling the ECM stiffness, in fact on soft ECM, cells differentiated in neural lineages or adipocytes; on stiff ECM, cells have an osteogenic fate, while on medium ECM, cells have myogenic fates (B. C. Heng et al., 2020).

  Studies conducted in mouse liver have shown that YAP is required in biliary epithelial cells and the loss of YAP during liver development leads to hypoplastic biliary ducts which are gradually lost over time (N. Zhang et al., 2010). On the other hand, mice carrying *LATS1/2* or *NF2* deletion showed a significant increment of biliary epithelial cells to the detriment of hepatocytes (Q. Chen et al., 2015a; Yi et al., 2016; N. Zhang et al., 2010). As a matter of fact, activation of YAP can lead to trans-differentiation of hepatocytes into biliary epithelium (Yimlamai et al., 2014).

  YAP has an important role also in nervous system; indeed, it maintains neural progenitors cell number in the embryonic neural tube and sustains proliferation and apical attachment of ependymal precursor cells, controlling ependymal integrity (Cao et al., 2008; Park et al., 2016). Moreover, YAP/TAZ operate also in peripheral nervous system regulating myelination and expression of laminin receptors in Schwann cells (Poitelon et al., 2016).

YAP has a pivotal role in epidermal stem cell proliferation and tissue expansion. Particularly in the skin, YAP expression is elevated in the basal epidermal progenitors and decreases in the suprabasal differentiated cells (Schlegelmilch et al., 2011b). Indeed, YAP knockout in the epidermis makes the skin thinner and fragile and leads to a lack of epidermal tissue in the distal part of the limbs. On the other hand, overexpression of YAP increases the amount of basal epidermal progenitors and inhibits terminal differentiation (Schlegelmilch et al., 2011b; H. Zhang et al., 2011).

In addition to the examples mentioned above, YAP/TAZ and in general the whole Hippo signaling pathway are involved in the regulation of cell differentiation of many other tissues and organs, such as the heart, kidneys, pancreas, lungs, salivary glands, mammary glands and blood vessels (S. Piccolo et al., 2014; Zheng & Pan, 2019). This therefore underlines the crucial role that the Hippo pathway has in cell fate decisions and differentiation and the great importance that the study of this pathway assumes to better understand all mechanisms.

- *Promoting Stemness and Regeneration*

  YAP/TAZ are involved in stem and progenitor cell fates in several tissue (J. Mo et al., 2014; Panciera et al., 2016). For instance, they are necessary for embryonic stem cells (ESCs) to maintain pluripotency and self-renewal, in fact activation of YAP prevents ESC differentiation (Lian et al., 2010; Varelas et al., 2008). However, it was demonstrated that YAP/TAZ are also crucial for the differentiation of ESCs (Azzolin et al., 2014a), even if the reasons are not well known, probably related to the different types of culture conditions.

  It was reported that transient activation of YAP leads differentiated cells into stem cells in different tissues such as neuronal tissue, pancreas or mammary gland (Panciera et al., 2016). YAP/TAZ are greatly expressed at the base of the crypts in mouse intestines, and their inhibition prejudices regeneration upon intestinal injury even if it has no perceptible effects on normal development (Azzolin et al., 2014a; Cai et al., 2010). In fact, upon injury, post-transcriptional activation of YAP is enhanced in order to provoke regeneration and increasing transient amplifying cells (Cai et al., 2010). Moreover, it was reported that YAP is active in self-renewing intestinal organoids at the proliferative crypt-like regions, while it is cytoplasmic at the differentiated regions (Gjorevski et al., 2016).

Interestingly, increased YAP activity can raise regeneration in the adult mice heart, where normally the regeneration levels are very low (Del Re et al., 2013; Heallen et al., 2013). During mice embryonic development, YAP increases cardiomyocytes proliferation; in this way new-born mice are able to regenerate cardiac tissue after injury. This ability is loss after birth because cardiomyocytes exit the cell cycle, but genetically increasing YAP activity leads to cardiomyocytes proliferation and ease the repair of hearts damaged by hearth attack (Del Re et al., 2013; Heallen et al., 2013). This heart regenerative ability after induction of YAP is very promising from a therapeutic point of view for heart-attack patients (Misra & Irvine, 2018). More generally, as also seen in the other tissues, the transient activation of YAP/TAZ could represent a valid tool for regenerative medicine.

- *Hippo pathway in skin fibroblasts*

  YAP/TAZ regulate proliferation in fibroblasts during skin development and repair. The activity of YAP/TAZ in the epidermis is in turn regulated by various signals, such as signalling downstream of adherens junctions and integrins, mechanical forces transduced by the cytoskeleton. These mechanisms cooperate with the Hippo signalling pathway in a context-dependent manner, although the prevalent signals controlling YAP/TAZ in fibroblasts appear to be mechanical ones (Rognoni & Walko, 2019). In skin fibroblasts, YAP is mostly nuclear in proliferative cells, while it is cytoplasmic in quiescent cells. Moreover, in adult dermal fibroblasts YAP is predominantly cytoplasmic, while it is nuclear and therefore expressed in dermal cells in and outside the wound bed in the early wound healing phase (Walko et al., 2017). Furthermore, inhibition of YAP/TAZ and TEAD4 through knockdown or treatment with verteporfin (a YAP inhibitor) leads to decreased proliferation in fibroblasts and increased apoptosis (M. J. Lee et al., 2014). Interestingly, Quan and colleagues demonstrated that Cyr61 (a classic YAP/TAZ target gene) is a mediator of aberrant collagen homeostasis in chronologically aged and photoaged human skin (T. H. Quan et al., 2006). In fact, Cyr61 expression is high in fibroblasts in chronologically aged and photoaged human skin *in vivo*, and its activity through different pathways leads to altered collagen homeostasis. Indeed, it appears that Cyr61 involves the transcription factor activator protein 1 (AP-1) in chronologically aged and photoaged human skin, as high levels of Cyr61 activate this factor in

human skin fibroblasts. Furthermore, Cyr61 controls collagen homeostasis by impairing the TGF-β pathway, acting on the first steps of this pathway.

In addition, in skin fibroblasts, it has been observed that deletion of Ezrin (a protein involved in organising the interphase between the plasma membrane and cytoskeleton) impairs proliferation by preventing YAP localisation in the nucleus and thus inhibiting the expression of its target genes (C. Quan et al., 2018). This underlines the connection between cell size and mechanical stimuli with YAP; in fact, Ezrin knockdown decreased fibroblast size and mechanical properties and this leads to a lower nuclear localisation of YAP. Further evidence of the relationship of YAP/TAZ with fibroblast cell size can be seen in another study. Qin and colleagues demonstrated that CTGF (one of the main target genes of YAP/TAZ) is downregulated in aged human dermal fibroblasts *in vivo*. In particular, reduced fibroblast size, which is a characteristic feature of aged human skin, leads to decreased CTGF expression. This decreased expression of CTGF linked to fibroblast cell size is mediated by reduced nuclear translocation of YAP/TAZ, highlighting how YAP/TAZ and their target CTGF are involved in human dermal ageing (Qin et al., 2022).

As mentioned earlier, YAP is also involved in skin repair and wound healing. Indeed, it has been observed that in dermal fibroblasts, YAP activation leads to the expression of CTGF and Cyr61, which through paracrine signalling promotes wound healing (Shome et al., 2020). Furthermore, in another study it was observed that YAP/TAZ and TEAD are implicated in skin scarring, and nuclear expression of these three factors is increased in fibroblasts of keloids and hypertrophic scars (Petrou et al., 2022). It had already been shown that YAP/TAZ are involved in skin wound healing (Fig. 4) through regulation of the expression of the TGF-β1 signalling pathway (M. J. Lee et al., 2014). Furthermore, the involvement of YAP, CTGF and TGF-β in wound healing can also be interestingly linked to diabetes. Indeed, diabetic patients have compromised wound healing combined with dermal fibroblast dysfunction. YAP expression in dermal fibroblasts of diabetic mice has been shown to be reduced associated with downregulation of CTGF and TGF-β (J. Yu et al., 2017). It therefore appears that reduced YAP activity leads to reduced expression of wound healing stimulators such as CTGF and TGF-β in dermal fibroblasts.

In conclusion, the strong connection of the Hippo pathway and in particular of YAP/TAZ with fibroblast proliferation, cell size and wound healing has recently emerged, emphasising the importance of the regulation of this pathway in skin development and repair and the implications it may have in various diseases.

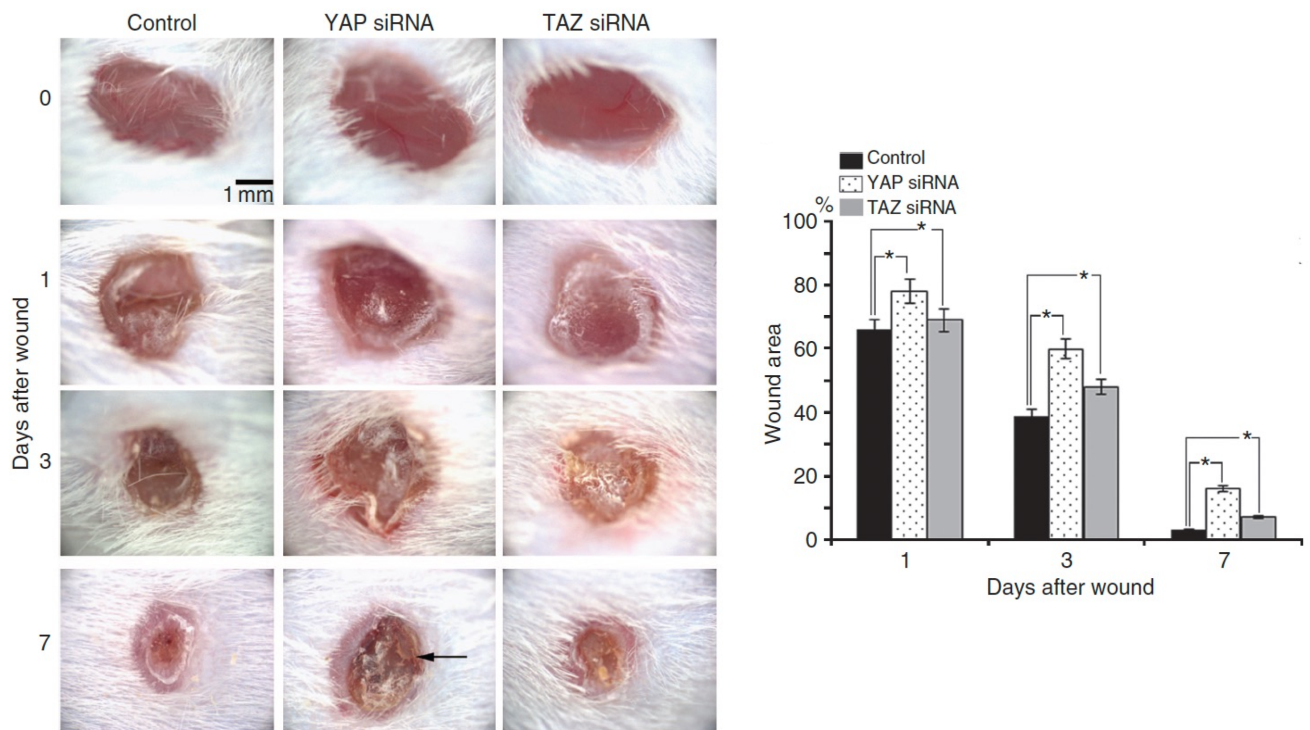


Figure 4. Skin repair during wound healing. Silencing of YAP and TAZ decreases wound healing. Figure taken from M. J. Lee et al., 2014

1.1.5 The Hippo Pathway and Disease

As seen previously, the Hippo pathway is implicated in many fundamental roles in cells and consequently in the organism; it therefore follows that it is implicated in various diseases. First of all, the most documented connection in literature is with cancer and tumorigenesis, given the critical role the Hippo pathway has in various processes that influence growth, survival, proliferation and in general in development and homeostasis. Besides cancer however, there are implications in other types of diseases as well including developmental abnormalities, autoimmune diseases, etc. Recently, involvement with several neurodegenerative diseases has also emerged.

- *The Hippo Pathway in Cancer*

  Deregulation of Hippo pathway have been observed many times in several mammalian tumors and Hippo signaling-YAP/TAZ has a well-documented role in promoting cancer cell proliferation (F. X. Yu et al., 2015; Zanconato et al., 2016). Along with the regulation of cell proliferation and cell death, Hippo pathway, especially YAP/TAZ, are also implicated in other aspects of tumorigenesis, such as EMT, cell migration, tumor microenvironment and cancer stem cell (Zanconato et al., 2016). Even though genetic mutations in Hippo pathway components are rarely observed in cancer, their dysregulations are common, especially YAP/TAZ but also core components as MST1/2, SAV, MOB and LATS1/2, promoting tumorigenesis (Harvey et al., 2013; Zanconato et al., 2016). Indeed, a recent study on 9 125 tumor samples showed diffused deregulation of signaling core components in different human cancers, classifying the Hippo pathway as one of the eight major signaling pathways principally altered in human cancers (Sanchez-Vega et al., 2018). Particularly, deletion of several HP components as MST1/2, SAV1, NF2, KIBRA, LATS1/2 or MOB1A/B or overexpression of YAP lead to hyperplasia and tumorigenesis in different tissues (Cai et al., 2010; Camargo et al., 2007; Q. Chen et al., 2015b; Dong et al., 2007b; Giovannini et al., 2000; Hermann et al., 2018; K. P. Lee et al., 2010; Lu et al., 2010; Nishio et al., 2016; H. Song et al., 2010; Yi et al., 2016; D. Zhou et al., 2009). Loss-of-function of *NF2* or its inactivation is observed in neurofibromatosis type 2, malignant mesothelioma, renal cell carcinoma (RCC) and in cervical squamous cell carcinoma (CSCC) (Mehra et al., 2018; Murakami et al., 2011; Rouleau et al., 1993; Sekido, 2011; Y. Wang et al., 2018). Moreover, in CSCC and RCC also other HP components such as LATS2 and SAV1 are mutated and *LATS1/2* mutations are even detected in malignant mesothelioma (Miyanaga et al., 2015; Murakami et al., 2011; Y. Wang et al., 2018). Besides the mutation of these HP core components, activation of YAP/TAZ is more preeminent in human cancers. Indeed, in different kinds of cancers as CSCC, hepatocellular carcinoma, medulloblastoma or oral squamous cell carcinoma, YAP gene locus is amplificated (Fernandez-L et al., 2009; Overholtzer et al., 2006; Y. Wang et al., 2018; Zender et al., 2006). Moreover, gain-of-function fusion proteins of YAP/TAZ, due to chromosomal translocations, were observed in epithelioid hemangioendothelioma, luminal breast cancer and ependymoma (Antonescu et al., 2013; S. Li et al., 2013; Pajtler et al., 2015; Parker et al., 2014; Tanas et al., 2011). Finally, crosstalk with

other oncogenic pathways can also lead to YAP/TAZ activation. For instance, in uveal melanomas, a gain-of-function mutations in *GNAQ/GNA11* lead to an activation of YAP/TAZ (Feng et al., 2014; F. X. Yu et al., 2014). YAP/TAZ are also activated in most human colorectal cancers carrying mutations in the adenomatous polyposis coli (*APC*) gene (Azzolin et al., 2014b; Cai et al., 2015). In addition, in pancreatic ductal adenocarcinoma and in lung cancer was observed YAP activation, due to oncogenic mutation of KRAS (Kapoor et al., 2014; Shao et al., 2014). In lung adenocarcinoma, YAP/TAZ activation is caused by inactivation of liver kinase B1(*LKB1*) (Mohseni et al., 2013). Interestingly, YAP/TAZ can be activated also by oncogenic viruses; in fact, it was observed that the YAP/TAZ activation in sarcoma is due to Kaposi sarcoma-associated herpesvirus, while in the Merkel cell carcinoma the activation is by Merkel cell polymavirus (G. Liu et al., 2015; Nguyen et al., 2014). On the contrary, it is important to highlight that loss of YAP has also been observed in multiple myeloma and even reduced expression of YAP is linked with poor prognosis in acute myeloid leukemia (Cottini et al., 2014). This is relevant, as it suggests that in some cancer types YAP may be a context-dependent tumor suppressor.

Another role that YAP/TAZ have in tumorigenesis is to promote cancer stem cell traits (Cordenonsi et al., 2011). Furthermore, YAP/TAZ are also able to promote metastasis in different ways: inducing EMT, expressing growth factors, matricellular proteins and cytokines which modify the tumor microenvironment (Calvo et al., 2013; X. Guo et al., 2017; Lamar et al., 2012; G. Wang et al., 2016; J. Zhang et al., 2009). Additionally, YAP/TAZ are able to provoke tumor immune evasion through regulatory T cells or PD-L1 (Helena et al., 2018; Ni et al., 2018). However, the role of Hippo pathway in cancer immunity is controversial, in fact it can have a dual role, both positive and negative. Indeed, the knockout of *LATS1/2* and *MST1/2* and the activation of YAP in mouse liver lead to the formation of an immunosuppressive microenvironment (X. Guo et al., 2017). On the other hand, *LATS1/2* knock out can inhibit tumor growth in syngeneic mouse models enhancing antitumor immunity (Moroishi et al., 2016). Therefore, the role of Hippo signaling in tumor immunity and how it influences the relationship between tumor and immune system needs further studies, as the regulation is very complex. This dual role as oncogene and oncosuppressor of YAP/TAZ in tumours is increasingly emerging. Indeed, other studies show an antitumour activity of YAP/TAZ, such as

in small cell lung cancer (SCLC) and in retinoblastoma where YAP/TAZ have a tumour suppressor function (Pearson et al., 2021; Rudin et al., 2019). In another study, it was also found that pharmacological inhibition of LATS leads to the repression of ESR1 and the growth of ER+ breast cancer cells and patient-derived tumour organoids (S. Ma et al., 2022). This suggests that LATS could be a possible therapeutic target for this type of tumour. Recently, a new binary classification of tumours has emerged and is divided into two main classes: YAP/TAZ-deficient (YAP^{off}) and YAP/TAZ-positive (YAP^{on}) (Fig. 5), based on whether YAP/TAZ are present or absent and whether they play a pro- or anti-oncogenic role (Pearson & Bremner, 2021). To the YAP^{off} class belong all blood tumours (leukaemia, lymphoma, and myeloma), neural tumours (retinoblastoma, low-grade glioma, neuroblastoma) and small cell and well-differentiated neuroendocrine tumours. This class can be further subdivided into YAP^{off} liquid cancers and YAP^{off} solid cancers. Interestingly, these two classes of tumours seem to have a different regulation of YAP-TEAD. Indeed, in YAP^{on} cancers, YAP-TEAD cooperate with AP1 to induce cell cycle genes; in contrast, in YAP^{off}, YAP-TEAD cooperate with homeobox and basic helix-loop-helix factors to induce integrin, extracellular matrix and other adhesion genes. They also have different epigenetic regulation and different drug vulnerability. Indeed, it has been seen that YAP^{off} tumours can switch to YAP^{on} and *vice versa* as a potential resistance mechanism to develop drug resistance (Pearson et al., 2021; Pearson & Bremner, 2021). These emerging data offer new insights into the regulation of different tumours and offer potential new therapeutic approaches.

Finally, it was observed that YAP/TAZ activation is also able to give cancer cells resistance to different chemotherapies (Y. Zhao & Yang, 2015). In conclusion, dysregulation in Hippo pathway act on several aspects of cancer and tumorigenesis and further studies are needed to fully understand all the mechanisms, given that it also appears to be context-dependent, and to use this pathway as a target for potential treatments.

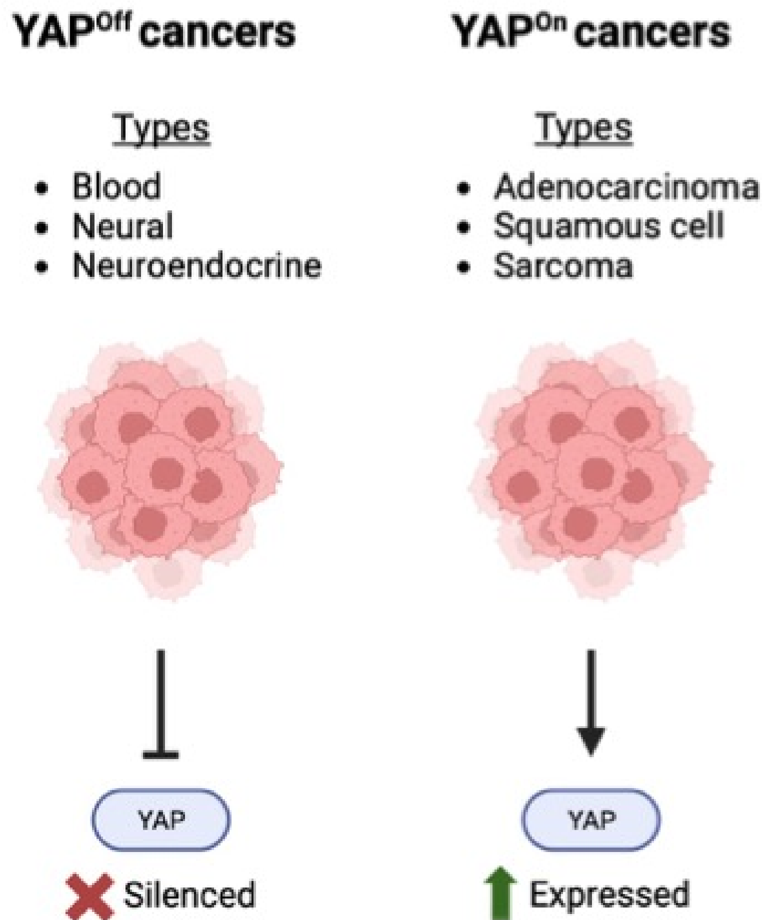


Figure 5. Graphical representation of the new cancer classification: YAP^{off} and YAP^{on}. YAP^{off} cancers include, for example, blood, neural and neuroendocrine cancers, while YAP^{on} cancers include, for example adenocarcinoma, squamous cell cancer and sarcoma.

- *The Hippo Pathway in Neurodegenerative Diseases*

  The Hippo signaling pathway is implicated in degenerative diseases, but interestingly in recent years the involvement of this pathway has also been discovered in a specific branch of these disorders: neurodegenerative diseases (Fig. 6). Indeed, it was observed that HP plays a crucial role in several neurodegenerative disorders including Huntington's disease (HD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), retinal degeneration disorders, Parkinson's disease (PD), Alexander disease, dentatorubral-pallidoluysian atrophy (DRPLA), Leber congenital amaurosis (LCA) and cerebral ischemia-reperfusion (Ahn et al., 2020; Jin et al., 2020; Sahu & Mondal, 2020; Tanaka et al., 2020).

Regarding the involvement of the Hippo pathway in Huntington's disease refer to the dedicated chapter 1.3.

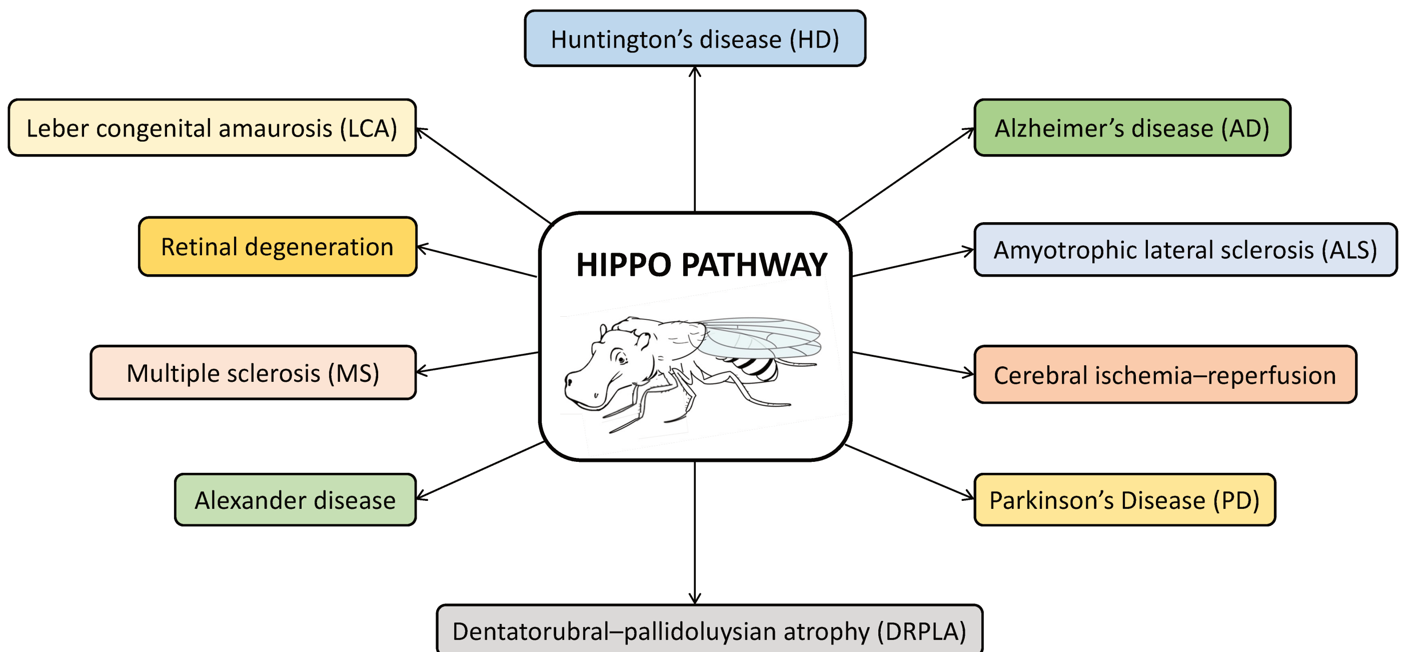


Figure 6. Graphical representation of the emerging involvement of the Hippo pathway in several neurodegenerative diseases; the figure shows the neurodegenerative diseases in which HP has recently been found to play a role.

Hippo pathway implications similar to those seen in Huntington's disease have been observed also in Alzheimer's disease. In fact, in AD it was detected neurodegeneration probably correlated to Transcriptional Repression-Induced Atypical cell Death (TRIAD), a particular Hippo-dependent necrotic cell death (details in chapter 1.3) and to nuclear YAP deprivation (Tanaka et al., 2020). In addition, in cortical neurons of AD patients they observed LATS1 activation and they found YAP into the Aβ aggregates. Besides necrosis, in AD is observed also apoptosis, linked with YAP as well, indeed nuclear YAP interact with p73 which induces apoptosis activating *Bax*, a proapoptotic target gene (Mao et al., 2016). Thus, in Alzheimer's disease neuron cell death goes through the entire degradation process, involving both apoptosis and necrosis, which are YAP and intracellular Aβ dependent. Another aspect AD has in common with HD is that S1P has a positive effect on ER instability in AD-iPSC-derived neurons (Tanaka et al., 2020).

Another neurodegenerative disease in which TRIAD has been observed is amyotrophic lateral sclerosis, although in this case it is non-identical, as the pro-survival form of YAP (YAPdeltaC) decreases in the course of neurodegeneration (Morimoto et al., 2009). However, full-length YAP and activated p73 level remain preserved until the late symptomatic stage. Despite the fact that the expression of total p73 decreases in parallel to the progression of the disease, the ratio of phosphorylated form of p73 to total p73 raised during the late symptomatic stage, indicating a crucial role of YAP-p73 deregulation in ALS neurodegeneration (Morimoto et al., 2009). Moreover, the presence of YAP in the nucleus of the motor cortex is significantly decreased in ALS patients compared to healthy controls, which lead to the consequent reduced expression of YAP target genes (Sadri-Vakili et al., 2018). Furthermore, it was found that neuronal death in ALS could be ameliorate with the lack of Hippo signaling activation, especially the lack of MST1 is neuroprotective, underlining the crucial role of Hippo pathway in ALS (Azuma et al., 2018; J. K. Lee et al., 2013).

The Hippo pathway is correlated with retinal detachment, in which photoreceptor cell death cause vision loss. It was observed that MST2 is a regulator of photoreceptor cell death in mouse model of retinal detachment, indeed *MST-/-* mice displayed reduced photoreceptor cell death. Furthermore, in these mice it was also shown a decrease of PUMA, FAS and activated caspase 3, which are proapoptotic molecules, and an inhibited nuclear relocalization of phosphorylated form of YAP (Matsumoto et al., 2014). All these data indicate that MST2 and the Hippo signaling could represent promising therapeutic targets for retinal detachment and highlight their neuroprotective role.

Recently, correlations have also emerged between the Hippo pathway and Parkinson's disease. In fact, it was demonstrated in PD a downregulation on Netrin1 (NTN1), a secreted laminin-related protein which has role in system development, axon guidance and growth (Ahn et al., 2020; J. Li et al., 2014; L. Lin & Isacson, 2006; Osborne et al., 2005). Reduction in NTN1 levels leads to the increased activation of MST1, which interacts and phosphorylates the netrin receptor UNC5B resulting in its apoptotic activation, responsible for dopaminergic neuronal loss, and causing a decrease of YAP levels and the consequent increased cell death. Ahn and colleagues demonstrated that the inhibition of UNC5B or the inhibition of its phosphorylation by MST1 are able to reduce neuronal apoptosis (Ahn et al., 2020).

In Alexander disease, deregulation of glial fibrillary acidic protein (GFAP) lead to actin reprogramming and stabilization. This deregulation induces the activation of a mechanosensitive signaling cascade, which in turn activates the YAP-Hippo regulation. Elevated YAP levels induce the expression of A-type nuclear lamina promoting brain stiffness, which deteriorate the pathology (L. Wang et al., 2018).

A study of large-scale transcriptional alterations in DRPLA (a spinocerebellar degenerative disorder), identified a downregulation of Fat in *Drosophila* (Calamita & Fanto, 2011; Napoletano et al., 2011). Fat is an upstream Hippo regulator and its downregulation leads to a decreased Hippo signaling, suggesting that is a potential mechanism of neuronal death even if the exact mechanisms have not yet been studied. However, the Fat-Hippo regulations is implicated in autophagy regulation in adult neurons, suggesting that DRPLA is related to dyregulated autophagy (Calamita & Fanto, 2011; Napoletano et al., 2011).

The loss of *Crb1* and *Crb2* or their mutation are the cause of LCA, with the consequent impaired retina, abnormal lamination and retinal thickening (Pellissier et al., 2013). Although the involvement of Crb in the regulation of the Hippo pathway is known, it has not yet been described whether Crb induces retinal abnormality through impaired Hippo signaling.

Taken together, all these studies demonstrate the pivotal role that Hippo pathway plays also in neurodegenerative disorders. Targeting this pathway could be crucial for the treatment of these diseases, although further studies are needed to understand the relationship between the Hippo pathway and neurodegenerative diseases and their mechanisms.

- <u>*The Hippo Pathway in Other Diseases*</u>

  Given the vast number of functions that the Hippo pathway performs in the cell, dysregulations and mutations of its components are obviously implicated in other diseases besides cancer. Focusing on mutations for example, YAP has a role in some eye pathologies; indeed, its activation in NF2 patients can lead to the formation of cataract (Kresak & Walsh, 2016). On the contrary, even the absence of its activity can lead to pathologies in the eye, such as a single mutation in *TEAD1* that perturbs the interaction with YAP, as observed in Sveinsson chorioretinal atrophy (SCRA), an autosomal dominant eye disease (Fossdal et al., 2004). Moreover, a heterozygous mutation of YAP is the cause of coloboma, a congenital malformation of the eye

(Williamson et al., 2014). Addedly, mutations of *MST1* are related with autosomal recessive primary immunodeficiency (Nehme et al., 2012).

In addition to genetic mutations, dysregulation of expression, phosphorylation and YAP/TAZ localization are also associated to various pathologies. For instance, arrhythmogenic cardiomyopathy (AC), a myocardial disease, is characterized by pathological Hippo pathway activation; in this disease cardiac myocytes are replaced with fibro-adipocytes and patients have cardiac disfunction and arrhythmia (S. N. Chen et al., 2014). Furthermore, in Stanford type A aortic dissection (STAAD) a low level of YAP protein was detected (Jiang et al., 2016); and disturbed blood flow induces YAP/TAZ promoting atherosclerosis (K. C. Wang et al., 2016; L. Wang et al., 2016). In pulmonary hypertension was observed an increased YAP protein level and a decreased YAP phosphorylation (Bertero et al., 2016). Moreover, an increased YAP activity, due to its nuclear accumulation, leads to cyst growth in polycystic kidney disease (Cai et al., 2018; Happé et al., 2011). The expression and localization of TAZ are also implicated in disease, for example, increased protein levels of TAZ induce liver inflammation and fibrosis in nonalcoholic steatohepatitis (NASH) (X. Wang et al., 2016). Sjogren's syndrome, an autoimmune disease, is instead characterized by a mislocalization of TAZ from the intercellular junctions to the nucleus (Enger et al., 2013).

In conclusion, it is important to underline that the exact mechanisms by which the Hippo pathway acts on these diseases are not yet well known and need to be investigated.

1.2 Huntington's Disease

1.2.1 Aetiology

Huntington's disease is an inherited autosomal dominant neurodegenerative disorder caused by a cytosine-adenine-guanine (CAG) trinucleotide expansion in the gene coding for huntingtin (*HTT*), located in the short arm of chromosome 4 (4p16.3) (MacDonald et al., 1993). The CAG expansion leads to the formation of a polyglutamine tract (polyQ) at the N-terminus of the protein. The normal range of CAG triplet in healthy population is between 6 and 26 repeats. When the number of triplets is more than 27 but less than 35, the individuals could rarely show cognitive impairment and behavioural symptoms (Cubo et al., 2016; Downing et al., 2016; Squitieri & Jankovic,

2012), but the most concerning consequence for these individuals is the further expansion of CAG during the meiosis and the consequent increase in the number of triplets in the progeny (McColgan & Tabrizi, 2018) (Fig. 7). Although the threshold beyond which the disease develops is normally considered 36 repetitions, the population with 36-39 repeats exhibits reduced penetrance, older age at onset, and a varied, reduced or missing clinical expression (Dayalu & Albin, 2015; Semaka et al., 2013). Meanwhile, individuals with more than 40 CAG repeats exhibit full penetrance and a complete clinical picture. Moreover, the higher the number of repetitions, the greater the severity and speed of disease progression (Aziz et al., 2009). Furthermore, the age at onset (AO) is inversely correlated to the number of trinucleotide repeats. A CAG repeat length > 60 provokes juvenile HD, characterized by a stronger phenotype and an anticipated AO (Squitieri et al., 2006). Although AO of juvenile HD is considered under 20 years old, two sub-categories have recently emerged: early infantile onset (AO < 10 years old) and juvenile onset (AO < 20 years old) (Fusilli et al., 2018).

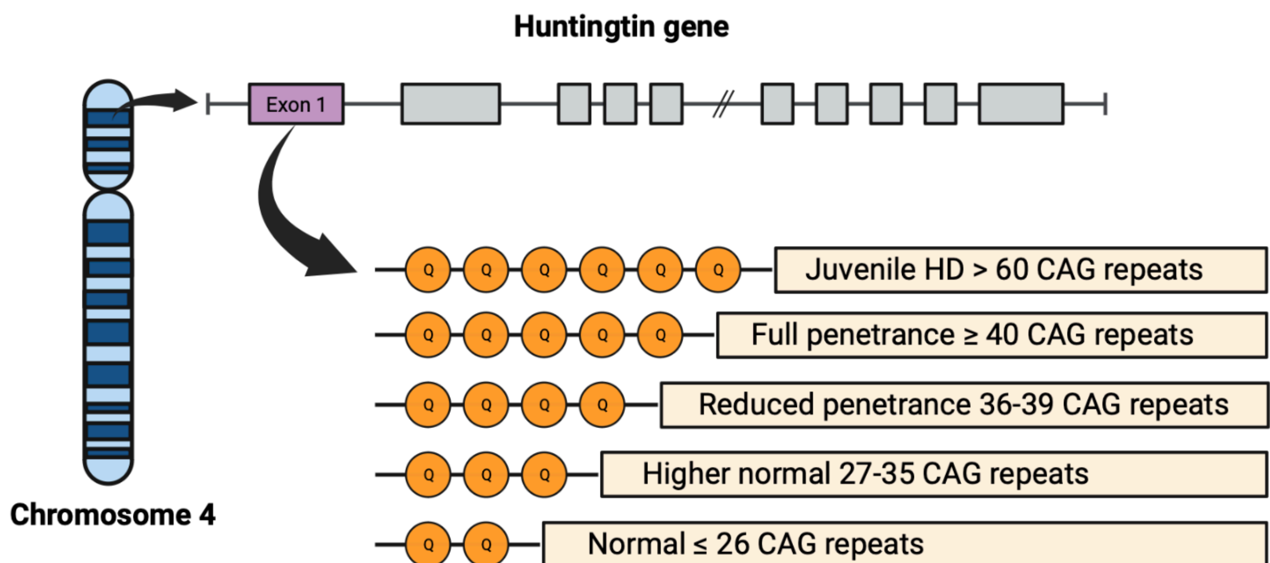


Figure 7. Graphical representation of the relationship between the number of CAG repeats in the first exon of the huntingtin gene located in the short arm of chromosome 4 and the penetrance of Huntington's disease. Image created with BioRender.com.

1.2.2 Epidemiology

The prevalence of Huntington's disease is considered 2.7 per 100 000 individuals (Pringsheim et al., 2012). Nevertheless, the number varies in different parts of the world.

Indeed, Western population exhibits a prevalence of 10.6-13.7 per 100 000 individuals, while in East Asia is lower with a frequency of 1-7 per million (Kay et al., 2018; Rawlins et al., 2016). In South Africa, black people exhibit lower rates compared to white population and mixed communities. General population of British Columbia, Canada, has a frequency of 13.7 per 100 000 individuals, instead the general Caucasian population has a higher rate of 17.2 per 100 000 individuals (Fisher & Hayden, 2014). These discrepancies are related to the differences in the HTT gene, indeed populations characterized by a higher frequency have longer average of trinucleotide repetitions (G. P. Bates et al., 2015).

1.2.3 The Huntingtin Protein

Huntingtin protein is a 348 kDa α-helix protein ubiquitously expressed, especially in the central nervous system (CNS) and testes (S. H. Li et al., 1993). The exon 1 of the *HTT* gene corresponds to the N-terminal segment, the poly-glutamine tract and a proline-rich domain (PRD) (Caterino et al., 2018). The protein also contains several HEAT [Huntingtin, Elongation factor 3 (EF3), proteinphosphatase 2A(PP2A), yeast kinase TOR1] repeats. These consensus areas are resistant to proteolytic cleavage and this is thought to give the protein its scaffold function, mediating protein-protein interaction (G. P. Bates et al., 2015). HTT undergoes several post-translational modifications, such as phosphorylation, ubiquitination, SUMOylation, acetylation and palmitoylation (Cong et al., 2011; Steffan et al., 2004; Thompson et al., 2009; Yanai et al., 2006). These post-translational regulations can impact on the half-life of the protein, the aggregation and degradation by ubiquitin-proteasome system (UPS). Moreover, HTT can be cleaved by many caspases, calpains and endopeptidases, generating different N-terminal fragments, including one corresponding to the first exon, considered critical for HD (G. P. Bates et al., 2015). Indeed, mutant huntingtin (mHTT) fragments, in contrast to wt HTT, generate β-sheets that are capable of forming aggregates, with an efficiency directly dependent on the number of CAG repeats (S. Chen et al., 2001; Ross & Tabrizi, 2011). Furthermore, mHTT aggregates into the nucleus, as opposed to wild-type HTT which is predominantly cytoplasmic (DiFiglia et al., 1997).

Although the role of huntingtin has not yet been fully defined and understood, the main function seems to be as a protein scaffold, but HTT is involved in numerous processes

given its ubiquitous expression. In fact, the protein has a pivotal role in brain development; it regulates transcriptional process balancing neurotrophic support and receptors; it contributes to the equilibrium of histone acetylation/deacetylation and glial activation; it has a role in mitochondrial functions and biogenesis; it regulates signaling pathways, axonal transport of organelles by microtubules and it is also involved in autophagy (Cisbani & Cicchetti, 2012; Franco-Iborra et al., 2021; Munoz-Sanjuan & Bates, 2011; Saavedra et al., 2018).

1.2.4 Pathogenesis

In Huntington's disease patients, the expanded polyQ tract causes misfolding of the huntingtin protein, which leads to the formation of soluble monomers of the protein that bind together producing oligomers. These oligomers aggregate to form mHTT fibrils and insoluble inclusions in the nucleus as well as in the cytoplasm of neurons (DiFiglia et al., 1997; Rüb et al., 2016). Despite previous assumption that these insoluble mHTT aggregates were pathogenic (Davies et al., 1998; Ross, 1997), it has been shown that inclusion does not provoke cell death (Arrasate et al., 2004; Saudou et al., 1998; Slow et al., 2005). Actually, subsequent studies have pointed out that the N-terminal mHTT oligomers are toxic and aggregates formation could have a protective role for the cell (Lajoie & Snapp, 2010; J. Miller et al., 2011; Nagai et al., 2007; Nucifora et al., 2012; Pieri et al., 2012; Sahl et al., 2012). Thus, endoplasmic reticulum stress is induced by oligomers before the appearance of the aggregates and the following formation of these inclusions rather reduces toxicity sequestrating oligomers (Leitman et al., 2013; Takahashi et al., 2008). In addition, mis-splicing of the mRNA (Landles et al., 2010; Neueder et al., 2017) or proteolytic cleavage of the full length mHTT by caspases and calpains (Sathasivam et al., 2013) induce the formation of a toxic N-terminal mHTT fragment (toxic exon 1 protein, TOPIC). Indeed, it has emerged that the level of TOPIC is higher in juvenile HD patients and in individuals presenting long CAG repeats (Neueder et al., 2017; Sathasivam et al., 2013). Furthermore, expression of this toxic exon 1 protein in mice leads to an early severe onset of the disease compared to those carrying the same number of CAG repeats but in full-length mHTT (Gillian. Bates et al., 2002).

Interestingly, it was observed that mHTT can diffuse between cells. Indeed, in *Drosophila* released mHTT from synaptic terminals is incorporated into adjacent neurons by endocytosis (Babcock & Ganetzky, 2015). Moreover, in *Drosophila* mHTT

can also be phagocytized by glial cells and may induce the wild-type HTT to form aggregates (Pearce et al., 2015). Pecho-Vrieseling and colleagues observed the spreading of mHTT between neurons through synapses in three different models: i. from induced pluripotent stem cells (iPSCs)-derived neurons of HD patients to wild-type (wt) mouse brain slices; ii. from HD mouse cortical neurons to medium spiny neurons in a wt mouse corticostriatal brain slice; iii. after injection of mHTT fragments into wt mouse cortex (Pecho-Vrieseling et al., 2014). It is important to note that the mHTT spreading is not a prion propagation, in fact until now it has not been observed in HD (Kovacs & Budka, 2008). Nevertheless, proof of these mHTT transfer in humans are restricted. One evidence comes from a post-mortem analysis of HD patient brains who received fetal striatal transplants from donors not carrying the HTT mutant gene. In this study mHTT has been found in the extracellular matrix of the transplants, hypothesising the releasing of the mutant protein by neurons even though inclusions were not observed within the cells (Cicchetti et al., 2014).
In HD patients, mHTT impacts several mechanisms despite the abnormal aggregation of the protein as shown by numerous *in vitro*, *in vivo* and post-mortem studies. Some of these embraces: altered gene transcription, excitotoxicity mediated by N-Methyl-D-Aspartate (NMDA) receptor, dopaminergic dysfunction, mitochondrial dysfunction and oxidative stress, altered autophagy and decreased trophic support (Gil & Rego, 2008; Naia et al., 2015, 2016; Ribeiro et al., 2014; Rosenstock et al., 2011). These dysfunctional processes are due to toxic gain of function of mHTT and loss of function of the wt HTT (A. Kim et al., 2021).

- *Excitotoxicity*

  The GABAergic medium spiny neurons (MSNs) are affected by striatal neurodegeneration and are the most impaired neuronal cells in HD patients. This degeneration can be caused by NMDA receptor-mediated excitotoxicity; indeed, MSNs have high level of this receptor (especially the NR2B subunit) than striatal interneurons (Landwehrmeyer et al., 1995). Notably, NR2B subunit increases permeability to the receptor and decreases its deactivation time. Since MSNs have increased level of NR2B, they are more sensitive to glutamate-mediated excitotoxicity (Sieradzan & Mann, 2001). *In vitro* studies have correlated higher excitotoxic cell death with the co-expression of NR2B and mHTT (Zeron et al., 2001); while *in vivo* studies demonstrated elevated degeneration of MSNs in murine

HD model overexpressing NR2B (M. Y. Heng et al., 2009). Moreover, in post-mortem human HD brains it has been detected augmented amounts of glutamate (Storey et al., 1992). Finally, increased activation of NMDA receptors results in modifications of intracellular calcium homeostasis and mitochondrial dysfunctions, leading to neurodegeneration and cell death (Gil & Rego, 2008; R. Rosenstock et al., 2010).

- *Dopaminergic Dysfunction*

  Dopamine (DA) levels are altered in HD patients, indeed dopaminergic pathways from the *substantia nigra pars compacta* (SNc) to the striatum are defective. More specifically, the levels of DA are higher in the early phase of HD, probably favouring hyperkinesia at this stage. On the contrary, in later stages of HD, DA is drastically reduced, which may promote akinesia (Garrett & Soares-da-Silva, 1992; Kish et al., 1987; Weeks et al., 1996; Yohrling IV et al., 2003a). Furthermore, dopamine favours the production of reactive oxygen species (ROS), with the consequent activation of the pro-apoptotic c-Jun N-terminal kinase (JNK)/c-Jun pathway, synergistically with mHTT (Charvin et al., 2005). mHTT is *per se* able to weakly induce JNK pathway (Garcia et al., 2004; Y. F. Liu, 1998; Y. F. Liu et al., 2000; Merienne et al., 2003) in a ROS independent manner; however, DA-induced ROS production enhances this activation (Charvin et al., 2005). Moreover, DA increase toxicity of mHTT, since high level of ROS inhibits autophagocytic degradation of the expanded protein, that in turn accumulates in the cells (Vidoni et al., 2016). Dopamine may also go through to enzymatic and non-enzymatic degradation and therefore be a source of ROS itself (Sulzer & Zecca, 2000). Elevated ROS levels, due to DA, provoke misfolded proteins production and their aggregation, lipoperoxidation of membranes and organelle leakage leading to cell death (Miyazaki & Asanuma, 2009). Finally, DA increases aggregate formation of mHTT in all cellular compartments via D2 receptor. Interestingly, D2 receptors are strongly expressed in MSNs, known as the first striatal neurons affected in HD (Reiner et al., 1988; Yohrling IV et al., 2003b).

- *Mitochondrial Dysfunction and Oxidative Stress*

  Defects in mitochondria have been reported in Huntington's disease due to direct or indirect effect of mHTT. These deregulations are characterized by changes in

glycolysis and increased oxidative stress, but also modifications in the oxygen consumption rate, disruption of mitochondrial membrane potential and alteration in the activity of the enzymatic complexes involved in mitochondrial respiratory chain. Additionally, the mitochondrial transcriptional regulation is altered, accompanied by changes in biogenesis, turnover and degradation by mitophagy (Carmo et al., 2018). Brains and lymphoblasts of HD patients exhibit high level of mitochondrial fragmentation (J. Kim et al., 2010; Panov et al., 2002). Actually, in HD the balance between mitochondrial fission and fusion is shifted to fission; indeed, mHTT has the ability to bind the fission promoter dynamin-related protein 1 (DRP1) stronger than wt HTT, enhancing its activity (W. Song et al., 2011). This disequilibrium contributes to neuronal death as it leads to reduced osmotic homeostasis and mitochondrial mobility, limited energy production, augmented oxidative stress and compromised Ca_2^+ buffering (Knott et al., 2008; Knott & Bossy-Wetzel, 2008).
Moreover, even the total number of mitochondria is reduced in HD brains (J. Kim et al., 2010), as well as the activity of enzymatic complexes and lymphoblasts mitochondrial membrane potential (Browne et al., 1997; Gu et al., 1996; Johri et al., 2013; Naia et al., 2015; Napoli et al., 2013).
Peroxisome proliferator-activated receptor-g coactivator-1 alpha (PGC-1α) is the master regulator of mitochondrial biogenesis and its expression has found to be decreased in cells and HD animal models (Cui et al., 2006a; Johri et al., 2013). Notably, mHTT is able to bind PGC-1α promoter and hinders cAMP response element-binding protein (CREB)/TATA-box binding protein associated factor 4 (TAF4), reducing PGC-1α expression (Cui et al., 2006b). PGC-1α has other functions beyond mitochondrial biogenesis; indeed, it is also involved in adaptive thermogenesis, repression of free radical production, etc... (Johri et al., 2013). As a consequence of that, in HD mice has been documented defective adaptive thermogenesis and high level of ROS (Perluigi et al., 2005; Weydt et al., 2006, 2018).
These mitochondrial dysfunctions lead to a decreased ATP production as observed in HD patient brains and in cellular and animal models of HD (Browne & Beal, 2004; Mattis et al., 2012; Mochel, Durant, et al., 2012; Mochel, N'Guyen, et al., 2012). Moreover, reduced level of glucose metabolism and increased lactate

concentration have been detected in the brains of individuals affected by HD (Antonini et al., 1996; Feigin et al., n.d.; Jenkins et al., 1993; Reynolds et al., 2005). mHTT binds to the mitochondrial outer and inner membranes, leading respectively to calcium emission, that in turn may cause cell death (Choo et al., 2004; Panov et al., 2002), and inhibition of the mitochondrial proteins import (Yablonska et al., 2019; Yano et al., 2014). In conclusion, all these consequences of mitochondrial dysfunction contribute to neuronal death.

- *Altered Autophagy*

  Autophagic dysfunction is a hallmark of HD. The wt HTT is degraded in two ways: ubiquitin-proteasome system or autophagy. Instead, mHTT undergoes post-transcriptional modifications and it becomes a better candidate for autophagy rather than UPS (Martinez-Vicente, 2015). Despite that, the clearance pathways are incapable to degrade the mutated protein once it forms aggregates (Pircs et al., 2018; Weihl, 2013). Additionally, mHTT aggregates sequestrate mammalian target of rapamycin (mTOR), which is a macroautophagy inhibitor, and this leads to an upregulation of macroautophagy (Ravikumar et al., 2004). Nonetheless, the formed autophagosomes are unable to identify their cytosolic cargoes, which are therefore not incorporated in the autophagosomes and consequently not degraded. Despite the high levels of energy consumed by the cells to produce autophagosomes, they fail in the clearance of mHTT aggregates (Martinez-Vicente et al., 2010). Dysregulated macroautophagy induces accumulation of lipofuscin into lysosomes, compromising their function (Terman et al., 2010).

  Wild-type HTT has a scaffolding role in the selective autophagy, stimulating cargo recognition and autophagy initiation. Deregulations of these processes could be due to the polyQ tract of mHTT (Rui et al., 2015). Moreover, even the chaperone-mediated autophagy (CMA) has found to be deregulated in HD; indeed, in younger 111QHtt knock-in mice, CMA is up-regulated, but it declines faster with age in HD mice compared to control mice at the same stage of their life (Koga et al., 2011). Thus, probably to counteract the deregulation of macroautophagy, cells increase CMA, but it fails for more reasons, one could be the loss of CMA efficiency due to age and another is the dysregulation of lysosome caused by lipofuscin accumulation. Therefore, the inefficiency of the entire autophagic system may participate in the HD neuronal death (Cortes & la Spada, 2014; Koga et al., 2011).

- *Decreased Trophic Support*

  Huntingtin is a fundamental regulator of brain-derived neurotrophic factor (BDNF), which modulates the synaptic plasticity, neuronal survival and differentiation (Anthony Altar et al., 1997; Baquet et al., 2004; Zuccato et al., 2001). HTT favours the vesicular anterograde and retrograde transport of BDNF along microtubules (MTs) (Gauthier et al., 2004). Instead, mHTT compromises BDNF transport along axonal microtubules, blocking the interaction between key components of the motor machinery complex to MTs, leading to a reduction in neurotrophic support and to neurotoxicity (Gauthier et al., 2004). Accordingly, in zQ175 knock-in HD mice, BDNF-containing vesicles move slower and travel shorter distances (C. Yu et al., 2018). Moreover, in these HD mice it was observed a reduced release of BDNF in cortical neurons (C. Yu et al., 2018).

  In several HD mouse models, it has been shown a decrease in mRNA and protein levels of BDNF (Duan et al., 2003; Gines et al., 2003; Giralt et al., 2011; Jin et al., 2015; Pang et al., 2006; Peng et al., 2008; Spires et al., 2004; Suelves et al., 2019; Zuccato et al., 2001). In parallel, a downregulation of BDNF gene transcription and protein levels has been detected also in HD patients (Ciammola et al., 2007; Krzysztoń-Russjan et al., 2013). Particularly, in these individuals a decrease in BDNF protein levels was observed in different parts of the brain such as striatum (Ferrer et al., 2000; Gauthier et al., 2004; Seo et al., 2004), cerebral cortex (Seo et al., 2004; Zuccato et al., 2001, 2008), cerebellum and *substantia nigra* (Seo et al., 2004). Notably, these variations are characteristic of the early phase of the disease, indicating a premature failure of trophic regulations contributing to neurodegeneration (Seo et al., 2004). Interestingly, HD individuals have low levels of BDNF in the serum, which are inversely correlated with a longer CAG repeat length and therefore the severity of the disease (Ciammola et al., 2007; Krzysztoń-Russjan et al., 2013; Squitieri et al., 2009).

- *Peripheral Pathology*

  Considering that wild-type HTT is ubiquitously expressed, it is not surprising that mHTT affects all cells and tissue of the body, not only the central nervous system (S. H. Li et al., 1993; van der Burg et al., 2009). Particularly, mHTT alters several tissues such as skeletal muscle, cardiovascular system, blood, skeleton, genital

system, digestive tract and immune system (van der Burg et al., 2009) (Fig. 8). Regarding muscular system, it has been shown that HD patients and HD mice exhibit skeletal muscle atrophy (Farrer & Meaney, 1985; Farrer & Pao-Io Yu, 1985; Ribchester et al., 2004; Trejo et al., 2004); additionally, HD mice show cardiac muscle atrophy too (Mihm et al., 2007). Nevertheless, heart failure is the second cause of death in HD patients after pneumonia (Lanska et al., 1988). Muscle atrophy affects also the important weight loss observed in patients at the last stage of HD (Goodman et al., 2008), as a matter of fact patients with elevated body weight exhibit slower disease progression (Myers et al., 1991).

The immune system is dysfunctional in HD, indeed high levels of proinflammatory cytokines and chemokines were detected in peripheral blood of patients (Björkqvist et al., 2008; Wild et al., 2011).

Genital system is also impacted, since testes have the highest expression of HTT after brain (S. H. Li et al., 1993; Strong et al., 1993). Despite fertility in HD is not impacted, testosterone levels and numbers of germ cells are decreased in HD patients that also experience an abnormal seminiferous tubule morphology (Markianos et al., 2005; van Raamsdonk et al., 2007). In addition, in HD mouse models it was observed testicular atrophy (van der Burg et al., 2009).

Another peripheral pathology observed in HD patients is osteoporosis and the severity is correlated with the number of CAG repeats. It was observed a decreased bone mineral density although the cause is not yet known, this could be due to the neuroleptic treatment used in the patients or directly to HD itself (van der Burg et al., 2009).

Interestingly, it has been observed that skin fibroblasts from HD patients exhibit reduced cell growth, without observable alterations in the cell cycle (Jędrak et al., 2018). Furthermore, these HD fibroblasts show a reduction in ATP levels concomitant with a decrease in mitochondrial metabolic activity, in addition to an increase in mitochondrial superoxide and the related increase in antioxidant enzymes as a possible defence mechanism. Another study performed on Juvenile HD skin fibroblasts, showed reduced mitosis and proliferations in these peripheral cells accompanied by elevated ROS and increased mitochondrial membrane potential, in parallel with increased proteasomal activity probably to increase cell survival and counteract mHTT (Aladdin et al., 2019). Dysregulation of mitochondria bioenergetics, increased oxidative stress and gene alterations in HD skin fibroblasts

have been observed in multiple studies (del Hoyo et al., 2006; Gardiner et al., 2018; Marchina et al., 2014a).

Thus, all these data underline the importance to study the effect of mHTT in other tissues and not only in nervous system.

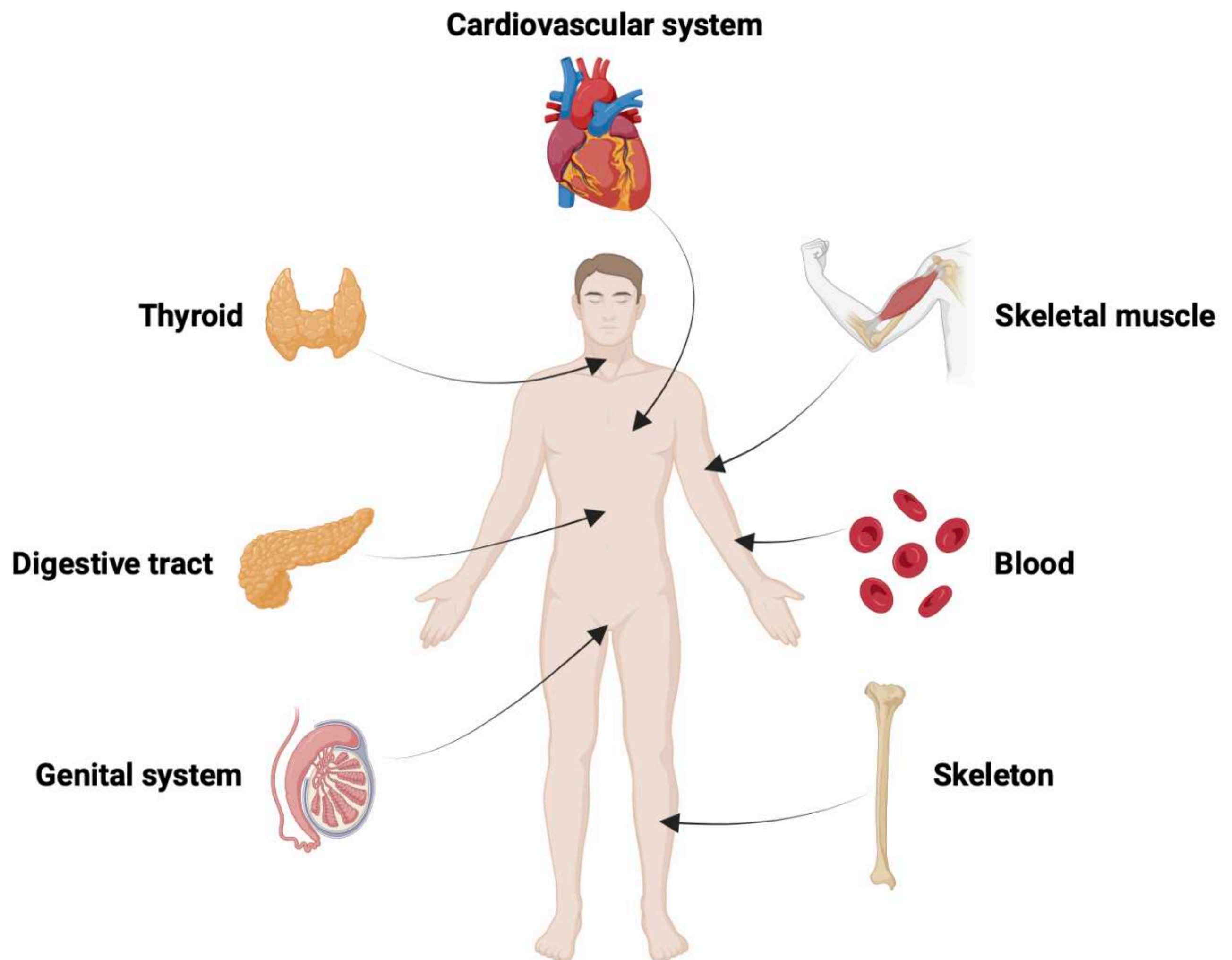


Figure 8. Graphic representation of peripheral organs and tissues altered by mHTT, where issues were observed in HD patients in addition to the nervous system. See text for detail. Image created with BioRender.com.

- *Genetic modulators*

  Recently, it has emerged that several genes influence HTT expression and age at onset, these genes are named HD genetic modifiers (Tabrizi et al., 2020). A genome-wide association study (GWAS) conducted on 4 082 HD patients discovered two

loci on chromosome 8 and 15 associated with AO (J. M. Lee et al., 2015). Potential modulator genes found in these two loci are: *FAN1*, *MTMR10*, the pseudogene *HERC2P10* on the chromosome 15, and *RRM2B* and *UBR5* on the chromosome 8; all linked to DNA repair pathways (Holmans et al., 2017; Jain & Chen-Plotkin, 2018). One example is the DNA repair enzyme FAN1, which overexpression delays AO and slows down progression of the disease; on the other hand, the knockout or RNA interference (RNAi) of *FAN1* raises the number of CAG repeats (Goold et al., 2019). These data suggest a new role of FAN1 in the protection against CAG instability. In fact, this emerging function of FAN1 is supported by another study carried on fragile X syndrome, characterized by CGG repeats, in which the knockout of FAN1 in mouse model expands the number of somatic repeats (X. N. Zhao & Usdin, 2018).

Following studies pointed out other genetic modified genes located on a chromosome 5 locus; they are all associated with DNA repair, transcription and splicing (Flower et al., 2019; J. M. Lee et al., 2019; Moss et al., 2017; Tabrizi et al., 2020). Thus, all these data underline that DNA repair activity has a pivotal role in HD pathogenesis, as variations in all the genetic modifiers affect repeat instability, the age of onset as well as the severity of the disease.

1.2.5 Clinical features

The major clinical features, characteristic of Huntington's disease, are a consequence of neurodegeneration and include motor impairment, cognitive and neuropsychiatric disturbances (McColgan & Tabrizi, 2018). Age at onset is around 35-45 years and the disease lasts 16-18 years, until the patients die, which is mainly caused by infections in the lungs. The severity with which patients display these symptoms varies from person to person and although a full clinical diagnosis is made following unexplained extrapyramidal movements, cognitive and psychiatric impairments appear years earlier than motor disturbances.

Movements impairments in HD are mainly characterised by a deleterious escalation in involuntary movements and an ever-increasing obstruction of voluntary movements (Ross et al., 2014). In the initial phase of the pathology, individuals show hyperkinetic symptoms, of which the main one is chorea (Dorsey et al., 2013). Conversely, the advanced stage of the disease is characterized by hypokinetic movement impairments, including bradykinesia, dystonia, rigidity, balance and gait disturbance. These

hypokinetic movements are associated with the duration of the disease and with the number of CAG repeats, as opposed to chorea (Rosenblatt et al., 2006). Moreover, difficulties can be observed when speaking and/or swallowing, which increase the risk of choking.

Patients with Huntington's disease also display a variety of cognitive and neuropsychiatric symptoms, the most common of which are apathy, irritability, anxiety, violence, nervousness, obsessive-compulsive disorder, and psychosis (in the final stage of the disease) (Andhale & Shrivastava, 2022; McColgan & Tabrizi, 2018). Interestingly, apathy is the only neuropsychiatric symptom correlated with the disease phase (Tabrizi et al., 2013). Furthermore, HD patients may exhibit impatience, guilt, fear, low self-confidence, but above all depression. Indeed, the second most frequent cause of death in HD patients is suicide. Moreover, difficulty in thinking, impaired sleep or daytime sleepiness are observed in patients (Glidden et al., 2020). In conclusion, given all the different symptoms mentioned above, the impact of HD on patients' quality of life is devastating and will lead individuals to need 24-hour care.

1.2.6 Treatments

Nowadays, the only treatments available for Huntington's disease are symptomatic therapy, focused to ameliorate as much as possible the quality of life of the patients, acting on motor, psychiatric and behavioural impairments (A. Kim et al., 2021). Some of these treatments acting on motor symptoms include: decreasing DA neurotransmission to counteract chorea; DA modulators and DA inhibitors, anti-glutamatergic drugs to improve hyperkinesia. Regarding hypokinesia, there are few and not always effective treatments, which are mainly DA agonists (A. Kim et al., 2021). The currently available treatments for non-motor symptoms target some of the cognitive and behavioural symptoms observed, such as depression, but for example there are no therapies to counteract apathy or dementia (A. Kim et al., 2021).

As yet, no disease-modifying treatment has been successful, despite the promising results obtained in preclinical studies (Estévez-Fraga et al., 2016). Probably the reason why all these trials have failed is because mHTT leads to several deregulations that form intricate and complex interconnections, and it is difficult to completely resolve a single pathway or acting on only one is not enough to improve the disease course. However, several strategies appear to hold great promise (A. Kim et al., 2021; Tabrizi et al., 2020). One strategy is called "HTT lowering" and aims to reduce mHTT expression; this

category includes RNAi (Ahmadzada et al., 2018; Ha & Kim, 2014; Setten et al., 2019), allele-specific oligonucleotides (ASOs) (Bennett & Swayze, 2010) and small molecule modulators of RNA processing. Another very promising strategy, which could give long-term effects, resolve all pathogenic mechanisms generated by mHTT and even prevent the disease from being genetically transmitted to offspring is DNA-targeting approach. In fact, these strategies are designed to solve the problem upstream by directly modifying the HTT gene sequence or its transcription; and the three most promising approaches are: zinc-finger nucleases (ZFNs) (Klug, 2010), transcription activator-like effector nucleases (TALENs) (Nemudryi et al., 2014) and Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (Adli, 2018). In addition, transplants of human fetal striatal tissue and stem cells transplants are also hopeful (Bachoud-Lévi, 2017; Cicchetti et al., 2009; Freeman et al., 2000). All these approaches and different strategies are very promising, but for the time being they still have problems with off-target effects, side effects, administration and delivery of the molecule to the target site. Therefore, they need further studies and tests before they can be used on HD patients (A. Kim et al., 2021; Tabrizi et al., 2020). Indeed, many of these strategies and others are now at the stage of clinical trials and pre-clinical trials. The current studies in clinical trial are: dopaminergic modulation, glutamatergic modulation, synaptic modulation, BDNF levels regulation, mitochondrial function and biogenesis activation, aggregate inhibition, antibody therapy, genetic manipulations, dietary supplementation, combined pharmacological therapies, stem cell therapies, deep brain stimulation (DBS) and physical activity (A. Kim et al., 2021). In addition, pre-clinical phase studies are: up-regulation of BDNF levels, modulation of other neurotrophic factors [glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CTNF), VEGF, insulin-like growth factor-1 (IGF-1)], autophagy regulation, epigenetic modulators [sirtuins, histone deacetylase (HDAC) inhibitors, lysine deacetylase (KDAC) inhibitors], nanotechnology and nanoparticles, stem cell treatment and genetic manipulations. All these approaches (available therapies, pre-clinical and clinical studies) are well reviewed in detail by Kim and colleagues (A. Kim et al., 2021). In conclusion, for the time being HD patients can only receive symptomatic and not always very effective treatments, but as seen, all these studies are very encouraging and promising and will lead, together with the discovery of new therapeutic approaches, to the cure of this devastating disease in the future.

1.3 Hippo pathway and Huntington's Disease

1.3.1 Deregulations of Hippo Pathway in Huntington's Disease

As seen above, the crucial role that the Hippo pathway plays in neurodegenerative diseases has recently emerged. An important case in point is Huntington's disease, indeed recent studies have revealed significant alterations in HP in several HD models (Sahu & Mondal, 2020; Sileo et al., 2022) (Fig. 9). Particularly, it was found that in both post-mortem human cortex and neural stem cells (NSCs) derived from HD patients, the presence of YAP in the nucleus is significantly lower than in controls (Mueller et al., 2018). The same authors did not observe a change in *YAP* transcription in HD, pointing out that the observed decrease is probably due to a mechanism at the post-transcriptional level, such as phosphorylation. Consistently, in HD post-mortem human cortex, the phosphorylated form of YAP (pYAP) is significantly increased, although total YAP levels remain unchanged. At the same time, the phosphorylated and active form of MST1/2 (pMST1/2) are also increased, again without increasing total levels, providing a consistent explanation for the increased phosphorylation of YAP and the consequent decrease in nuclear localization found in HD patient brains. In parallel, the authors also obtained comparable results in a murine model of HD, Htt CAG knock-in mice $Hdh^{Q111/Q111}$, in which both phospho-YAP and phospho-MST1/2 were increased compared to the $Hdh^{Q7/Q7}$ control. These deregulations probably lead to the decreased YAP/TEAD interaction observed in HD post-mortem human cortex compared to controls, suggesting a consequent alteration in gene expression. In accordance with this, *Cyr61*, a HP target gene, is downregulated in HD cortex. In addition, other HP genes are also deregulated: *LATS2*, *MEIS1* and *SAV1* (Mueller et al., 2018). These results mirror those obtained in another study, where it was already observed that nuclear YAP was decreased in human HD patient brains. Furthermore, in these post-mortem HD brains and in mutant Htt-KI mice, phospho-LATS1 was increased compared to controls (Yamanishi et al., 2017). Remarkably, these authors confirmed previous findings (Hoshino et al., 2006; Mao et al., 2016), observing that in HD human brains and in mouse HD model brains there is a particular form of necrotic cell death named TRIAD. This atypical necrosis shows several phases: (i) the destruction of the cistern structures of ER, (ii) the enlargement and vacuolization of ER, (iii) the detachment of the outer nuclear membrane from the inner membrane, (iv) the release of ER content into the cytosol, (v) the ballooning of the cell body, the shrinkage of the nucleus, and enucleation

(Mao et al., 2016; Yamanishi et al., 2017). Interestingly, TRIAD is TEAD/YAP-dependent and mHTT exacerbates this necrosis. Indeed, knockdown of YAP induces cell death, whereas knockdown of *LATS1/2* repress it (Mao et al., 2016). This leads to the hypothesis that either nuclear YAP induces TRIAD repression, or it is the lack of YAP in the nucleus that promotes this necrosis. As proof of this, LPA and S1P, which as previously seen are ligands of GPCR receptors and activate YAP, repress cell death. On the other hand, inactivating YAP by induction of HP, acting on GPCR receptors, leads to increased cell death. Furthermore, *TEAD1* knockdown also increases cell death. Intriguingly, it was observed that polo-like kinase 1 (Plk1), a serine/threonine-protein kinase which promote apoptosis, is able to phosphorylate YAP at T77, thereby decreasing its interaction with TEAD, as this phosphorylation leads to increased binding of YAP to p73. Thus, phosphorylation of YAP by Plk1 determines its partner from TEAD to p73, while its phosphorylation by LATS inhibits interaction with both of these partners. Interestingly, HTT seems to play a role in this regulation, as huntingtin is able to bind YAP with an affinity proportional to the length of the polyQ tract. Surprisingly, it was observed that YAP is present in HTT aggregates in HD mouse models. These results led to the idea that mHTT may inhibit YAP/TEAD-mediated transcription by interacting with YAP, and thus acting on HP could result in a decrease or inhibition of TRIAD. Indeed, it has been shown that treating htt-KI mice with LPA and S1P leads to a suppression of ER instability and cell death simultaneously with motor functions amelioration in these mice (Mao et al., 2016; Yamanishi et al., 2017). Finally, in transgenic HD *Drosophila*, it was observed that overexpression of Yorkie improves polyQ-mediated toxicity in eye imaginal disks and recovers visual functions. Intriguingly, the positive effect due to YAP overexpression appears to be regulated by several mechanisms such as cell cycle activation, regulation of immune deficiency, and modulating Toll pathway (a pathway that regulates humoral immunity to counter bacterial and fungal infections) (Dubey & Tapadia, 2018).

All these data showed a close connection between the Hippo pathway and Huntington's disease, paving the way for a new therapeutic approach for this devastating neurodegenerative disorder.

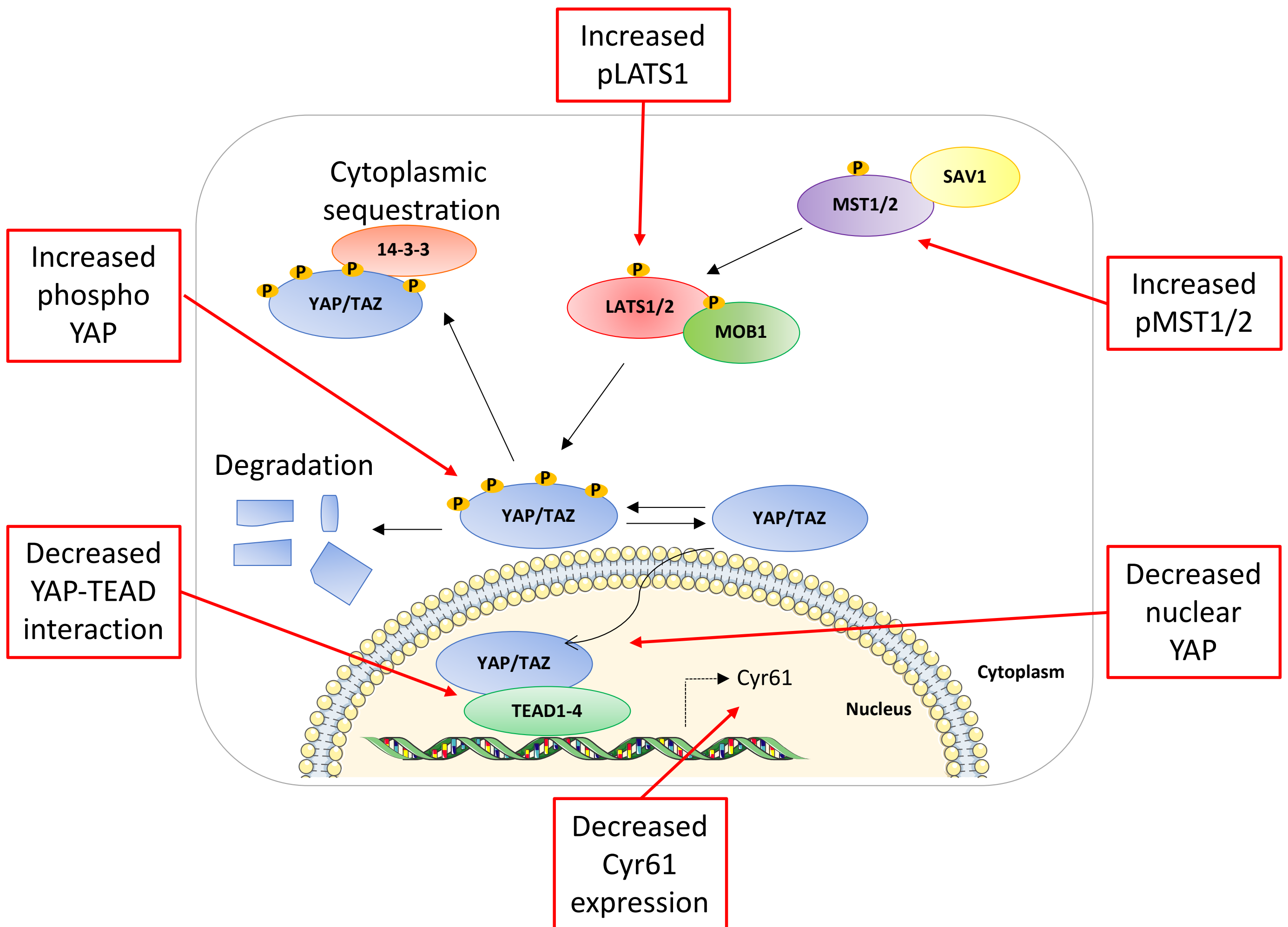


Figure 9. Schematic representation of Hippo pathway deregulations observed in HD models and post-mortem HD brains. See text for detail. Figure adapted from Sileo et al., 2022.

1.3.2 Pharmacological Activation of YAP/TAZ-TEAD Interaction

There are two main strategies for activating the YAP/TAZ-TEAD interaction and they consist of either decreasing the phosphorylation of YAP/TAZ, thereby favouring their nuclear localisation, or increasing their interaction via TEAD ligands (Sileo et al., 2022). Thanks to a study on the phosphorylation of YAP/TAZ, conducted by over-expressing different members of GPCRs, the first activators and inhibitors of YAP/TAZ activity were discovered (F. X. Yu et al., 2012). The most useful inhibitors of YAP/TAZ phosphorylation are LPA and S1P (Fig. 10) which, as mentioned above, promote translocation of YAP/TAZ into the nucleus and its consequent TEAD binding, activation of target genes and inhibition of cell death (Mao et al., 2016).

The second class of YAP/TAZ phosphorylation inhibitors are MST1/2 or LATS1/2 inhibitors. As they bind to those kinases in their ATP pocket, these inhibitors have moderate selectivity and side-effects are due to inhibition of other kinases. To better understand the potency of these kinase inhibitors and to have a good yardstick, the experimental ATP concentration will be mentioned after each IC_{50}. This gives a clear idea of the potency of these compounds by elucidating the difference between enzyme inhibition and cellular activity, considering that the normal cellular ATP concentration is 1-10 mM. Otherwise, a nanomolar IC_{50} measured on the purified enzyme at low ATP concentration has no relevance at cellular level.
A high-throughput screening conducted on an in-house compound library led to the discovery of a molecule, called XMU-MP1 (Fig. 10), capable of inhibiting MST1/2 (Fan et al., 2016). This compound is a competitor of ATP and is capable of inhibiting both MST kinases, with an IC_{50} of 164 nM for MST1 and 34 nM for MST2 (with 10 μM ATP). In HepG2 cells, XMU-MP1, increases the amount of YAP in the nucleus without altering its total levels at micromolar concentration. Furthermore, it enhances the transcription of HP target genes *Cyr61* and *CTGF* by twofold at 1 μM concentration and fourfold at 3 μM. Moreover, it was observed that this molecule, following intraperitoneal injection in mice at concentrations of 1 to 3 mg/kg, is able to increase intestinal repair, liver repair, and regeneration in acute or chronic liver injury. Finally, it was shown that XMU-MP1 can also recover astrocyte senescence and cognitive functions (X. Xu et al., 2021), stimulate glial scar formation and improve demyelination and inflammatory infiltration (Xie et al., 2020; J. Zhang et al., 2021).
Other potential pharmacological targets of the Hippo pathway are LATS1/2, and inhibitors of these kinases already exist. TRULI (Fig. 10) is one of these LATS1/2 inhibitors, and is able to inhibit both kinases with an IC_{50} of 0.2 nM (with 10 μM ATP) (N. Kastan et al., 2021). In utricles cells from mice, this molecule is able to increase nuclear translocation of YAP, reduce its phosphorylation and induce cell proliferation, after 24 h of treatment at a concentration of 10 μM. With the same treatment time and at the same concentration, TRULI also reduces phosphorylation of YAP (at S127, the LATS phosphorylation site) in MCF 10A mammary epithelial cells. Furthermore, in late embryonic (E17.5) utrical supporting cells, it induces up-regulation of cell cycle-related genes, especially those related to S and G2/M phases. On the other hand, it induces down-regulation of pro-apoptotic and inflammatory genes. Interestingly, TRULI

reduces phosphorylation of YAP and induces proliferation in cardiomyocytes, as well as increasing proliferation of Müller cells in organoids (N. Kastan et al., 2021). Very recently, TRULI was tested in HD human neurulation model to prove that LATS represents a promising druggable target in Huntington's disease (F. M. Piccolo et al., 2022). Interestingly, it has recently been observed that the Hippo pathway is one of the most deregulated pathways in systemic lupus erythematosus keratinocytes, leading to a decrease in nuclear YAP and the subsequent increased UV-mediated apoptosis (Hile et al., 2023). Inhibition of the kinase activity of LATS1/2 (and thus phosphorylation of YAP) by TRULI treatment led to decreased apoptosis at baseline and a decrease in apoptosis following UVB exposure (Hile et al., 2023). Finally, the authors who discovered TRULI are already chemically modifying it to produce a new generation of more effective LATS inhibitors (TDI-011536, Fig. 10) (N. R. Kastan et al., 2022). Another LATS inhibitor, VT02956 (Fig. 10), emerged from a high-throughput screening of 17 000 compounds with the aim of identifying potential new molecules for the treatment of breast cancer. *In vitro* studies have shown that this compound inhibits LATS1/2, with an IC_{50} of 0.76 nM for LATS1 (400 μM ATP) and 0.52 nM for LATS2 (6000 μM ATP) (S. Ma et al., 2022). Further high-throughput screening to identify small molecules that induce cell proliferation uncovered another LATS inhibitor: GA-017 (Aihara et al., 2022). This molecule inhibits LATS1 and LATS2 with an IC_{50} of 4.1 nM and 3.9 nM (400 μM ATP) respectively. Treatment with GA-017 at 5-20 μM leads to an increase in nuclear YAP and the subsequent increase in the expression of the target genes *Cyr61*, *CTGF* and *Ankrd1*, inducing the formation of SKOV3 (ovarian adenocarcinoma) and A431 (squamous carcinoma) spheroids and mouse intestinal organoids.

A recent study showed a new compound capable of increasing YAP/TEAD interaction using HEK293A-TEAD-luc cells. This molecule, called PY-60, releases annexin A2 from membranes, thereby inhibiting the phosphorylation of YAP (Shalhout et al., 2021). Through biolayer inferometry, a micromolar affinity for annexin A2 (ANXA2) was observed, and the activation of the target genes *CTGF*, *Ankrd1* and *Cyr61* at 1-10 μM was also seen. Thanks to this study, annexin A2 emerged as a potential component of the Hippo pathway, further characterising its complex regulation.

Small TEAD ligands that stimulate YAP/TAZ-TEAD binding have recently been discovered and are Q2 and B22 (Pobbati et al., 2019). Q2 leads to increased expression

of the target genes *CTGF*, *Cyr61* and *Ankrd1*, as well as accelerating *in vivo* cutaneous wound healing in mice. Another small molecule that increases YAP-TEAD binding is eicosapeptide 4E, which is a VGLL4 inhibitor and leads to increased expression of CTGF, Cyr61, Ankrd1 and SEPINE1 in human cardiomyocytes and also accelerates wound healing (Adihou et al., 2020).

TEADs are S-palmitoylated at a conserved cysteine residue situated at the entry of a hydrophobic pocket of the C-terminal pocket. These palmitoylation is considered to be responsible of the TEAD stability and favour YAP/TAZ interaction with the C-terminal domain of TEADs (Liberelle et al., 2022). It has been recently reported that depalmitoylase such as APT2 are responsible of TEAD depalmitoylation (N. G. Kim & Gumbiner, 2019a). An inhibitor of APT2 such as ML-349 could therefore be considered as an activator of YAP/TAZ-TEAD interactions (Won et al., 2016).

As seen, several compounds and numerous strategies have recently emerged to target different components of the Hippo pathway, activate YAP/TAZ-TEAD binding and lead to the activation of specific target genes. These molecules could represent potential new pharmacological therapeutic approaches for the treatment of diseases where these elements are downregulated.

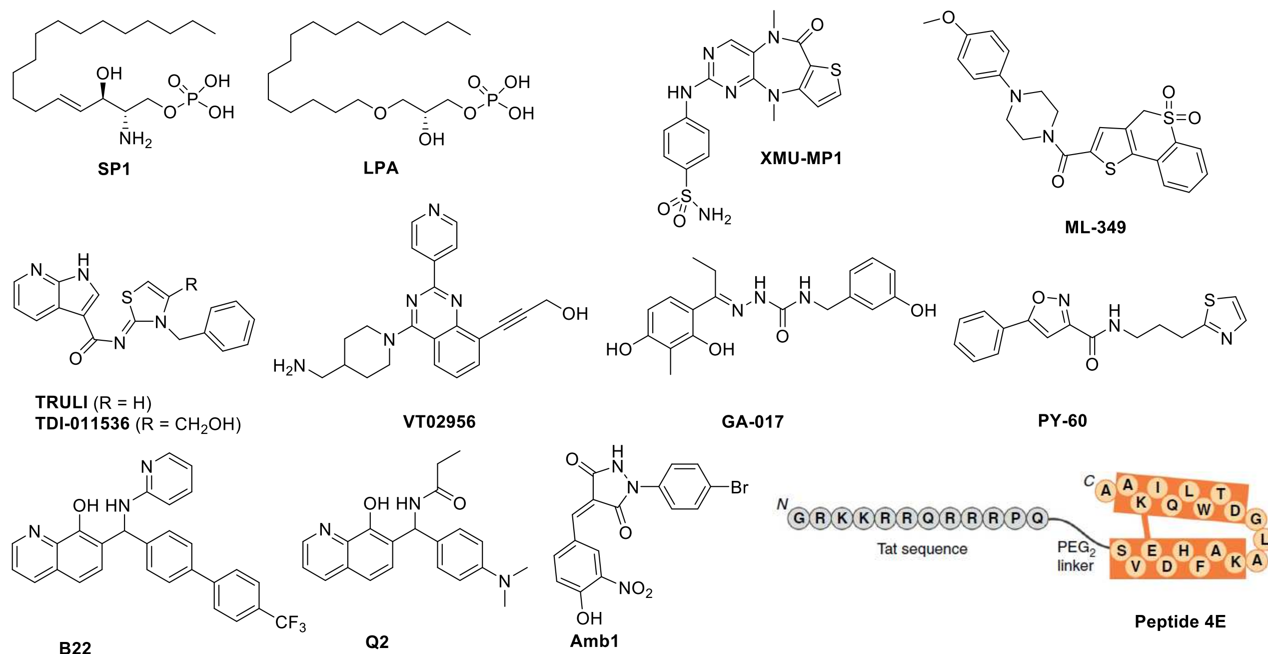


Figure 10. Structures of the molecules able to increase YAP/TAZ-TEAD interaction.

2. Objectives

In the last decades, researches have been mostly focused on the pro-oncogenic role of YAP/TAZ-TEAD in tumorigenesis and cancer (Zanconato et al., 2016). Hovewer, very recently, it is emerged that YAP/TAZ can play also an anti-oncogenic role and increasing their activity could have a beneficial role in this type of cancer (X. Guo et al., 2017; S. Ma et al., 2022; Moroishi et al., 2016; Pearson & Bremner, 2021; Rudin et al., 2019). Furthermore, the important role that the Hippo pathway plays in the context of neurodegenerative diseases has become increasingly evident in the recent years; indeed, the involvement of HP in numerous neurodegenerative disorders has been discovered and reviewed (Ahn et al., 2020; Jin et al., 2020; Sahu & Mondal, 2020; Sileo et al., 2022; Tanaka et al., 2020). In particular, HP appears to play a pivotal role in Huntington's disease as evidenced by recent findings, as several deregulations of HP actors and decreased YAP/TAZ-TEAD activity have been identified in HD (Mao et al., 2016; Mueller et al., 2018; Yamanishi et al., 2017). Indeed, these studies showed an increased activity of Hippo pathway with the consequently decrease in YAP nuclear localisation and YAP/TEAD binding. These deregulations lead to increased cell death in HD mouse models and post-mortem HD cortex. Additionally, Mao et colleagues showed that inhibition of HP suppresses cell death in HD mice cerebral cortex and improves motor function of HD model mice (Mao et al., 2016). These data suggest that targeting HP could represent a new therapeutic approach. Furthermore, reduced YAP levels via Hippo/MST1/LATS and the resulting increase in cell death were also shown in Parkinson's disease post-mortem brain and in PD mice models (Ahn et al., 2020). Moreover, increasing YAP/TAZ-TEAD activity could have a positive effect in increasing regeneration and wound healing (Cai et al., 2010; Del Re et al., 2013; Heallen et al., 2013; M. J. Lee et al., 2014; Misra & Irvine, 2018; Shome et al., 2020; Walko et al., 2017). In fact, it was shown that YAP/TAZ are involved in skin wound healing and in dermal fibroblasts YAP activation promotes this process (M. J. Lee et al., 2014; Shome et al., 2020)

Recently, several molecules capable of activating and increasing the YAP/TAZ-TEAD interaction have been discovered and developed. These different drugs act on different targets and at different steps of the Hippo pathway. There are compounds that act more upstream while others act downstream by targeting different players of the Hippo pathway. For instance, there are molecules that act upstream on GPCRs such as LPA and S1P (Mao et al., 2016). Other compounds act on the kinase cascade such as XMU-MP1 which is an

MST1/2 inhibitor and TRULI, VT02956 and GA-017 which are LATS1/2 inhibitors (Aihara et al., 2022; Fan et al., 2016; Kastan et al., 2021; Ma et al., 2022). Finally, molecules that act further downstream directly on YAP/TAZ-TEAD binding are Q2, B22 and 4E peptide (Adihou et al., 2020; Pobbati et al., 2019). In addition, new strategies are emerging to activate and increase YAP/TAZ-TEAD binding such as ML349, which acts on TEAD depalmitoylation (Kim & Gumbiner, 2019; Vujic et al., 2016), and PY60 involving annexin A2 (Shalhout et al., 2021), further expanding the understanding of the complex regulation of HP.

Therefore, the objective of this thesis is to compare different activators of YAP/TAZ and TEAD, select the best molecule in increasing their activity and test it in different cellular models by analysing its effect on cell growth, proliferation and its impact on RNA and protein levels of YAP/TAZ-TEAD targets. The strategy is divided into three parts:

1. The first part is dedicated to select the most promising molecules (available at the beginning of this study) in activating YAP/TAZ-TEAD binding by screening different compounds. Selection was conducted in HEK293T cells by luciferase assay dose response, using a reporter which is a YAP/TAZ-responsive synthetic promoter driving luciferase expression.

2. The second part is focused on the validation of the effect of these compounds on cell growth, RNA and protein expression of YAP/TAZ-TEAD targets in HEK293T cells and in small molecule neuronal precursor cells (smNPCs). We analysed, following treatment with the most promising molecule, the RNA and protein expression of the two classical HP target genes *CTGF* and *Cyr61* by RTqPCR and Western blot, respectively. We measured the cell growth of the two cell lines by Incucyte.

3. Finally, in the third part, we analysed fibroblasts from HD patients, which as reported in the literature have reduced proliferation and mitosis compared to fibroblasts from healthy people (Aladdin et al., 2019; Jędrak et al., 2018). In these cells, we measured cell growth, proliferation and expression of several HP target genes at basal level by comparing healthy and HD fibroblasts with Incucyte, BrdU assay and RTqPCR, respectively, to verify that HP is deregulated. We then evaluated the effect of the previously selected compound also in fibroblasts by analysing cell growth, proliferation and expression of HP target genes in samples taken from healthy individuals.

3. Material and Methods

3.1 Models

3.1.1 Cellular models

Three cell models were used for the different experiments: HEK293T, smNPCs and human fibroblasts. Luciferase assay experiments were performed on HEK293T cells (single dose was applied for six activators of YAP/TAZ-TEAD interaction and one inhibitor, dose-response curves were measured for five activators). The HEK293Ts and small molecule neuronal precursor cells (smNPCs), thanks to the collaboration with Pr. Rejko Krüger from University of Luxembourg, were used for cell growth assay experiments, RTqPCR and Western blot. Human Fibroblasts were obtained according to the French ethics regulations (Lille Ethics Committee, Protocole Convergence, CPP/2008/009). These cells were used for cell growth experiments, BrdU assay and quantification of gene expression by RTqPCR.

- HEK293Ts were grown in Dulbecco's modified Eagle's medium containing high glucose 4500 mg/L (ThermoFisher, USA, Réf. 11965092) and 10% fetal bovine serum (FBS), HEPES 25 mM (Life technologies, Réf. 15630056), Penicillin-Streptomycin 10000 U/mL (ThermoFisher, USA, Réf. 15140122) at 37°C, in a humid atmosphere containging 5% CO_2 and maintained in T75 flasks.
- smNPCs were grown in basal medium N2B27 with the addition of three factors: Ascorbic acid (Sigma), CHIR 99021 (Axon) and Purmorphamine (PMA) (Sigma-Aldrich) at 37°C, in a humid atmosphere containging 5% CO_2 and maintained in T75 flasks, pre-coated with Geltrex (basement membrane matrix containing growth factors, Fisher Scientific). The basal medium is composed of 50% DMEM-F12 (Life/Technologies, USA) and 50% NeuroBasal medium (Life technologies, USA) containing GlutaMAX Supplement (Life technologies, USA), Penicillin-Streptomycin 20U/mL (ThermoFisher, USA), B27 Supplement (minus vitamin A) (Life technologies, USA), N2 Supplement (100x) (Life Technologies). smNPC culture medium needs to be freshly prepared and warmed at 37°C and it is composed with basal medium plus Ascorbic acid (Stock solution as 300nM, use 1:2000), 3µM CHIR (Stock solution as 6mM, use 1:2000) and 0.5µM Purmorphamine (Stock solution as 1mM, use 1:2000).
- Fibroblasts were grown in Dulbecco's modified Eagle's medium containing high glucose 4500 mg/L (ThermoFisher, USA) and 15% fetal bovine serum, HEPES

25mM (Life technologies, Réf. 15630056), Penicillin-Streptomycin 10000 U/mL (ThermoFisher, USA, Réf. 15140122) at 37°C in a humid atmosphere containging 5% CO_2 and maintained in T75 flasks.

3.1.2 Drug Treatments

Amb1 was purchased by Ambinter c/o Greenpharma (Orléans, France), TRULI and Dasatinib were purchased by Seleckchem (Souffelweyersheim, France), XMU-MP1 was purchased by CliniScience (Nanterre, France), ML-349 and Q2 were purchased by Sigma-Aldrich (Saint-Quentin-Fallavier, France). All the drugs were dissolved in DMSO at 10 mM.

3.1.3 Cell Transfection

HEK293Ts were co-transfected with TEAD-Luc reporter (8xGTIIC-luciferase) and pCMV-βGal vector (for details see chapter 3.2.1). Transfection was performed with Lipofectamine 2000 according to manufacturer instructions (Thermo Fisher Scientific, Réf. 11668019). After adding Lipofectamine 2000 to Optimem medium and incubating for 5 minutes at room temperature (RT), a mix consisting of the two vectors and Optimem was added to Lipofectamine and incubated 30 minutes at RT. For TEAD-Luc reporter and pCMV-βGal vector, 100 ng and 10 ng were used, respectively. The control, on the other hand, was only transfected with 30 ng pcDNA. After the 30-minute incubation, the mix consisting of Lipofectamine 2000 and DNA was added to the cells, and placed in the incubator 6 h at 37°C 5% CO2, before changing the culture medium.

3.1.4 Cell fixing

One day after the treatment with TRULI (5 µM and 10 µM) or DMSO for control and Bromodeoxyuridine (BrdU) in DMEM Medium 0.2% FBS, the medium was discarded and fibroblasts were rinsed 3 times with PBS 1X (Invitrogen). After the washing, Paraformaldehyde (PFA) 4% (Sigma-Aldrich) was added to the cells and incubate for 15 minutes in the dark at RT. The incubation with PFA was following by 3 washes with PBS 1X. Fixed cells were stored at 4°C in PBS Azide 0.02% and covered with parafilm.

3.2 Molecular Biology

3.2.1 Luciferase assay – Dose response

Luciferase assay was made after the co-transfection of HEK293T with pCMV-βGal vector and TEAD-Luc reporter (8xGTIIC-luciferase) (addgene, Réf. Plasmid #34615). The YAP/TAZ-responsive synthetic promoter driving luciferase expression possess synthetic TEAD-binding sites upstream of the luciferase gene. The cells were treated for 18 h with the molecules in DMEM Medium 0.2% FBS at the concentration: DMSO (control) 10 µM, XMU-MP1 3 µM, TRULI 1 µM, Amb1 5 µM, Q2 10 µM, B22 10 µM, ML349 1 µM and Dasatinib 1 µM. After 18 h the cells were lysed and were analysed with the Varioskan in combination with Neolite (Neolite Reporter Gene Assay System, 1000 Assay Points; Perkin Elmer, Réf. 6016711) to measure the luminescence. The signal of β-galactosidase (absorbance at 420 nm) was used as an internal control of the transfection. The luciferase signal of each sample was then normalized to the β-galactosidase signal. Dose response assay was done for: TRULI, ML349, Amb1, Q2 and XMU-MP1 at different concentrations:

-TRULI: 50 nM, 100 nM, 500 nM, 1 µM, 2.5 µM, 5 µM and 10 µM;

-ML349: 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 1 µM and 5 µM;

-Amb1: 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 1 µM and 5 µM;

-Q2: 25 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM and 10 µM;

-XMU-MP1: 25 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM and 10 µM.

3.2.2 RTqPCR

- *RNA Extraction*

  One day after the treatment with TRULI (5 µM and 10 µM) and DMSO for control, in DMEM Medium 0.2% FBS, cells were lysed in RLT buffer (QIAGEN, Réf. 79216) and the RNA extraction was made with the “NucleoSpin RNA” (Macherey-Nagel, Réf. 740955.50) according to manufacturer instructions. Following this procedure, a purification-amplification step is performed using the “NucleoSpin RNA Clean-up XS2” (Macherey-Nagel, Réf. 740903.50) kit according to manufacturer instructions. The total RNA samples were stored at -80°C. The concentration of the various extracted RNAs was determined by measuring the absorbance at 260 nm with a Nanodrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Absorbance measurements at 230 nm and 280

nm are also performed to assess the purity of the RNA samples. The A_{260} nm/A_{280} nm ratio assesses contamination and must be close to 2 for the RNAs to be considered pure. The A_{260} nm/A_{230} nm ratio is an indication of the presence of salts and peptide bonds and should be between 1.8 and 2.

- *Retro-transcription*

  The retro-transcription (RT) reaction was performed with “High-Capacity cDNA Reverse Transcription Kit” (Life Technologies, Réf. 4368813) according to manufacturer instructions. For each sample, 250 ng of RNA diluted in 10 µL of nuclease-free water was added to 10 µL of RT mixture containing deoxynucleotides, RT buffer, random primers and MultiScribe reverse transcriptase. The RT reaction was performed on the thermal cycler (Mastercycler Pro, Eppendorf) in the following steps: 10 minutes at 25°C; 120 minutes at 37°C; 5 minutes at 85°C.

- *Protocol RTqPCR*

  Quantitative RT-PCR for all the genes was executed using PowerUP SYBR Green (Thermo Fisher Scientific, Réf. A25778). 1 µL of the reverse-transcript was added to a 9 µL PCR mixture for 40 cycles. Each cycle included 95°C for 15 s, 10 cycles of Touch Down PCR from 70°C to 60°C for 15 s, and 72°C for 30 s, followed by 30 cycles of 60°C for 15 s and 72°C for 30 s, to conclude with 5 min at 72°C. All samples, including negative controls in which no DNA was added, were analysed in duplicate or triplicate, and the MAN2B1 gene was used as reference genes. All the RTqPCR were performed with StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Réf. 4376600) and analysed with StepOnePlus software. The quantitative expression of the genes was expressed as a Cycle threshold (Ct). For each sample, this value was normalized to the expression of the reference gene as delta Ct represented by 2-Δ (ΔCt) taking into account the difference in PCR efficiency. The normalised expression is therefore the ratio: (1/Ct of target gene) / (1/Ct of reference gene).

- *List of primers*

Gene	Forward 5’-3’	Reverse 5’-3’	Reference/Source
CTGF	AATGCTGCGAGGAGTGGGT	GGCTCTAATCATAGTTGGGTCT	(Nagaraja et al., 2012)
Cyr61	GAGTGGGTCTGTGACGAGGAT	GGTTGTATAGGATGCGAGGCT	(P. P. Chen et al., 2007)
AXL	GAGGTACATTGGCTTCGGGAT	GTGTCGGAAAGCTGCAGGGAG	This work
BIRC5	AGGACCACCGCATCTCTACAT	AAGTCTGGCTCGTTCTCAGTG	(Q. Li et al., 2020)
MYC	GCCACGTCTCCACACATCAG	TGGTGCATTTTCGGTTGTTG	(Hahm et al., 2016)
CENPF	ACCTTCACAACGTGTTAGACAG	CTGAGGCTCTCATATTCGGCA	(Shen et al., 2020)
BUB1B	AAATGACCCTCTGGATGTTTGG	GCATAAACGCCCTAATTTAAGCC	(Tian & Wang, 2020)
MAN2B1	GGAGAGGAGGCGGGATTA	GGAGCGGTCAGTCAGCAC	(Nkiliza et al., 2016)

Table 1. List of primers used for the RTqPCRs and their source.

3.2.3 Western Blot

- *Protocol*

  One day after the treatment with TRULI (5 µM and 10 µM) and DMSO for control, in DMEM Medium 0.2% FBS, cells were lysed with IP lysis buffer (Tris-HCl 1M, NaCl 4M, EDTA 500 mM, Triton 20%, glycérol 100%, H2O q.s. 50 mL) containing protease and phosphatase inhibitors at 150 µL. The lysates were rotate for 30 minutes at 4°C, centrifuged for 15 minutes at 15 000 G and then the supernatant was recovered and the pellet discarded. The samples were quantified with Pierce BCA protein assay kit (Thermo scientific, USA, Réf. 23227). After quantification, samples were prepared using 10 ng of protein with NuPAGE LDS sample buffer 4X (Life technologies, USA, Réf. NP0008) and reduced 10 min at 95°C. Samples were separated using Nu PAGE 4-12% Bis-Tris mini gels (Thermo Fisher Scientific, Réf. NP0329BOX) into NuPAGE MOPS SDS 1X running buffer (Life technologies, Réf. NP0001) and run for 45 min at 200 V.

Afterwards, the proteins from the gel were transferred on 0.45 µm nitrocellulose blotting membrane (Amersham, Réf. GE10600002) for 1 hour and 40 minutes at 30 V in cold 1X Tris-Glycine transfer buffer (14.4 g/l of glycine, 3 g/l of Tris Base and 5% methanol). After the transfer, membranes were blocked in BSA or milk 5% in TNT 1X (15 mM Tris base, 0.14 M NaCl, 0.5 M tween 20, pH 8) for 1 h at RT. Once the blocking was completed, the membranes were incubated with the respective primary antibodies (Table 2) overnight at 4°C. After that, 3 washings in TNT 1X followed and then incubation with the specific HRP-conjugated secondary antibodies for about 2 hours at RT. Following 3 washings in TNT 1X, the membranes were incubated for 5 minutes at RT with ECL prime (Amersham, Réf. GERPN2232) and then imaged with Amersham™ ImageQuant™ 800 biomolecular imager.

ImageQuant software (GE Healthcare Life Sciences, USA) was used to quantify the bands obtained and all analysed proteins were normalized to the loading control protein α-tubulin.

- *List of antibodies and conditions of use*

Name	Type	Concentration	Supplier	Blocking solution
CTGF	Monoclonal	1:1000	Cell signaling Technology, Rèf. 86641S	BSA
Cyr61	Monoclonal	1:1000	Cell signaling Technology, Rèf. 39382S	BSA
α-Tubulin	Monoclonal	1:10000	Novus Biologicals, Réf. NB100-690	Milk

Table 2. List of antibodies used for Western blots and their characteristics and mode of use.

3.3 Proliferation Assay

3.3.1 Incucyte

The cells are treated with the molecules and then incubated in the Incucyte (Sartorius, UK). During the incubation, several photos were taken at 1- to 3-hour intervals with a 20X objective and at the end of the experiment the cells were counted with the Incucyte2020B-Incucyte2022B software by comparing the number of cells treated with the molecules with the control treated only with DMSO.

3.3.2 BrdU assay

Fibroblasts derived from skin biopsies were cultured and then treated with TRULI for 24 h. After the treatment the cells were fixed in paraformaldehyde. After washing with PBS, fibroblasts were incubated with PBS-HCl 2 N for 30 minutes, followed by 10 minutes of incubation with sodium tetraborate decahydrate. After washing with PBS, a blocking solution of PBS 3% NGS (Natural Goat Serum) was added to the cells, followed by incubation overnight at 4°C with rat primary antibody for BrdU (Abcam, Réf. ab6326). The day after, fibroblasts were washed with PBS 1% NGS and incubate for 1 hour with fluorescent secondary antibody Alexa Fluor 488 goat anti-rat at 4°C (life technologies, Ref. A11006). The cells were washed with PBS and incubate 4 minutes with a solution of PBS DAPI at RT and then washed again with PBS. Images of the fluorescent cells were taken with Zeiss Cell Discoverer 7 and analysed with Zeiss software counting the number of total DAPI-positive cells and the number of BrdU-positive cells.

3.4 Statistical Analysis

All data presented in this manuscript represent at least three independent replicates for each experiment. Data were considered significant at $p<0.05$. Results are expressed as mean value with standard error on the mean (Mean +/- SEM). Significance is assessed by Ordinary one-way ANOVA test when the data met the criteria of normality and equal variances. Where it was not possible to meet those criteria, the Mann-Whitney or Kruskal-Wallis tests were used. All statistical analysis were performed with GraphPad Prism software version 9.0 (San Diego, USA) and all the p-values are indicated in each caption.

4. Results

4.1 Selection of YAP/TAZ-TEAD Activators

4.1.1 Molecules Screening with Luciferase assay

There are several molecules capable of activating or increasing YAP/TAZ-TEAD binding, and these can act at different levels of the Hippo pathway both upstream and downstream. LPA and S1P act very much upstream at the level of GPCRs (Mao et al., 2016). Then there are compounds that inhibit the kinases of the HP cascade, such as XMU-MP1 that inhibits MST1/2 (Fan et al., 2016) or TRULI, VT02956 and GA-017 that inhibit LATS1/2 (Aihara et al., 2022; N. Kastan et al., 2021; S. Ma et al., 2022). Finally, there are molecules such as Q2, B22 and 4E that act further downstream, directly activating or increasing YAP/TAZ-TEAD binding (Adihou et al., 2020; Pobbati et al., 2019). The aim was therefore to select which of the compounds available at the beginning of this study was best able to activate the YAP/TAZ-TEAD interaction. We decided not to use S1P and LPA, as they are molecules that act very upstream and could lead to side effects. Indeed, it was shown that S1P induce histamine release from mast cells (F. X. Yu et al., 2012), while LPA is involved in multiple steps of cancer such as stimulating cancer cell proliferation and migration (Balijepalli et al., 2021) and its use could be dangerous. Therefore, we tested XMU-MP1, TRULI, Q2 and B22. In addition, we chose Amb1, which is an in-house TEAD ligand, and also ML349 which is an inhibitor of APT2, responsible for depalmitoylation of TEAD, thus, inhibition of APT2 increases TEAD activity (N. G. Kim & Gumbiner, 2019a; Vujic et al., 2016). As negative controls, we used cells treated only with DMSO (in which the other molecules are dissolved) and cells treated with Dasatinib, which is a compound known to promote phosphorylation of YAP/TAZ and thus inhibit TEAD binding (Lamar et al., 2019; Oku et al., 2015).

To directly assess the efficiency of these molecules in activating HP in HEK293T, we employed a synthetic TEAD luciferase reporter (Dupont et al., 2011). This reporter is a YAP/TAZ-responsive synthetic promoter driving luciferase expression, therefore when the concentration of YAP in the nucleus is increased, the binding to TEAD is enhanced and thus the luminescent signal generated is greater. We initially chose to test the concentrations of the molecules generally used in the literature: XMU-MP1 at 3 μM, Amb1 at 5 μM, Q2 at 10 μM, B22 at 10 μM, ML349 at 1 μM and Dasatinib at 1 μM. On the other hand, due to a mistake during the process, we used TRULI 1 μM instead

of 10 μM as used by the authors (N. Kastan et al., 2021); however, it was efficient even at this lower concentration. Indeed, we observed that TRULI significantly increases TEAD activity compare to the control (DMSO) (Fig. 11). However, the other molecules did not lead to an increase in YAP/TAZ-TEAD activity, with even B22 decreasing luciferase activity. As expected, Dasatinib, decreased luciferase activity.

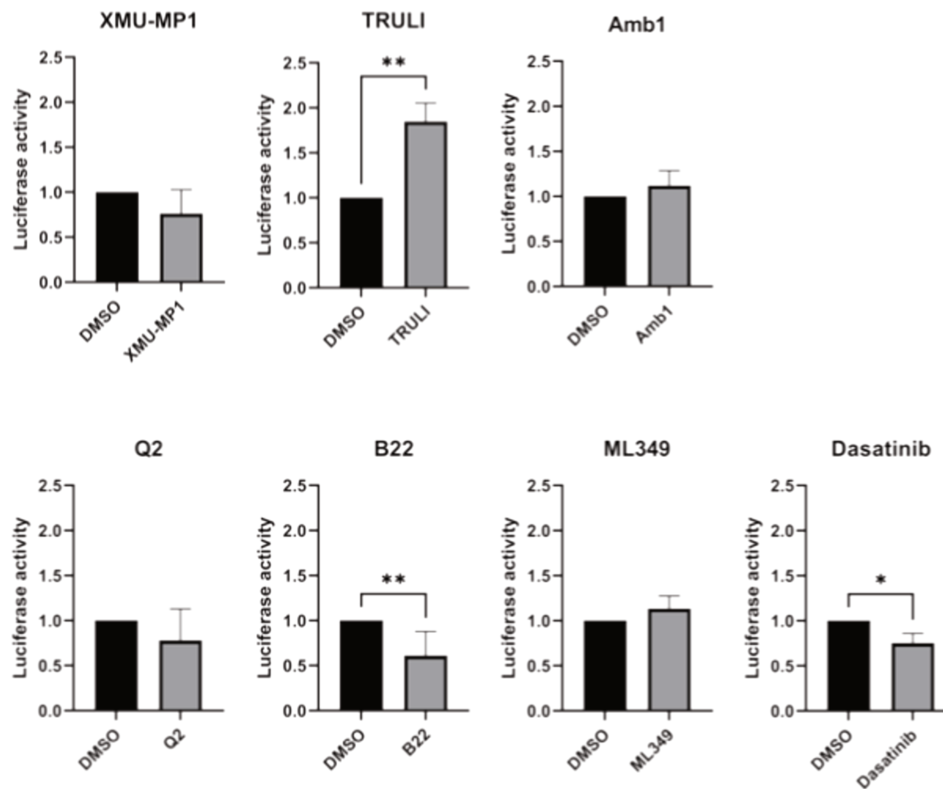


Figure 11. Test of activation of TEAD promoter by Luciferase assay. The value of the luciferase luminescence was normalized on the β-galactosidase data (internal control of transfection). TEAD activity was measured using a luciferase reporter after treatment of HEK293T with the following compounds: XMU-MP1 (3 μM), TRULI (1 μM), Amb1 (5 μM), Q2 (10 μM), B22 (10 μM), ML349 (1 μM) and Dasatinib (1 μM). TRULI significantly increases the expression of TEAD (p=0.0079, Mann-Whitney test), while B22 decrease luciferase TEAD activity (p=0.0079, Mann-Whitney test). The other molecules have no significant effects. Dasatinib functions properly as an inhibitor by significantly decreasing TEAD activity (p=0.0179, Mann-Whitney test). Error bars show standard deviation of at least three independent experiments (*p <0.05; **p<0.01).

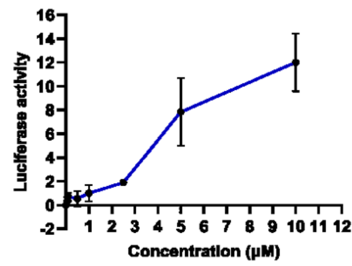
Luciferase data indicated TRULI as the most promising compound, while the other molecules did not yield the expected results. However, their inefficiency may be linked to the concentration and the conditions used in our experiment. The next step therefore was to test other concentrations of these molecules.

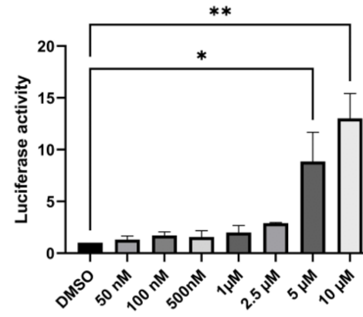
4.1.2 Dose response assay of the molecules

After the first screeing, the next step was to identify the optimal concentration for these molecules in activating TEAD activity. Specifically, for TRULI we wanted to test different concentrations to see if they are more effective than 1 μM, it was previous used in literature at 10 μM. For the other molecules, we wanted to identify other concentrations that might be more effective than those used. We performed several dose response luciferase assays with the same approach described before. From the initial molecules, we chose to exclude B22 as it belongs to the same chemical family as Q2 (7-substituted-8-hydroxyquinolines, see Figure 8), thus we decided to continue with the latter. We select a set of seven concentrations between 25 nM to 10 μM for the dose response assay.

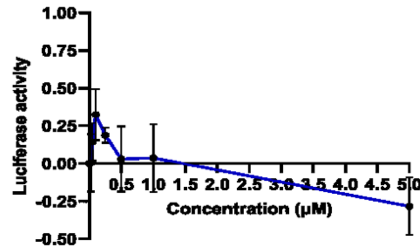
The results of the dose response assays showed that TRULI was again the compound that most increased TEAD activity in a dose-dependent manner, especially at concentrations of 5 μM and 10 μM, which increased luciferase signal approximately 9- and 13-fold, respectively (Fig. 12 A). The dose response assay for Amb1, Q2 and ML349 did not give encouraging results, as even when testing different concentrations of these molecules no significant activation of luciferase activity was observed (Fig. 12 B, 12 C and 12 E). Surprisingly, XMU-MP1 decreases TEAD activity and even in a dose-dependent manner (Fig. 12 D). Thus, it was clear from these dose response luciferase assays that the best molecule in activating TEAD activity is TRULI, especially at concentrations of 5 μM and 10 μM. The other molecules unexpectedly did not increase the activity of YAP-TAZ/TEAD, while XMU-MP1 even decreased TEAD activity. Thus, at this point, we selected TRULI, among all the molecules we tested, as the best YAP/TAZ-TEAD activator and also identified the best concentrations (5 μM and 10 μM) in activating this interaction to test its effect on cell growth.

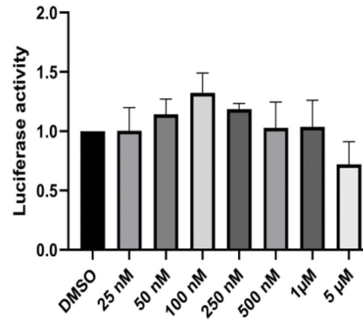
A. TRULI



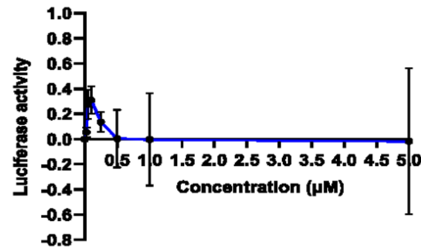


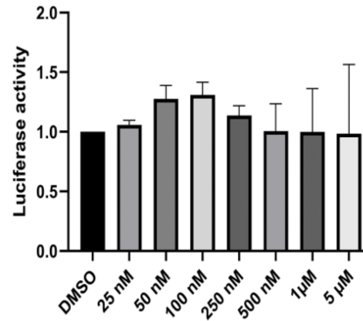
B. Amb1



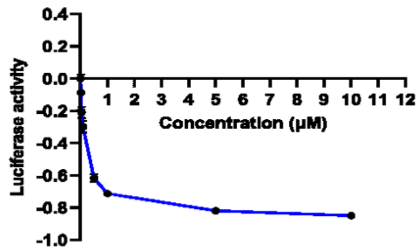


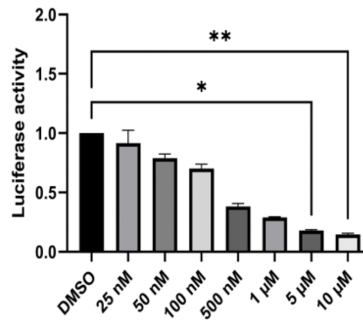
C. ML349



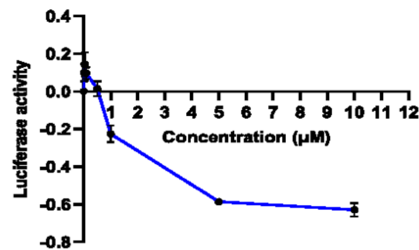


D. XMU-MP1





E. Q2



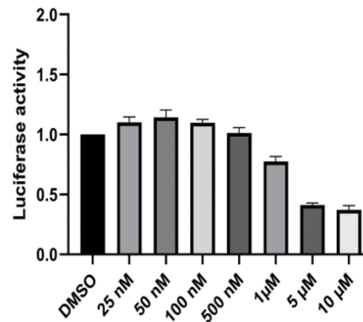


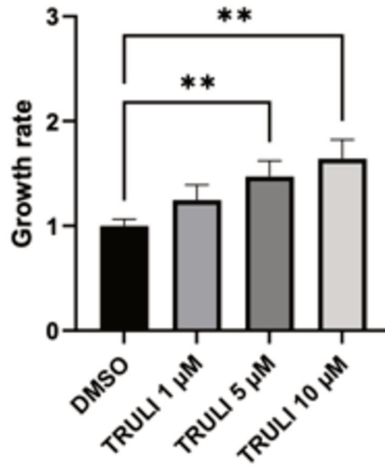
Figure 12. Test of activation of TEAD promoter by dose response luciferase assay. For each molecule, the graph on the left represents the dose response curve, while the graph on the right represents the scatter plot with bars. **A.** TRULI: the compound increases TEAD activity in a dose-dependent manner. 5 μM and 10 μM are the concentration that statistically increase TEAD activity (DMSO vs TRULI 5 μM p=0.0221, DMSO vs TRULI 10 μM p=0.0052; Kruskal-Wallis test); **B-C-E.** Amb1, ML349 and Q2: these molecules have no significant effect on TEAD activity; **D.** XMU-MP1: the compound decreases TEAD activity in a dose-dependent manner (DMSO vs TRULI 5 μM p=0.0225, DMSO vs TRULI 10 μM p=0.0037; Kruskal-Wallis test); Each experiment is normalized on its own control DMSO. Error bars show standard deviation of at least three independent experiments (*p<0.05; **p<0.01).

4.1.3 Effect of TRULI on the cellular growth

We treated the HEK293Ts at three different concentrations of TRULI: 1 μM, 5 μM and 10 μM, and analysed cell growth by incubating the cells in the Incucyte, a special incubator capable of taking pictures at set time intervals. Using the dedicated software, we counted the number of cells and compared those treated with TRULI with the control treated only with DMSO, and created a growth curve of these cells (Fig. 13 B). Analysing this curve, it became clear that the 5 μM and 10 μM concentrations increased cell growth in HEK293T; these data mirrored the results obtained with the dose response luciferase assay, in which these two concentrations increased YAP-dependent TEAD activity more.

Furthermore, it can be seen from the curve that the best result in increasing cell growth with TRULI is obtained after 24 hours of treatment, consistent with what the authors have shown in their study (N. Kastan et al., 2021). Therefore, data analyzed at 24 h of treatment showed that TRULI 5 μM and 10 μM significantly increased cell growth (Fig. 13 A). Finally, after selecting TRULI as the best YAP/TAZ-TEAD activator, and identifying the best concentrations to use, we demonstrated that at concentrations of 5 μM and 10 μM it is able to increase cell growth.

A



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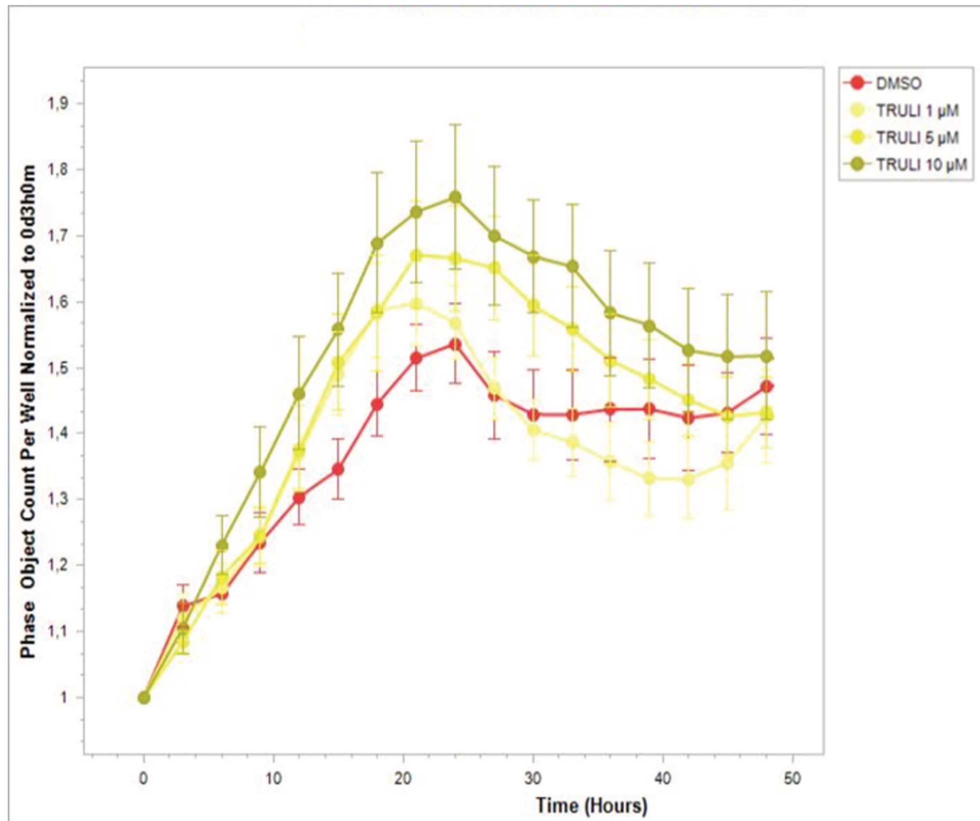


Figure 13. A. The treatment with TRULI (5 μM and 10 μM) statistically increase the cellular growth of HEK293T after 24 h treatment (DMSO vs TRULI 5 μM p=0.0093, DMSO vs TRULI 10 μM p=0.0014; Ordinary one-way ANOVA test). Error bars show standard deviation of at least three independent experiments (**p<0.01). **B.** Cell growth curve generated after software analysis of photos acquired every 3 hours via Incucyte. Treatment with TRULI increases cell growth in a dose-dependent manner. The peak of growth occurs after 24 hours of treatment.

4.1.4 Conclusions

With the aim of identifying the best YAP/TAZ-TEAD activator, we started by selecting different available compounds that acted at different steps of the Hippo pathway. We tested molecules that acted upstream on different players in the kinase cascade such as XMU-MP1 and TRULI, and molecules that acted downstream directly on YAP/TAZ-TEAD binding such as Amb1 and Q2. By testing the ability of these molecules to increase YAP-dependent TEAD activity and using different concentrations for each of them, we identified using a TEAD-depending luciferase reporter, TRULI as the best candidate in activating YAP/TAZ-TEAD binding, especially at concentrations of 5 μM and 10 μM. These results were corroborated by subsequent cell growth experiments in HEK293T, in which consistent with dose response luciferase assays, TRULI 5 μM and 10 μM significantly increased cell growth. At this point, we demonstrated that TRULI, which is a LATS1/2 inhibitor, was the molecule that showed the most promising results in both activating YAP/TAZ-TEAD binding and increasing cell proliferation. Therefore, the next step was to show that the activation of YAP/TAZ-TEAD by TRULI actually led to the activation of their target genes and that therefore the increase in cell growth was due to an increase in their expression.

4.2 Effect of TRULI on Hippo Pathway Target Genes, Protein Levels and cellular growth

Data obtained from the luciferase assay and proliferation assay demonstrate that the most promising concentrations of TRULI are 5 μM and 10 μM, thus, using these two concentrations, we analysed the effect that they have on the RNA and protein expression of HP target genes *Cyr61* and *CTGF*, and verify that TRULI actually induces the expression of these target genes by activating YAP/TAZ-TEAD interaction. We performed these experiments in 3 different cell lines: HEK293T, small molecule Neural

Precursor Cells (smNPC) wt and smNPC carrying a triplication of SNCA gene (gene involved in Parkinson's Disease) (Srinivasan et al., 2021). We started with HEK293T first as they represent a well-known, easy-to-handle cell model in which all the protocols used were well optimised. More importantly, in these cells we had already seen how well TRULI activated YAP/TAZ-TEAD binding and increased proliferation, so we needed to verify that these observed effects matched the expected increase in HP target gene expression. Then, in order to test the effect of TRULI on RNA and protein expression of HP targets on a model that was closer to neural cells, we decided to use smNPCs. These cells are derived from human fibroblasts, reprogrammed into iPSCs by retroviruses and then re-differentiated into neuronal precursors (Reinhardt et al., 2013). This particular type of cell has several advantages, as they are capable of immortal self-renewal, are easy to handle, culturable at a wide range of cell densities and using only inexpensive small molecules. Therefore, smNPCs represent a robust and affordable tool for neurodegenerative disease modelling. In collaboration with Professor Kruger, we used smNPCs wt, derived from healthy people, and smNPCs with triplication of the SNCA gene, derived from patients with Parkinson's disease (Barbuti et al., 2020). Therefore, we analysed the effect of TRULI in a neural model from healthy persons and from patients suffering from a neurodegenerative disease. Indeed, as described in the introduction, the role of the Hippo pathway in such other neurodegenerative disease is increasingly emerging (Ahn et al., 2020; Jin et al., 2020; Sahu & Mondal, 2020; Tanaka et al., 2020).

4.2.1 Effect of TRULI on RNA Level of HP Target Genes in HEK293T

After testing the various compounds in HEK293T and observing that TRULI greatly activated YAP/TAZ-TEAD binding and increased proliferation, we wanted to asses that YAP/TAZ-TEAD activation actually led to increased expression of HP target genes in this cell model. Therefore, we analysed by RTqPCR the mRNA levels of two of the most common HP target genes: *Cyr61* and *CTGF*. We chose to treat the cells with the two concentrations that gave the best results in activating YAP/TAZ-TEAD and proliferation, 5 µM and 10 µM (Fig. 12). For the duration of treatment, we chose 24 hours as this is in line with the literature and what we observed in the analysis of cell growth (Fig. 13). As expected, RTqPCR results showed increased RNA levels of both *Cyr61* and *CTGF* compare to the control DMSO (Fig. 14). Consistent with previous

results, TRULI 10 μM significantly increase the expression of the two target genes. Specifically, TRULI 5 μM increases the expression of Cyr61 by approximately 2-fold, whereas TRULI 10 μM increase the expression of both genes by approximately 3-fold. These data confirm that treatment with TRULI leads to increased expression of YAP/TAZ-TEAD target genes, as it increases the RNA levels of both *Cyr61* and *CTGF* with both concentrations tested.

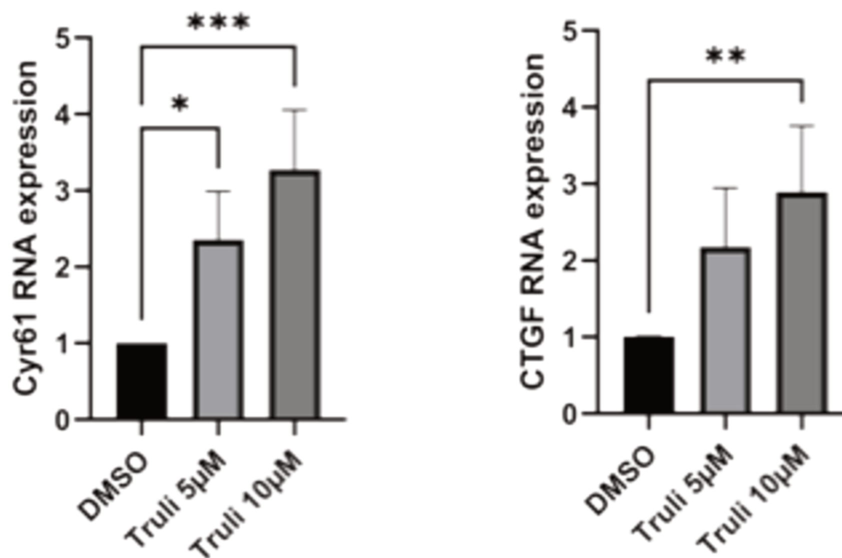


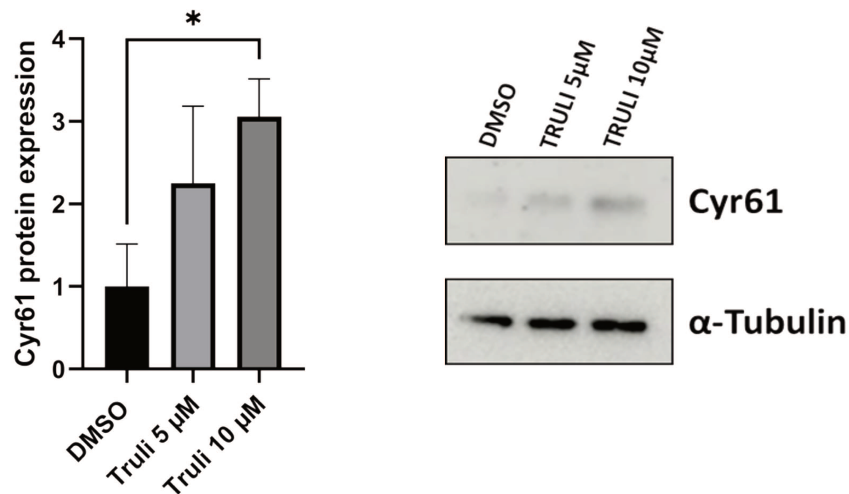
Figure 14. Effect of TRULI on RNA expression of *Cyr61* and *CTGF* in HEK293T after 24 h of treatment. The graph on the left is representative of Cyr61 RNA levels. TRULI 5 μM and 10 μM increase expression by approximately 2-fold and 3-fold respectively (DMSO vs TRULI 5 μM p=0.0169, DMSO vs TRULI 10 μM p=0.0007; Ordinary one-way ANOVA test). The graph on the right is representative of CTGF RNA levels. For CTGF TRULI 10 μM increase expression by approximately 3-fold (DMSO vs TRULI 10 μM p=0.0057; Ordinary one-way ANOVA test). Data are normalized on housekeeping gene MAN2B1. Error bars show standard deviation of at least three independent experiments (*p <0.05).

4.2.2 Effect of TRULI on Protein Level of HP Target Genes in HEK293T

Once we assessed that TRULI is able to induce YAP/TAZ-TEAD binding, increase Cyr61 and CTGF expression and in turn proliferation of the HEK293T cells, the final step was then to verify that it also leads to increased protein levels of these targets. We used the same conditions as previously used for RTqPCR, i.e., we treated the cells with TRULI 5 μM and 10 μM for 24 hours and then performed Western blot experiments to

analyse protein levels. Consistent with what we have seen in RTqPCR, Western blot data showed that TRULI 10 μM significantly increases Cyr61 protein level by about 3-fold (Fig. 15 A). As for CTGF, although we observed that the RNA level increased, we could not detect the protein levels in the Western blot. This is probably due to the fact that CTGF, but to a lesser extent also Cyr61, is very poorly expressed in HEK293, as can be seen from the expression graphs in The Human Protein Atlas (Fig. 15 B). In these graphs, it can be seen that the expression of CTGF is almost zero. In particular, in the first graph there are several cell lines belonging to different organs of the body and in HEK there is no CTGF expression. In the second graph there are 60 non-cancerous cell lines, distributed in descending order according to CTGF expression, and as can be seen HEK293 is the cell line that expresses the lowest levels of CTGF. It is possible that even if RNA levels are increased approximately 3-fold with TRULI 10 μM, this is not enough to lead to the production of enough protein to be detected by Western blot, a much less sensitive technique than RTqPCR. Ultimately, we have shown how TRULI in HEK293T increases YAP/TAZ-TEAD binding, this in turn induces increased expression of their target genes *Cyr61* and *CTGF*, which results in increased protein levels, at least for Cyr61. Finally, this probably leads to an increase in cell proliferation.

A



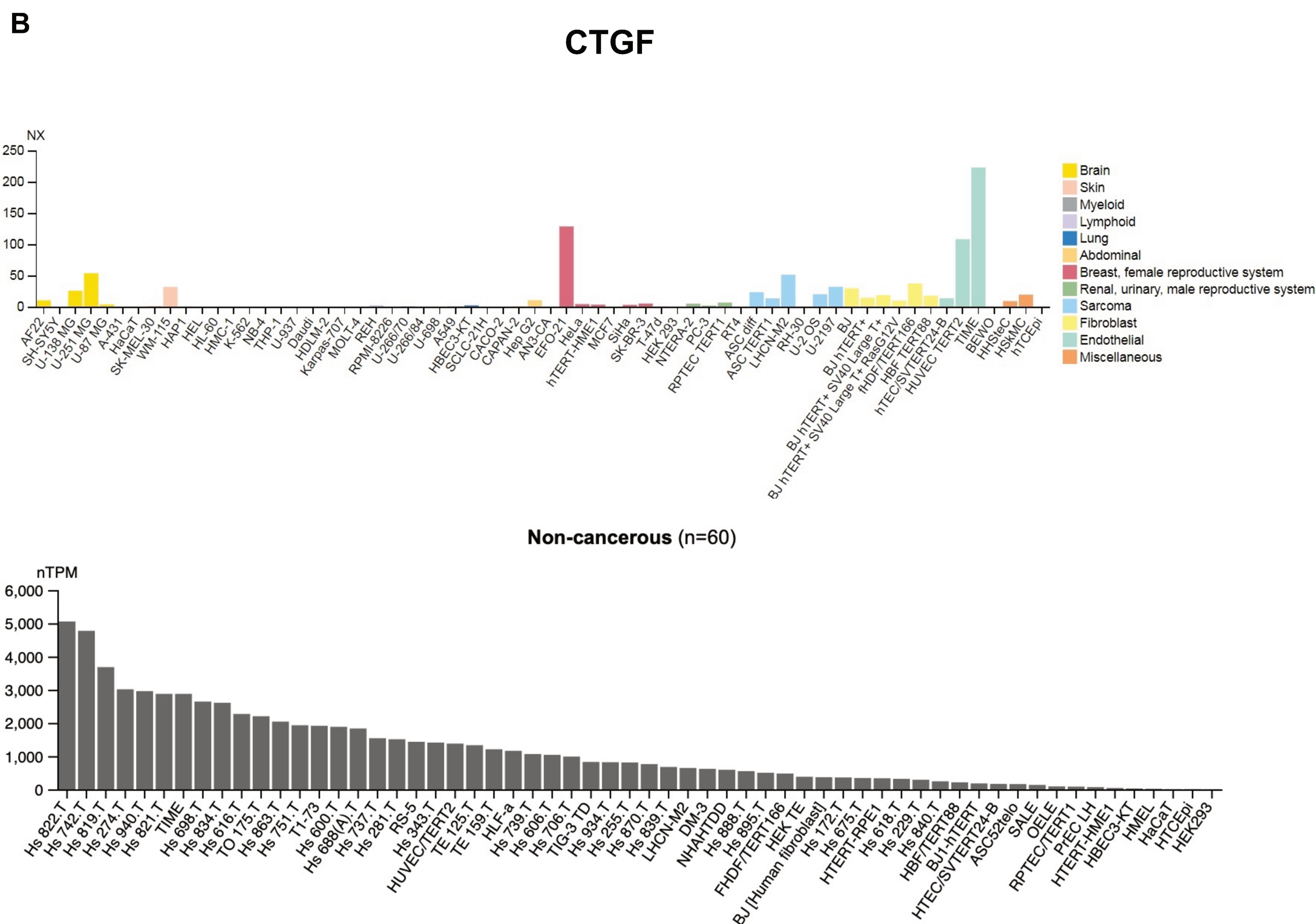


Figure 15. A. Effect of TRULI on protein level of Cyr61 in HEK293T after 24 h of treatment. TRULI 10 μM increases protein level by approximately 3-fold compare to the control DMSO (DMSO vs TRULI 10 μM p=0.0341, Kruskal-Wallis test). Data are normalized on tubulin. Error bars show standard deviation of at least three independent experiments (*p<0.05). **B.** Graphs representing CTGF expression in different cell lines. Protein expression of CTGF in HEK293 is almost absent. The diagram above is representative of different cell lines divided by colour, according to the part of the body to which they belong. The bottom graph is representative of 60 non-cancerous cell lines distributed according to CTGF expression levels, with HEK293 being in last place. (Graphs from The Human Protein Atlas).

4.2.3 Effect of TRULI on RNA Level of HP Target Genes in smNPCs

So far, we have shown that TRULI is a highly efficient inducer of YAP/TAZ-TEAD binding, it is able to increase RNA expression of *Cyr61* and *CTGF*, protein expression of at least Cyr61, and increase proliferation in HEK293T. At this point, however, our goal was to demonstrate that TRULI was able to increase the expression of the target genes in a model more similar to neuronal cells, so we tested the molecule in smNPCs.

We tested TRULI in parallel in smNPCs wt and smNPCs derived from patients carrying a triplication of the SNCA gene (implicated in Parkinson's disease). We analysed the RNA levels of *Cyr61* and *CTGF* after treatment with 5 μM or 10 μM of TRULI for 24 hours. As shown in figure 16 A and B, in this cell line TRULI is able to increase the expression of *Cyr61* and *CTGF* more potently than in HEK293T, in both smNPC wt and smNPC with triplication (tripl). In particular, the increase in *Cyr61* expression is statistically significant after treatment with 10 μM TRULI in smNPCs wt, while in smNPCs tripl the increase is obtained with both concentrations. Closer analysis of data pointed out that TRULI at 5 μM and 10 μM induce an increase of CTGF RNA expression of about 5-fold and 10-fold respectively in both smNPC lines; while for Cyr61 the increase is about 5-fold with TRULI 10 μM. Furthermore, the increase in CTGF expression following treatment is dose dependent in both smNPC lines, with TRULI 10 μM having a stronger effect than 5 μM. Thus, it is interesting to note that the effects of TRULI on *CTGF* and *Cyr61* RNA levels are comparable in wt and tripl smNPCs, this suggests that the positive effect of TRULI in increasing YAP/TAZ-TEAD target genes in disease cells is similar to wt cells.

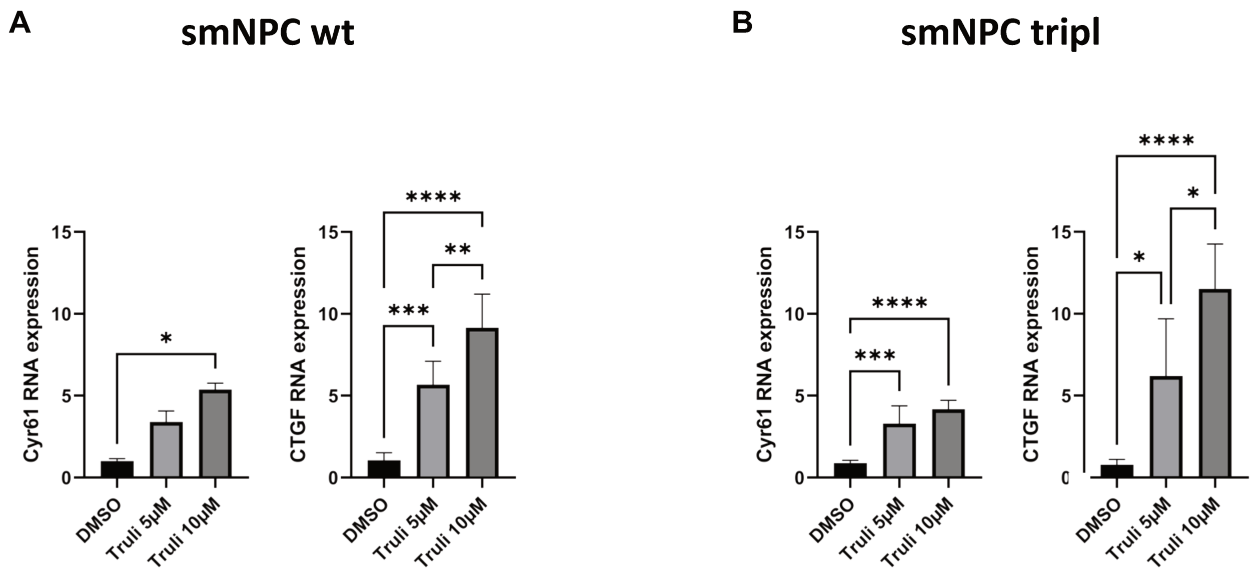


Figure 16. A. Effect of TRULI on RNA expression of *Cyr61* and *CTGF* in smNPC wt after 24 h of treatment. The graph on the left is representative of *Cyr61* RNA levels. TRULI 10 μM increases RNA expression (DMSO vs TRULI 10 μM p=0.0146; Kruskal-Wallis test). The graph on the right is representative of *CTGF* RNA levels. For CTGF TRULI 5 μM and 10 μM increase expression in a dose dependent manner (DMSO vs TRULI 5 μM p=0.0009, DMSO vs TRULI

10 µM p<0.0001, DMSO vs TRULI 5 µM p=0.0009, DMSO vs TRULI 10 µM p<0.0001, TRULI 5 µM vs TRULI 10 µM p=0.0073; Ordinary one-way ANOVA test; Ordinary one-way ANOVA test). **B.** Effects of TRULI on RNA expression of *Cyr61* and *CTGF* in smNPC tripl after 24 h of treatment. The graph on the left is representative of *Cyr61* RNA levels. TRULI 5 µM and 10 µM increase RNA expression (DMSO vs TRULI 5 µM p=0.0004, DMSO vs TRULI 10 µM p<0.0001; Ordinary one-way ANOVA test). The graph on the right is representative of *CTGF* RNA levels. For CTGF TRULI 5 µM and 10 µM increase expression in a dose dependent manner (DMSO vs TRULI 5 µM p=0.0009, DMSO vs TRULI 10 µM p<0.0001, TRULI 5 µM vs TRULI 10 µM p=0.0169; Ordinary one-way ANOVA test). Data are normalized on housekeeping gene *MAN2B1*. Error bars show standard deviation of at least three independent experiments (**p<0.01).

4.2.4 Effect of TRULI on Protein Level of HP Target Genes in smNPCs

Previous results have shown that TRULI increases the RNA levels of HP target genes, *Cyr61* and *CTGF*, also in smNPCs, both wt and tripl. Therefore, the next step was to see whether this increase also translates into an increase in the protein levels of Cyr61 and CTGF. Therefore, we treated the cells with TRULI 5 µM and 10 µM for 24 hours and then analysed the protein levels of Cyr61 and CTGF by Western blot. As expected, treatment with the molecule increases the protein levels of Cyr61 and CTGF in both wt and tripl smNPCs compare to control DMSO (Fig. 17 A-B). However, the increase is not of the same proportions observed with RNA. Furthermore, TRULI 10 µM significantly increases the protein levels of both Cyr61 and CTGF in both cell types. Moreover, in smNPC wt TRULI increases Cyr61 protein level at both concentrations, with 10 µM increasing protein expression more than with 5 µM. Unlike HEK293T, however, in smNPCs we were also able to detect increased CTGF protein levels. Thus, we have shown that the increase observed at the RNA level also translates into an increase in the protein levels of Cyr61 and CTGF in both wt and tripl smNPCs.

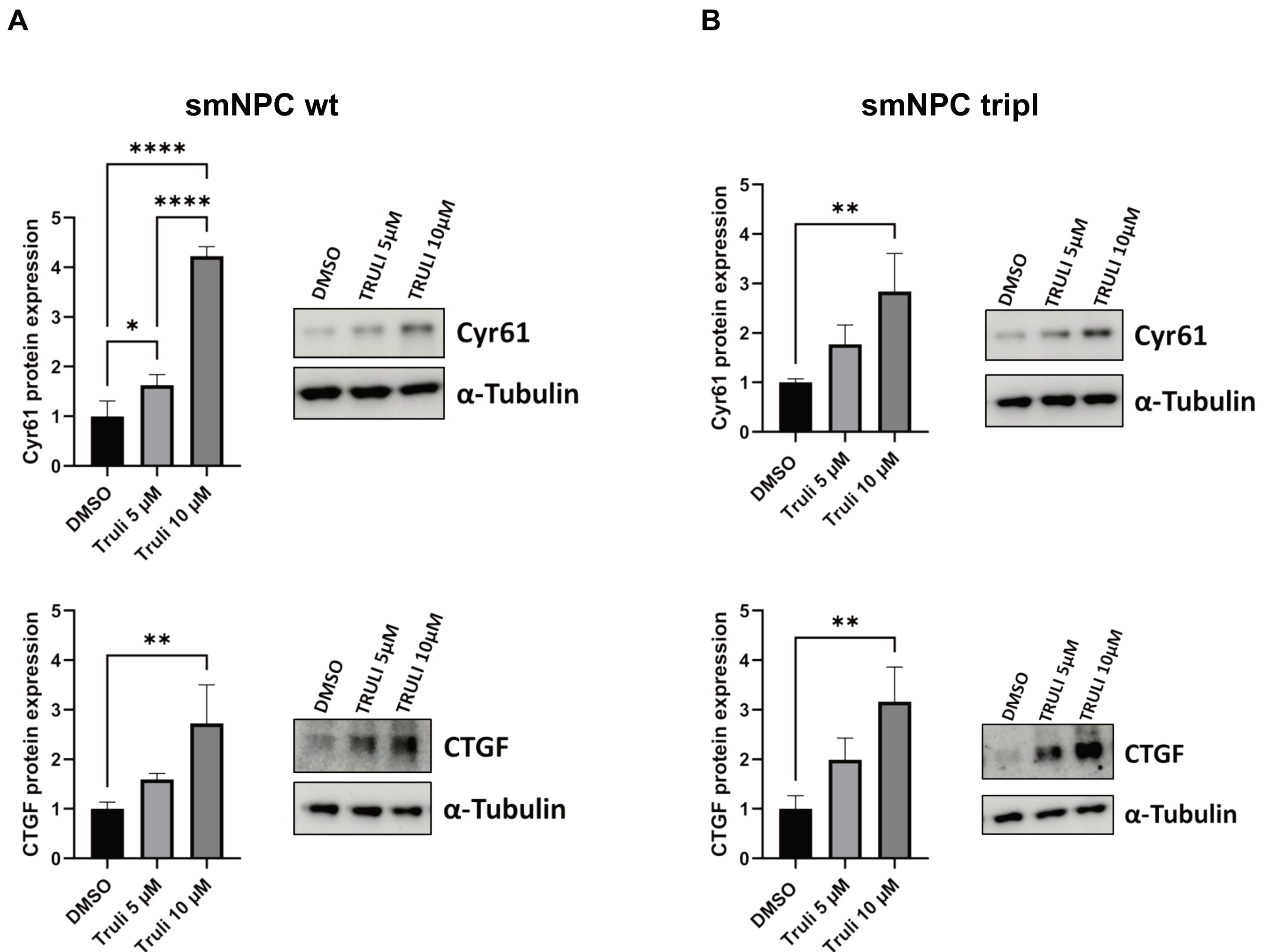


Figure 17. A. Effect of TRULI on protein level of Cyr61 and CTF in smNPC wt after 24 h of treatment. For Cyr61 TRULI 5 µM and 10 µM increase protein expression in a dose dependent manner (DMSO vs TRULI 5 µM p=0.0452, DMSO vs TRULI 10 µM p<0.0001, TRULI 5 µM vs TRULI 10 µM p<0.0001; Ordinary one-way ANOVA test) For CTGF TRULI 10 µM increases protein expression (DMSO vs TRULI 5 µM p=0.0067; Ordinary one-way ANOVA test). **B.** Effects of TRULI on protein level of Cyr61 and CTF in smNPC tripl after 24h of treatment. TRULI 10 µM increases the level of both proteins compare to the control DMSO (Cyr61 DMSO vs TRULI 10 µM, p=0.0071, CTGF DMSO vs TRULI 10 µM, p=0.0032; Ordinary one-way ANOVA test). Data are normalized on tubulin. Error bars show standard deviation of at least three independent experiments (*p<0.05).

4.2.5 Effect of TRULI on Confluence in smNPCs

After showing that treatment with TRULI increases the RNA and protein levels of Cyr61 and CTGF also in smNPCs, we wondered whether this effect also translated into

increased cell growth in these cells. Therefore, we analysed cell growth following treatment with TRULI 5 µM and 10 µM with Incucyte in both smNPC lines. However, it was difficult for these cells to be analysed using Incucyte software, because these cells tend to grow in aggregation and the software cannot distinguish individual cells and makes errors when counting them. Therefore, in order to analyse cell growth following TRULI treatment, we changed our strategy. Instead of trying to count individual cells, because of the difficulty, we decided to analyse the confluence of the cells using the Incucyte software. The software is able to quantify over time the area of the well covered by the cells compared to the area of the total well. Thus, if cells grow faster or increase in number after treatment, the area of the well covered by cells will be larger than the area covered by cells in DMSO control. In this way, we were able to create a growth curve for both smNPC lines. Analysing these curves, we observed that in both smNPCs, TRULI treatment increases confluence compared to the DMSO control (Fig. 18 A-B). In particular, we noticed that the increase in confluence occurred more after 24 hours, so we analysed 3 different treatment time intervals: 24 hours (condition used for all experiments so far), 36 hours and 48 hours. Specifically, in smNPC wt treatment with TRULI statistically increased confluence at 10 µM at all 3 times examined (Fig 18 A). In contrast, in smNPC tripl both 5 µM and 10 µM increase the confluency at all 3 times examined and the effect of the two concentrations of TRULI is comparable, (Fig. 18 B). Thus, it appears that the effect of treatment on confluence differs in the two smNPC lines and that in the smNPC tripl both concentrations yield the same effect, whereas in the smNPC wt a significant increase in confluency is obtained only with TRULI 10 µM.

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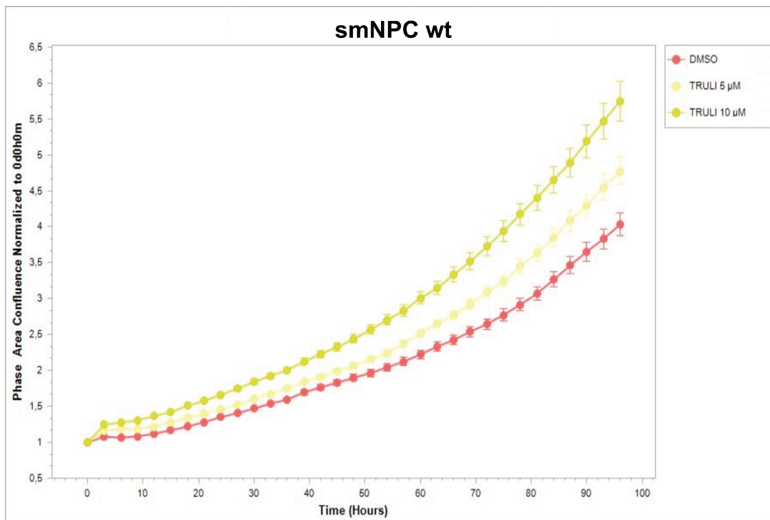

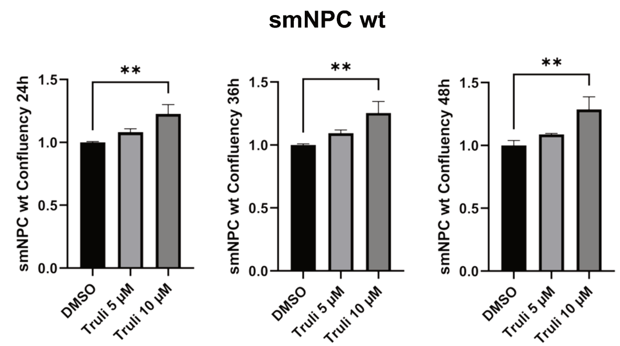

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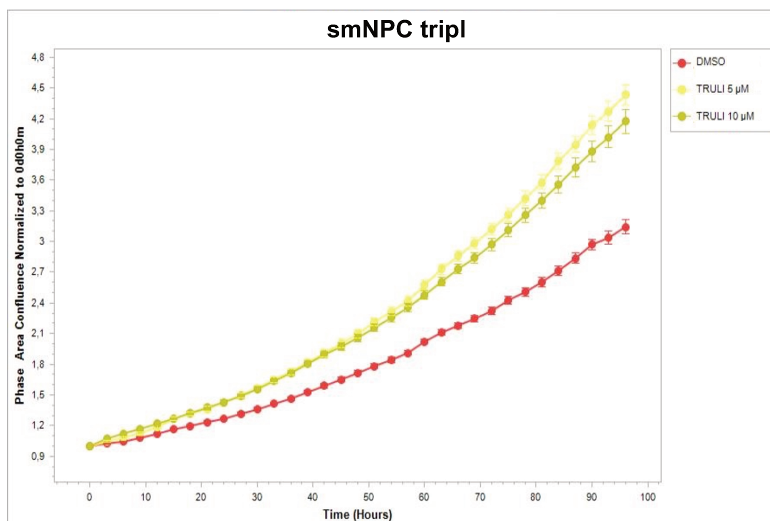

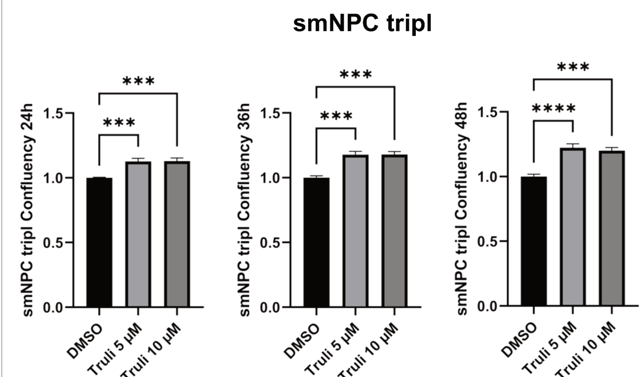

Figure 18. Confluence curve of smNPC wt and tripl generated after software analysis of photos acquired every 3 hours via Incucyte. **A.** Treatment of smNPC wt with TRULI 10 µM statistically increases confluence in the 3 selected time points of treatment: 24 h, 36 h, 48 h. (24 h DMSO vs TRULI 10 µM p=0.0015, 36h DMSO vs TRULI 10 µM p=0.0023, 48 h DMSO vs TRULI 10 µM p=0.0024; Ordinary one-way ANOVA test). **B.** Treatment of smNPC tripl with TRULI 5 µM and 10 µM statistically increase confluence in the 3 selected time points of treatment: 24 h, 36 h, 48 h (24 h DMSO vs TRULI 5 µM p=0.0004, 24 h DMSO vs TRULI 10 µM p=0.0004, 36 h DMSO vs TRULI 5 µM p=0.0001, 36 h DMSO vs TRULI 10 µM p=0.0001, 48 h DMSO vs TRULI 5 µM p<0.0001, 48 h DMSO vs TRULI 10 µM p=0.0001; Ordinary one-way

ANOVA test). Error bars show standard deviation of at least three independent experiments (**p<0.01; ***p<0.001; ****p≤0.0001).

4.2.6 Conclusions

In this section, we analysed the effects of TRULI on YAP/TAZ-TEAD target genes *Cyr61* and *CTGF* on both RNA and protein levels. Using TRULI at the two selected concentrations, 5 μM and 10 μM, we treated three different cell lines: HEK293T, smNPC wt and smNPC carrying a triplication of the SNCA gene. Treatment with the molecule induces an increase in RNA levels of both target genes. However, there are differences between cellular models; in smNPCs the increase in expression of both targets is much greater than the increase observed in HEK293T. It therefore appears that the effect of TRULI on Cyr61 and CTGF is cell dependent.

Regarding protein levels, in HEK293T, TRULI induces an increase consistent with that observed at the RNA level, although we were only able to detect Cyr61 and not CTGF, probably due to the low expression of this in HEK293T. On the other hand, in the two smNPC lines we observed an increase in protein level of both targets.

In addition, in the two neural lines we also measured cell growth by measuring the increase in cell confluence following treatment with TRULI and as expected the molecule induces an increase in confluence. However, in smNPC there are differences between wt and tripl; in smNPC wt the increase is visible only with TRULI 10 μM, whereas in smNPC tripl there are no difference in the effects induced by the two concentrations, suggesting a better effect of TRULI 5 μM since the same effect is obtained using half the concentration. In conclusion, after having identified the best activator of YAP/TAZ-TEAD binding among the molecules we had selected and the best concentrations to use, we showed that this compound is able to increase both the RNA and protein levels of the two YAP/TAZ-TEAD targets Cyr61 and CTGF in different cellular models, including neural models. We have also shown that TRULI is able to increase cell growth in these models. We have therefore demonstrated that TRULI works well as an activator and also has a positive effect on cells by increasing their growth. The next step was to test this compound on fibroblasts taken from patients with Huntington's disease to see that it is equally effective.

4.3 Comparison of cellular growth, proliferation and HP target genes expression in healthy and HD Skin Fibroblasts and effect of TRULI on healthy fibroblasts

Having shown that TRULI is able to induce YAP/TAZ-TEAD binding, increase their target genes and growth rate in both HEK293T and smNPC, our aim was to use fibroblasts to see the effect that the compound has on these cells. We chose to use fibroblasts taken from healthy individuals and HD skin fibroblasts taken directly from HD patients. Even though this model is not a neuronal model, fibroblasts have the advantage to be primary cells directly from patients, and therefore not a constructed model, considering how difficult is to obtain HD post-mortem brains. Moreover, since mHTT is expressed ubiquitously, molecular changes detected in fibroblasts may reflect peripheral mechanisms induced by the disease or could mirror processes that also take place in the brain (Marchina et al., 2014b; Petersen et al., 2014; Pierzynowska et al., 2019; Sassone et al., 2009; Squitieri et al., 2010). Furthermore, it has been shown that in HD fibroblasts exhibits reduced proliferation and mitosis (Aladdin et al., 2019; Jędrak et al., 2018) which could be partly caused by dysregulation of the Hippo pathway, as seen in the post-mortem brains of HD patients (Mao et al., 2016; Mueller et al., 2018; Yamanishi et al., 2017). Indeed, it has been observed that inhibition of YAP/TAZ in fibroblasts decreases cell proliferation and increases apoptosis (N. Gao et al., 2022). We selected fibroblasts from 4 different HD patients of different ages, aware of the fact that age can affect fibroblast cell growth; in combination we selected fibroblasts taken from 4 healthy people that matched the ages of the patients. Information about the fibroblasts chosen (age, gender, number of CAG repeats and symptomatology) are summarized in Table 3.

NAME	SEX	AGE	N° CAG	Symptomatology
HTT1	1	35	48	Symptomatic
CTL1	2	34	Not specified	Healthy
HTT2	1	50	44	Symptomatic
CTL2	1	55	Not specified	Healthy
HTT3	2	62	42	Symptomatic
CTL3	2	63	Not specified	Healthy
HTT4	2	35	44	Asymptomatic
CTL4	1	33	Not specified	Healthy

Table 3. Table representing the 8 types of fibroblasts used for the experiments: 4 taken from HD patients and 4 taken from healthy individuals. The lines are written in order of HTT and CTL pairing having the same age. The table provides information regarding the sex of the individuals: 1 for male and 2 for female. Age refers to the age of the individual at the time of collection. N° CAG represents the number of CAG repeats present in HD patients, in healthy individuals the number of CAG repeats is not known. In addition, it is indicated whether the patients at the time of cell harvesting already showed symptoms of the disease or not.

We treated the samples with DMSO (control), TRULI 5 μM and TRULI 10 μM for 24 h. For each of these samples, we first analysed the basal cell growth (cell incubated only with DMSO) with Incucyte by comparing HD fibroblasts with control fibroblasts, to verify the slow growth associated with HD fibroblasts. In addition, we performed BrdU assay in order to specifically analyse only proliferation, to distinguish it from cell survival; this tool allow us to check whether HTT cells actually proliferate less than controls, or whether the observed effect is due to lower cell survival. BrdU is a thymine analogue that when added to cells is incorporated in place of the nucleotide base by the reproducing cells. After fixing the cells, an antibody that recognises BrdU in the DNA strands can be conjugated and using a fluorescently labelled secondary antibody, it is possible to observe with a fluorescence microscope the cells that were proliferating at that precise moment, because only those cells will have incorporated BrdU instead of thymine. Furthermore, we analysed the expression levels of several HP target genes to see whether, as in the neural cells of HD patients, they are downregulated in fibroblasts. After analysing cell growth, proliferation and basal expression of HP target genes, we

then analysed the effects of TRULI treatment in fibroblasts from healthy individuals to test the effects of the molecule also in these cells in addition to HEK293T and smNPCs. Thus, we analysed the effects of the compound on cell growth, proliferation and the expression of HP target genes. We also verified the RNA expression of YAP and TEAD1 in fibroblasts by analysing their expression in the CTL1, comparing their RNA level by treating the cells with DMSO alone and with TRULI 5 µM and 10 µM for 24 h, observing that both genes are expressed in fibroblasts and there were no changes in their gene expression following treatment with the molecule (Supplementary Fig.1)

4.3.1 Comparison of cellular growth, proliferation and HP target genes expression in healthy and HD Fibroblasts

- Analysis of growth rate and proliferation in control and HD fibroblasts

To assess whether as it has been shown in literature, HD fibroblasts grow more slowly than healthy ones (Aladdin et al., 2019; Jędrak et al., 2018), we analysed the growth of these cells with Incucyte. We analysed each cell type individually and then calculated the number of cells after 24 h with DMSO considering all controls together vs. all patients. In contrast with previously published data, our analysis showed that there is not significative difference in cellular growth between control and HD fibroblasts (Fig. 19 A). An example of the growth curve of each cell type is shown Supplementary Data (Supplementary Fig. 2). The next step is to check specifically the cell proliferation and not the growth curve, to verify that indeed the cells from patients proliferate less than those from controls.

To calculate cell proliferation in these fibroblasts, we performed BrdU assays. After 24 h, we analysed the number of BrdU-positive cells compared to the number of total cells, thus obtaining a percentage of how many cells were proliferating at that time compared to the total. The results of these experiments were again unexpected, indeed BrdU data showed no differences in proliferation between control and HD fibroblasts (Fig. 19 B-C), in accordance with cell growth results. The next step then, was to analyse the expression of the Hippo pathway target genes to see if they are deregulated or can explain this discrepancy in observed results.

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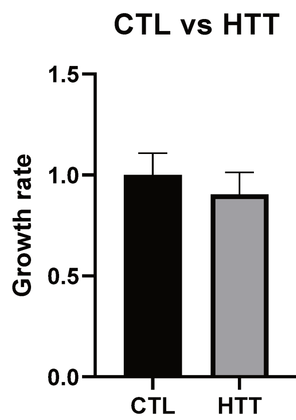

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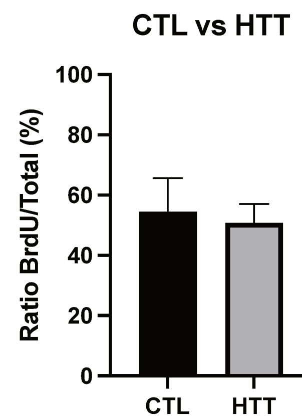


C

DAPI BrdU DAPI+BrdU

CTL1 DMSO

HTT1 DMSO

Figure 19. A. Cell growth analysis of healthy fibroblasts (CTL) versus patient fibroblasts (HTT) after 24-hours incubation with DMSO in Incucyte. The graph is representative of the 4

controls versus the 4 patients. The cell growth between control and HD fibroblast is comparable. Error bars show standard deviation of at least three independent experiments. **B.** Proliferation analysis by BrdU assays of healthy fibroblasts (CTL) versus patient fibroblasts (HTT) after 24-hour incubation with DMSO. The graph represented the percentage of the number of BrdU-positive cells compared to the number of total DAPI-positive cells. The graph is representative of the 4 controls versus the 4 patients, showing no difference between them. **C.** Photos acquired by Cell Discoverer 7 representative of CTL1 and HTT1 treated for 24 h with DMSO. DAPI is represented by the blue filter, BrdU by the green filter, and the merge by both.

- <u>Analysis of HP target genes RNA level in control and HD fibroblasts</u>

As observed in the literature, post-mortem brains of patients were found to have deregulated HP with reduced expression of target genes (Mueller et al., 2018). To test whether this is also reflected in peripheral cells, we started by analysing the RNA levels of the two major representative HP targets: *Cyr61* and *CTGF* in the previously mentioned 8 types of fibroblasts. As before, we incubated the cells for 24 h and then analysed the expression level of these two genes. The results of the RTqPCRs, however, showed no variation in RNA levels of these two target genes in HD fibroblasts compare to control (Fig. 20).

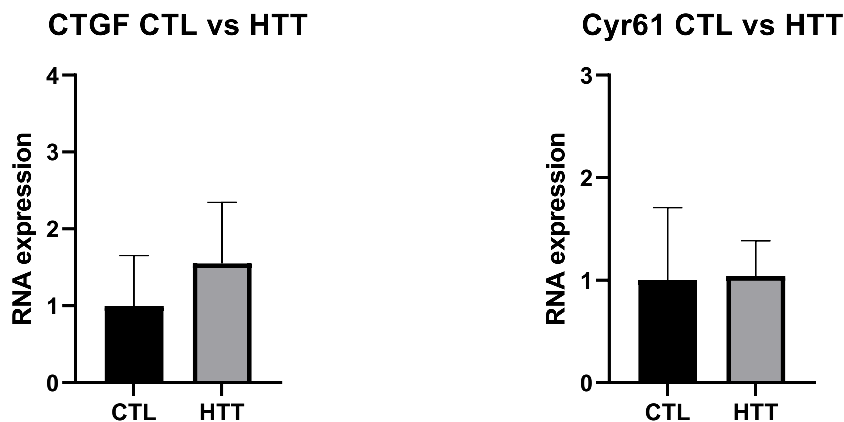


Figure 20. RNA expression of *CTGF* (graph on the left) and *Cyr61* (graph on the right) in control fibroblasts versus HD fibroblasts after 24 h with DMSO. RTqPCRs show no significative difference in RNA expression of the two target genes between HD and control fibroblasts. Data are normalized on housekeeping gene *MAN2B1*. Error bars show standard deviation of at least three independent experiments.

Given the results of CTGF and Cyr61, we searched for other potential targets of Hippo pathway involved in proliferation, cell growth and survival, to observe whether these are deregulated in HD fibroblasts (Fig. 21).

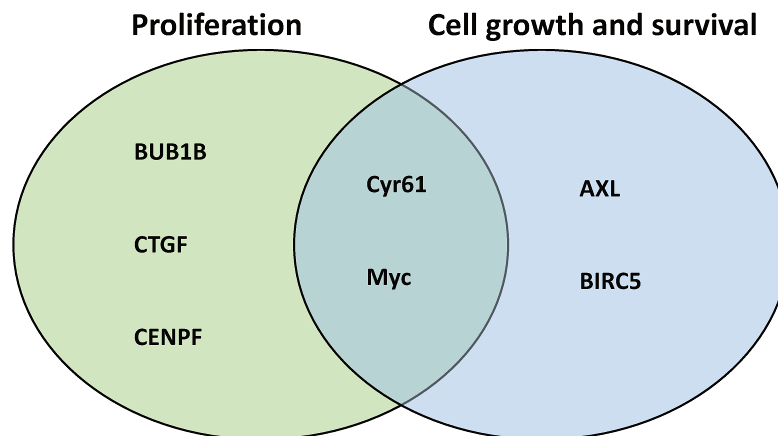


Figure 21. Graphical representation of the analysed HP target genes, divided according to their involvement in cell proliferation and/or cell growth and survival.

We selected AXL Receptor Tyrosine Kinase (Axl), which is another known target of HP (M. Z. Xu et al., 2011), and is involved in cell growth and survival (Axelrod & Pienta, 2014). Moreover, we selected baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5, called also Survivin), which is also a well-known target and it is involved in cell survival and inhibition of apoptosis (Felley-Bosco & Stahel, 2014; F. Li et al., 1998; K. Ma et al., 2016). In addition, through a cross-search of the literature and using the bioinformatic tool DAVID (https://david.ncifcrf.gov/home.jsp) (D. W. Huang et al., 2009; Sherman et al., 2022), we selected other known targets of Hippo pathway that are also interestingly upregulated following TRULI treatment (N. Kastan et al., 2021). These targets are: MYC Proto-Oncogene (Myc), Centromere Protein F (CENPF) and Mitotic Checkpoint Serine/Threonine Kinase B (BUB1B) (Zanconato et al., 2015). These targets are all involved in cell proliferation, cell growth or survival. In particular, Myc regulates proliferation, growth and apoptosis (D. M. Miller et al., 2012); CENPF is involved in chromosome segregation during cell division (Liao et al., 1995; Varis et al., 2006) and BUB1B is involved in spindle checkpoint function and chromosome segregation (G. K. T. Chan et al., 1999), so the latter two are also markers of proliferation. We analysed the RNA levels of these targets in the four HD fibroblast types and their respective controls with the same condition used for CTGF and Cyr61.

Interestingly, no downregulation of RNA levels emerged for these target genes either. (Fig. 22). Thus, we can state that with regard to this small pool of 8 samples we analysed (4 patients vs. 4 controls) we did not find a dysregulation of the HP target genes and that their expression is generally comparable between the patient and control fibroblasts, at least at this stage of the disease.

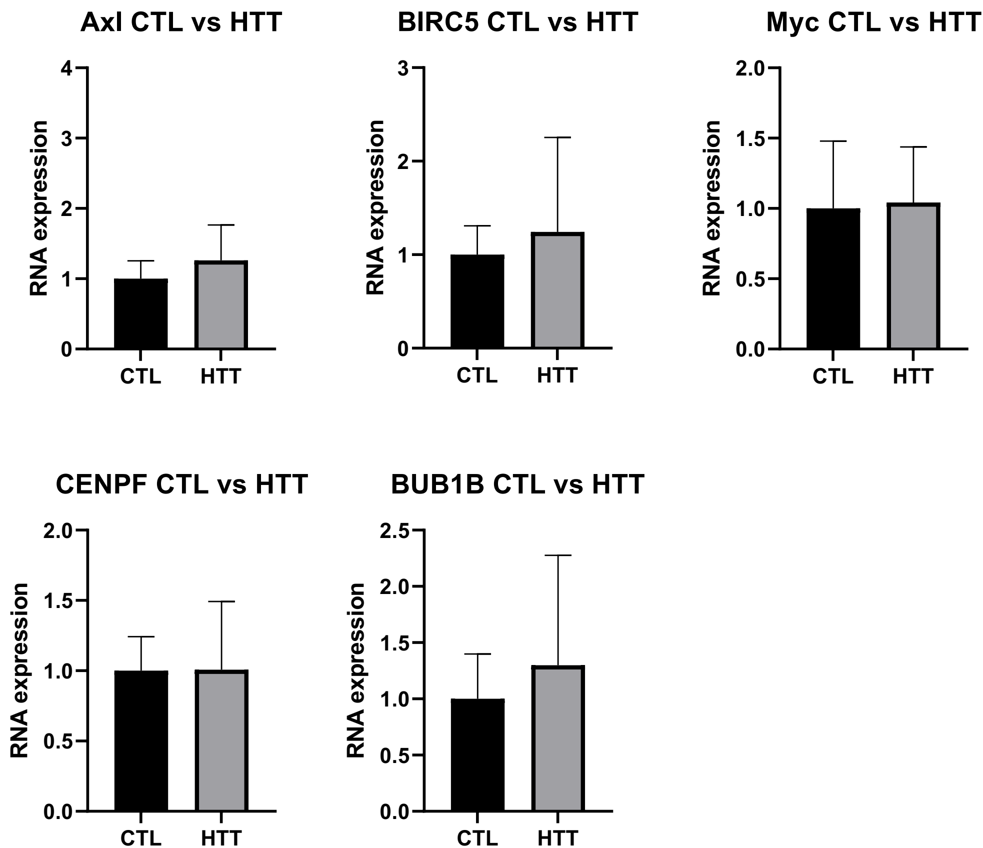


Figure 22. RNA expression of HP target genes (*Axl*, *BIRC5*, *Myc*, *CENPF* and *BUB1B*) in control fibroblasts versus HD fibroblasts after 24 h with DMSO. The graphs are representative of the 4 controls versus the 4 patients. No significant changes in the expression levels of these genes were observed between HD fibroblasts and controls. Data are normalized on housekeeping gene *MAN2B1*. Error bars show standard deviation of at least three independent experiments.

Contrary to previous published studies, our data show no differences in cell growth, proliferation and no HP target genes deregulations in HD fibroblasts compare to healthy fibroblasts. However, a limitation of this study is that the number of samples at our

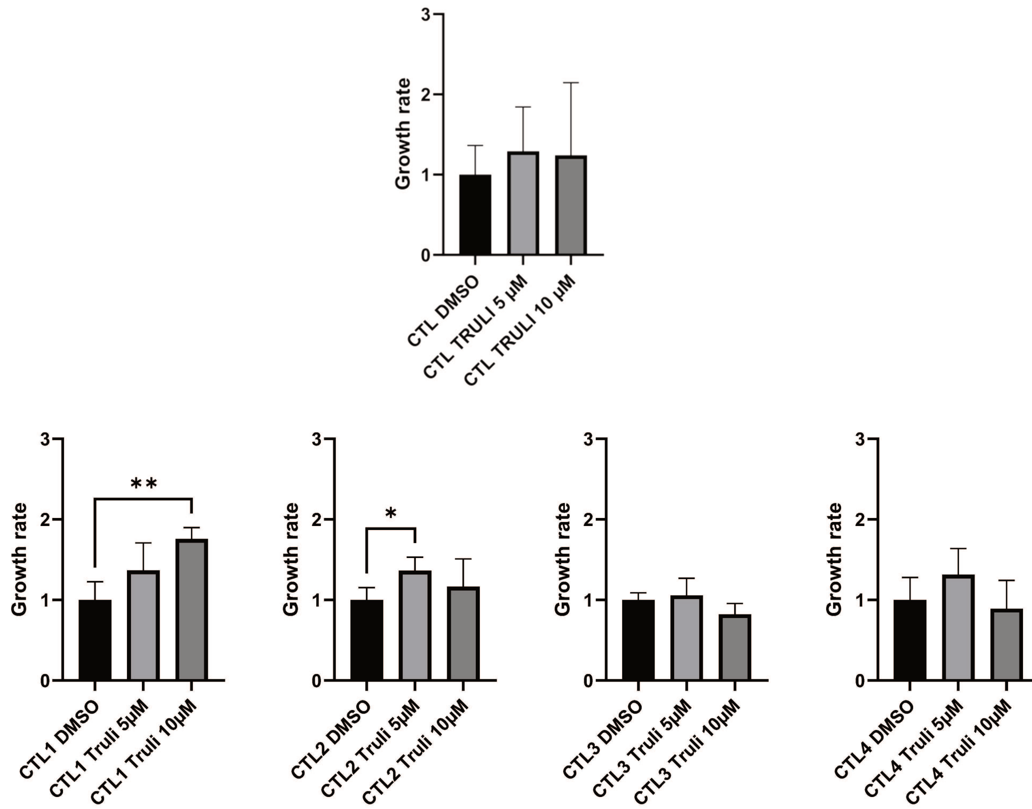
disposal was restricted; therefore, the number of fibroblasts to be analysed should be expanded in order to confirm these results.

Having observed that in fibroblasts taken from HD patients, cell growth and proliferation are not reduced and that the expression of HP target genes is not deregulated, we analysed the effect of TRULI only on fibroblasts taken from healthy individuals to see the effects of this molecule on fibroblasts and to confirm that it is able to increase these parameters in this cell type as well.

4.3.2 Effect of TRULI on Cellular Growth in Fibroblasts

Given the unexpected results obtained in HD fibroblasts, in which we showed that in our analysed samples cell growth and proliferation were not reduced and that the expression of HP target genes was not altered, we proceeded to analyse the effect of TRULI on control fibroblasts and demonstrate that even in this cell type the molecule is able to increase cell growth and the expression of HP target genes as observed previously in HEK293T and smNPCs. Therefore, in parallel with the analysis of the basal cell growth, we also observed how these cells behaved following treatment with TRULI 5 µM and TRULI 10 µM. We therefore treated the 4 control samples with TRULI at the two selected concentrations, as well as with DMSO, and analysed cell growth after 24 hours of treatment. Our results revealed that in general, when analysing all 4 samples together, there was no significant increase in cell growth following treatment with TRULI (Fig. 23 A-B). However, observing the great variation in the results obtained in the 4 different samples, evidenced by the large standard deviation obtained, we specifically analysed the effect of TRULI in the individual samples and noted that this is not equal. In CTL1, it can be seen that TRULI 10 µM significantly increases cell growth, while in CTL2, it is TRULI 5 µM that increases it, and for the other two samples, no significant difference in cell growth is observed following treatment (Fig. 23 A). From these results, it is evident that the effect of TRULI treatment is strictly cell-dependent (at least for this small pool of individuals observed), indeed, in half of the samples tested, the molecule increased cell growth with at least one of the two concentrations tested, while in the other two, no significant effects were obtained. It is therefore clear that it is necessary to increase the number of samples to be analysed in order to verify these results. The next step is to observe the effect of TRULI on proliferation of these samples observed by BrdU assay.

A



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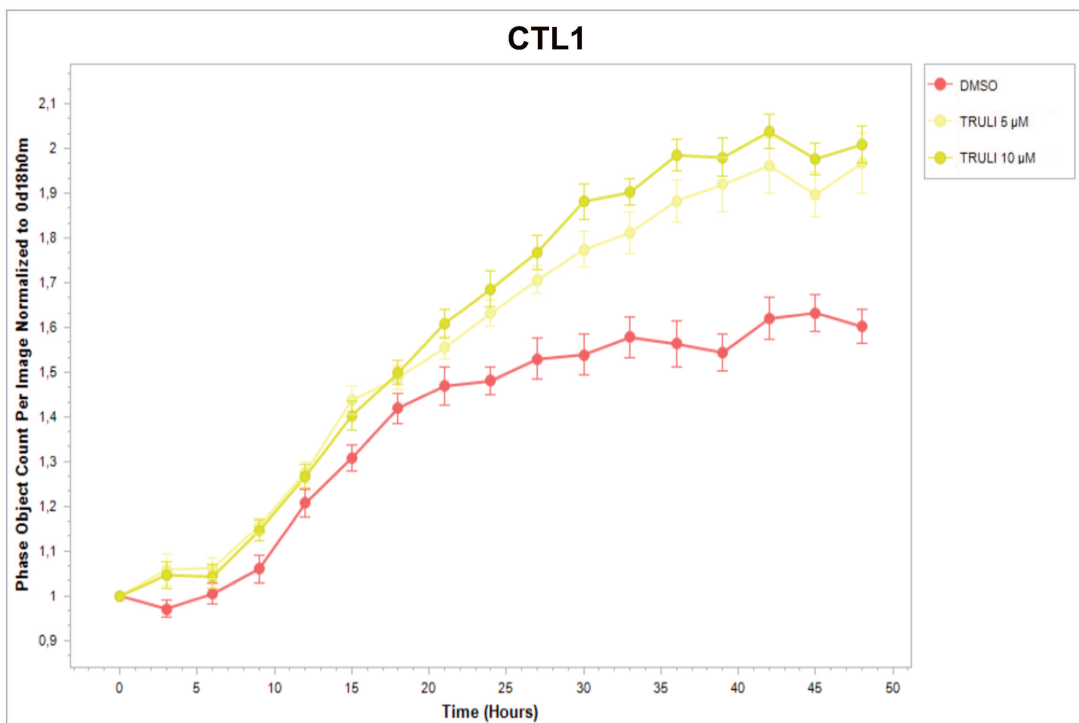

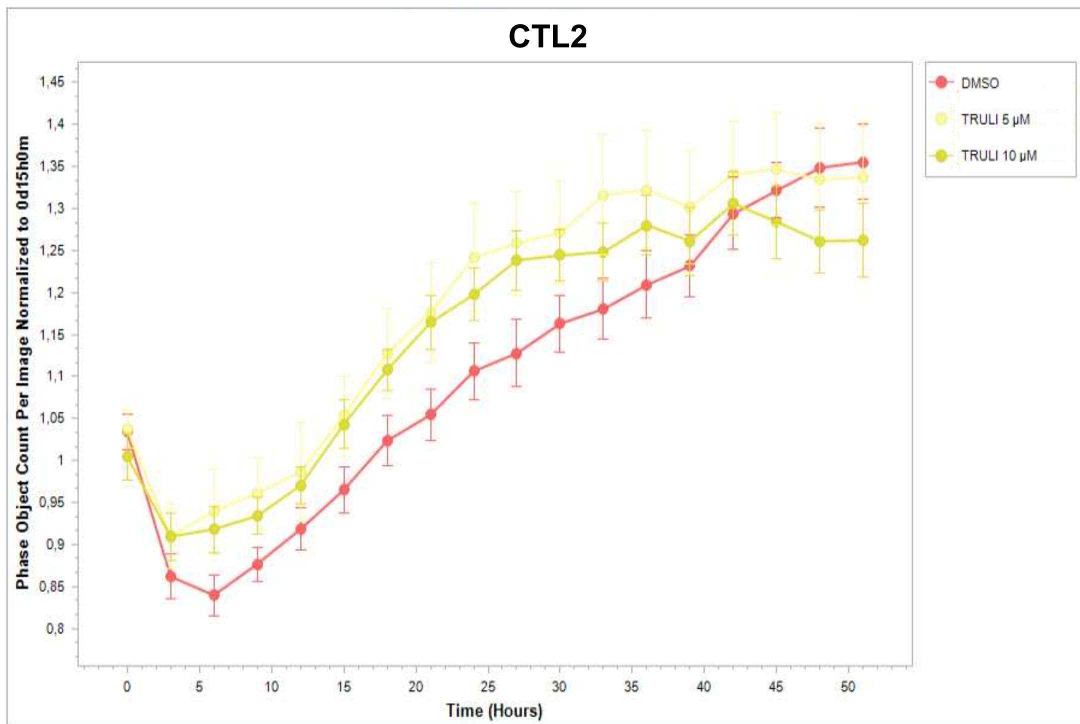
CTL2
DMSO
TRULI 5 µM
TRULI 10 µM
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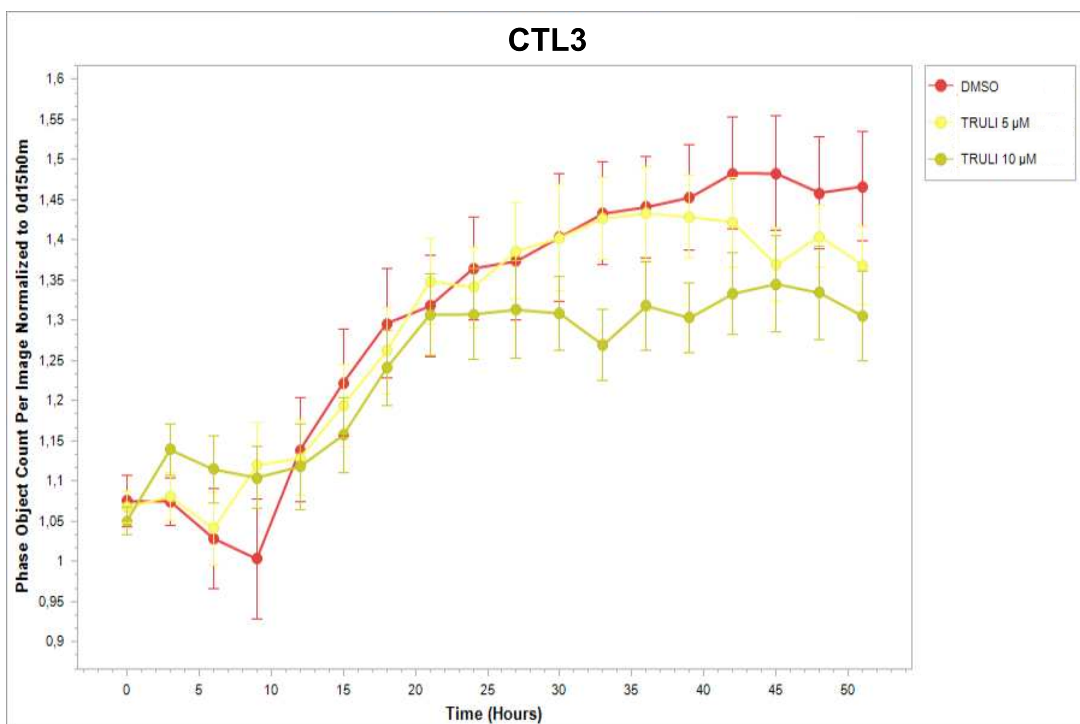
CTL3
DMSO
TRULI 5 µM
TRULI 10 µM
Phase Object Count Per Image Normalized to 0d15h0m
Time (Hours)
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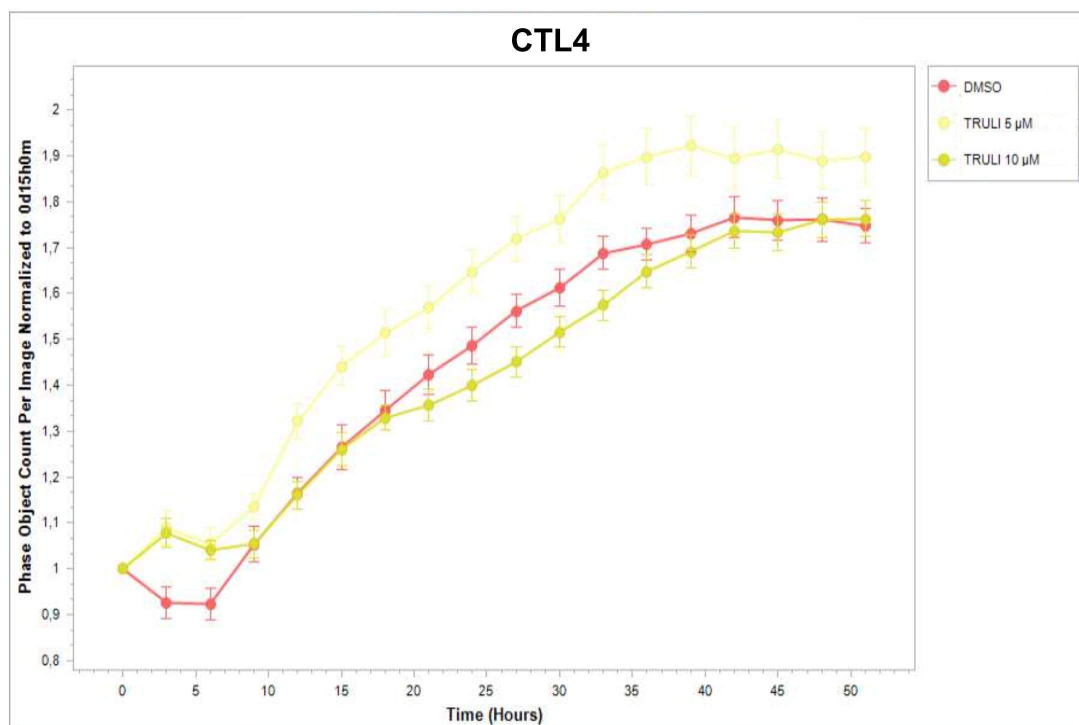
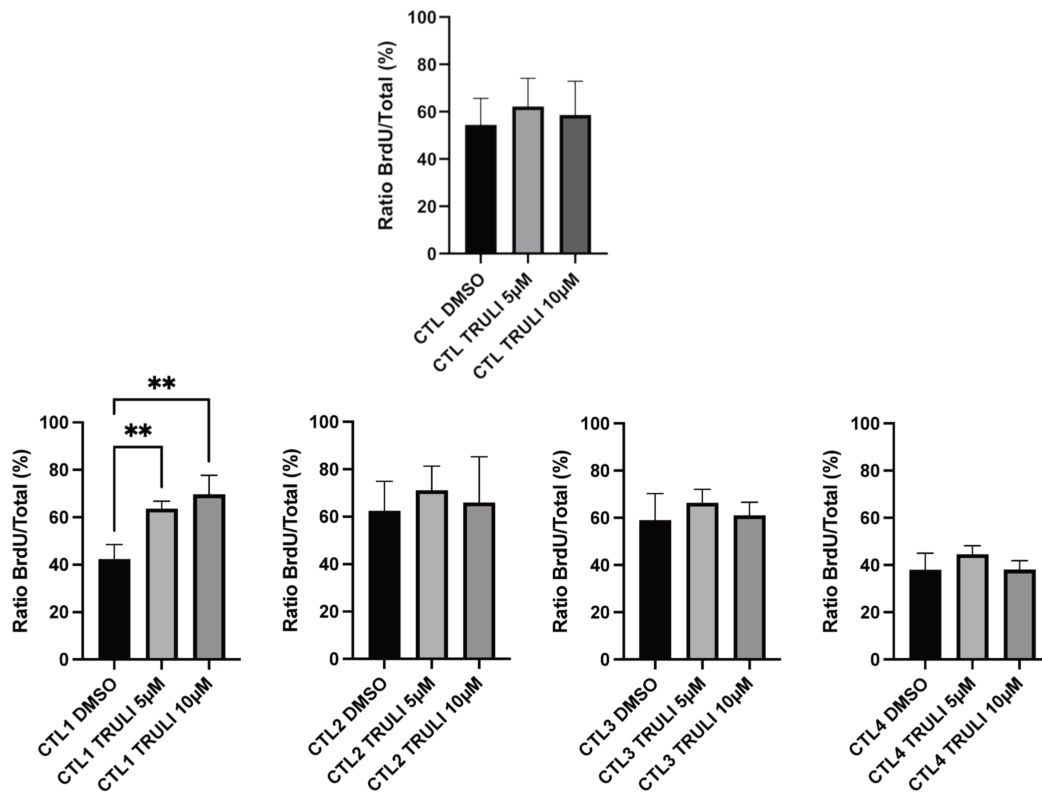


Figure 23. A. Cell growth analysis of healthy fibroblasts (CTL) after 24-hour incubation with DMSO, TRULI 5 μM and 10 μM in Incucyte. The graph on top is representative of the 4 controls; while the graphs below represent the treatment in each sample individually. Each graph is normalized to its respective control DMSO. CTL1 have significantly greater cell growth with 10 μM TRULI treatment (CTL1 DMSO vs CTL1 TRULI 10 μM p=0.0039; Ordinary one-way ANOVA test). CTL2 have greater cell growth with 5 μM TRULI treatment (CTL2 DMSO vs CTL2 TRULI 5 μM p=0.0291, Kruskal-Wallis test). Error bars show standard deviation of at least three independent experiments (*p<0.05; **p<0.01). **B.** Cell growth curve generated after software analysis of photos acquired every 3 hours via Incucyte. The figure shows 4 graphs representing the cell growth of the 4 control lines (CTL1, CTL2, CTL3, CTL4) treated with DMSO, TRULI 5 μM and TRULI 10 μM.

4.3.3 Effect of TRULI on Proliferations in Fibroblasts

In order to assess the effects of TRULI on fibroblasts proliferation and verify that the effects on cell proliferation correspond with those seen previously on cell growth, we performed BrdU assays on the 4 samples from healthy individuals after treatment with TRULI 5 μM and TRULI 10 μM for 24 h; treatment with DMSO was used as a negative control. Analysing then the BrdU-positive cells, we observed that in general considering the 4 samples, TRULI treatment appears to have no significant effect on proliferation

(Fig. 24 A). Specifically, when analysing each sample individually, we noticed that only in CTL1 TRULI led to a significant increase in proliferation, with both concentrations used; whereas in the other 3 samples we did not observe a significant effect on proliferation (Fig. 24 A). This again suggests that the effect of TRULI on fibroblasts is cell-dependent, in fact in the four samples analysed only in one treatment with the molecule lead to an increase in proliferation. The last step then is to check how the expression of HP target genes in these fibroblasts is influenced by the treatment with TRULI, and whether as observed so far these also vary between individuals.



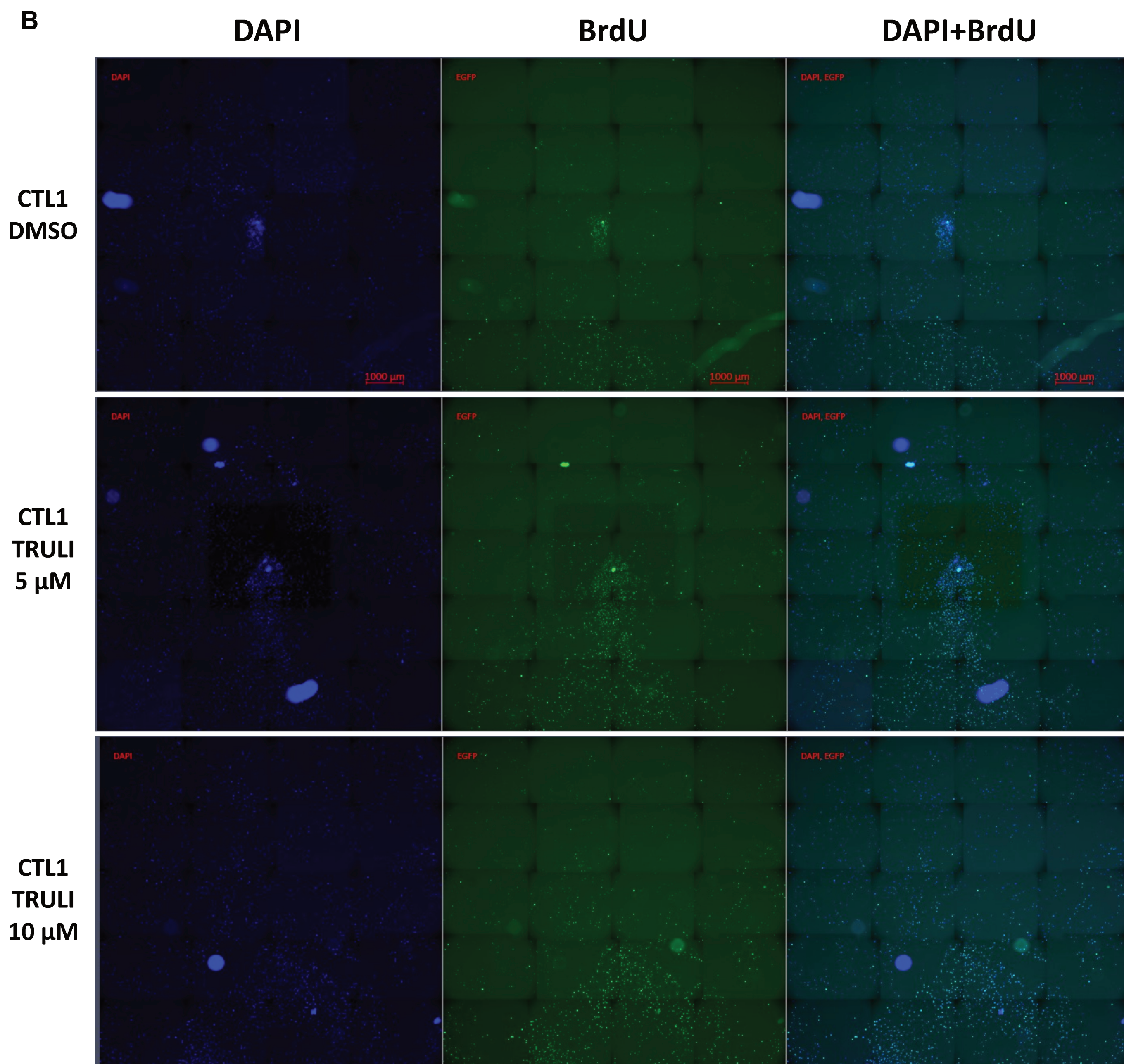


Figure 24. A. Proliferation analysis by BrdU assays of healthy fibroblasts (CTL) after 24-hours treatment with DMSO, TRULI 5 µM and TRULI 10 µM. The graphs represented the percentage of the number of BrdU-positive cells compared to the number of total DAPI-positive cells. The first graph is representative the treatment of the 4 controls analysed together and normalized on CTL DMSO; while the graphs below represent the treatment in each sample individually. Each graph is normalized to its respective control DMSO. In CTL1, the effect of TRULI is dose dependent and statistically increases proliferation at 5 µM and more at 10 µM (CTL1 DMSO vs CTL1 TRULI 5 µM p=0.0095, CTL1 DMSO vs CTL1 TRULI 10 µM

p=0.0028; Ordinary one-way ANOVA test). Error bars show standard deviation of at least three independent experiments (*p<0.05; **p<0.01). **B.** Photos acquired by Cell Discoverer 7 representative of CTL1 treated for 24h with DMSO, TRULI 5 μM and TRULI 10 μM. DAPI is represented by the blue filter, BrdU by the green filter, and the merge by both. Compared to the DMSO-treated control, treatment with TRULI increases the number of BrdU-positive cells compared to the total cell number.

4.3.4 Effect of TRULI on HP target genes RNA level in Fibroblasts

In order to observe the effect that TRULI has on the gene expression of the HP targets, we analysed the mRNA levels of all target genes seen previously after treatment with DMSO, TRULI 5 μM and TRULI 10 μM, for 24 hours. The RTqPCR data were surprising for two main reasons: the first is that the effect of the compound on the HP target genes is not the same in the various cells but can vary from individual to individual, where in some samples the effect is greater than in others and *vice versa*; the second is that the RNA levels of the two main target genes *CTGF* and *Cyr61* decrease following treatment with TRULI, sometimes even drastically (Fig. 25). In general, for all genes we noticed how the expression of these varies greatly sample by sample, as can be seen by observing the large standard deviation of the graphs in which we analysed all control together (Fig. 25). Even tough there is not significative change in RNA level between treatment and DMSO, looking gene by gene, *CTGF* RNA levels drop drastically and significantly with TRULI treatment in CTL2, CTL3 and CTL4 (Fig. 25 A). In contrast, in CTL1 there are no significant changes in the expression among the treatments. However, it is evident how surprisingly *CTGF* expression decreases in almost all the fibroblasts analysed following treatment with TRULI, which should instead increase its expression, as also seen previously in other cell models, such as HEK293T and smNPC.

Similar to *CTGF*, the overall expression of *Cyr61* upon treatment does not change, but analysing the effect of TRULI on the samples individually, the RNA levels decrease dramatically in CTL2, CTL3 and CTL4 (as observed also for CTGF) (Fig. 25 B). Specifically, we observed a decrease in *Cyr61* RNA levels in CTL2 and CTL4 with both concentrations, while in CTL3 only TRULI 5 μM reduces significantly *Cyr61* expression. Taken our findings together, we observed how surprisingly the expression levels of the two major target genes of YAP/TAZ-TEAD, *CTGF* and *Cy61*, decrease

following treatment with TRULI, suggesting that other mechanisms may be involved in this regulation.

For *Axl*, the effect on expression is different from *CTGF* and *Cyr61*, as there are no significative differences in RNA levels after the treatment (Fig. 25 C).

On the other hand, the results obtained analysing the expression levels of *BIRC5*, were drastically different compared to the data obtained for *CTGF* and *Cyr61* as TRULI 5 μM increases *BIRC5* RNA levels considering all controls together (Fig. 25 D). Specifically, the analysis of the individual samples revealed an increase of *BIRC5* RNA level in CTL1 and CTL3 with both concentrations, while in CTL4 only with 5 μM (Fig. 25 D).

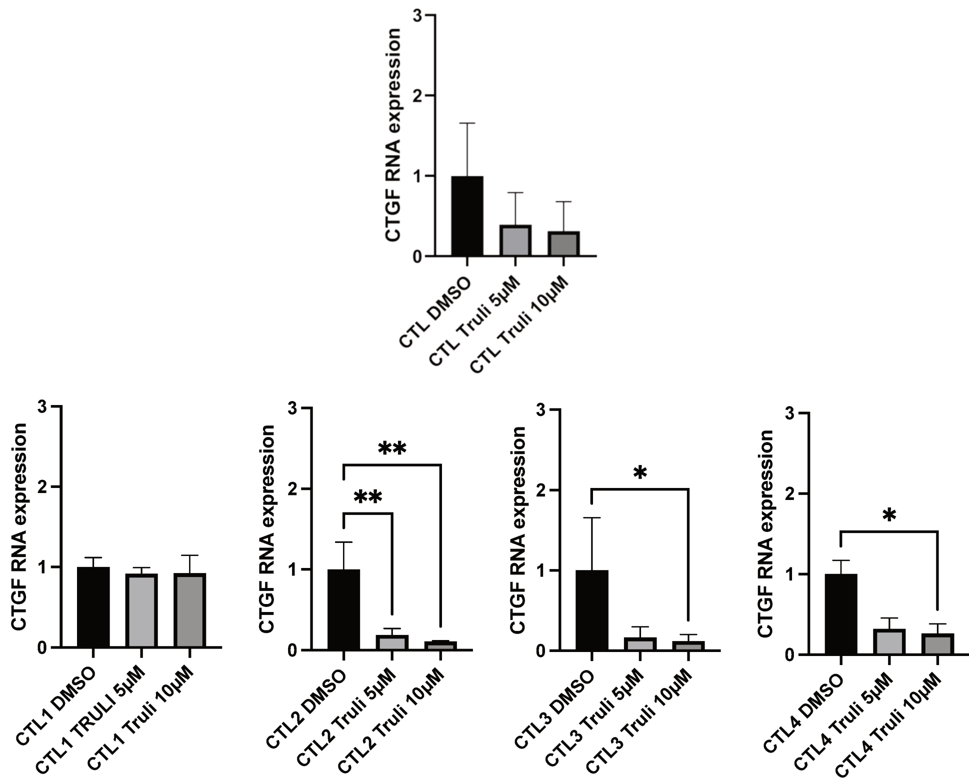
Myc expression levels does not change compare to DMSO, indeed the RNA level significantly increases only in CTL3 with TRULI 10 μM (Fig. 25 E).

Similar to *BIRC5*, *CENPF* expression levels increase after treatment with TRULI (Fig. 25 F). Indeed, both concentrations of the molecule generally increase the expression of this target gene. In particular, RNA levels increase significantly in CTL1, CTL3, CTL4, both with TRULI 5 μM and 10 μM.

Finally, TRULI increases *BUB1B* expression levels in half of the samples (Fig. 25 G). Indeed, we observed a significant increase in CTL1 and CTL4 with both concentrations, even tough analysing all controls together the increase upon treatment is not significative compare to DMSO.

In conclusion, the RTqPCR data gave us interesting and unexpected results. Firstly, we observed that for all the genes analysed, treatment with TRULI produces an effect that is not always the same in the different samples, suggesting that the observed differences are due to individual variability. Furthermore, we showed that in the majority of fibroblasts examined, treatment with TRULI leads to a surprising decrease in the expression of *CTGF* and *Cyr61*, unlike the other cellular models observed previously, suggesting that some other mechanisms are involved in this regulation. On the other hand, we showed that the expression of the target genes *BIRC5*, *CENPF* and *BUB1B* increases following TRULI treatment.

A



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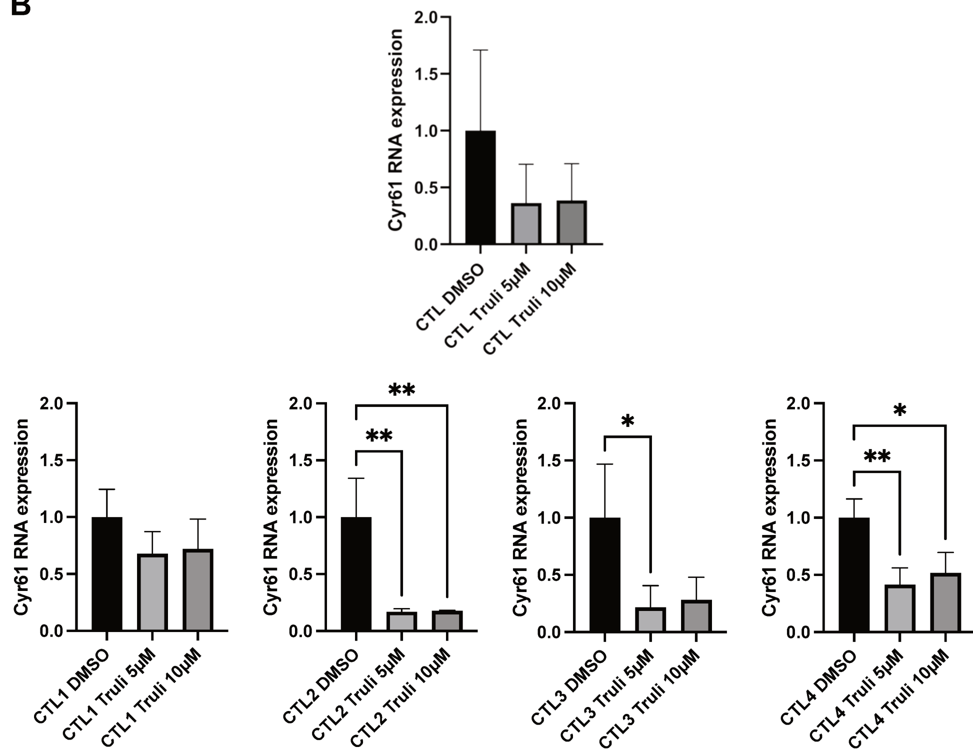

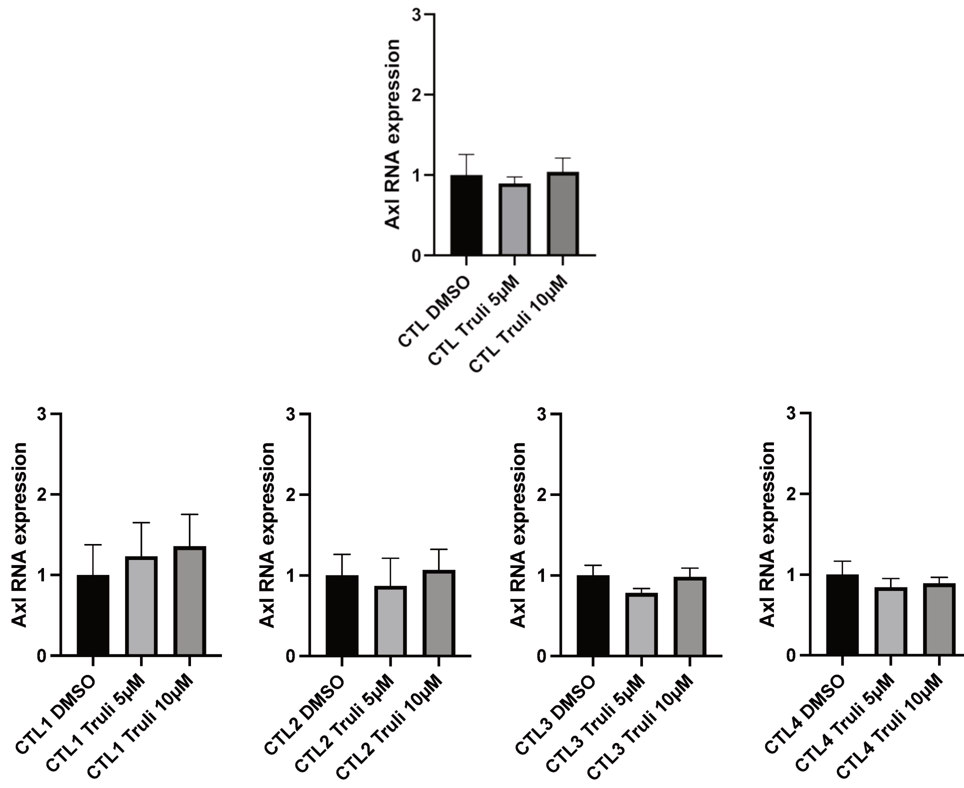
C
Axl RNA expression
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2
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CTL Truli 10µM
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CTL1 Truli 5µM
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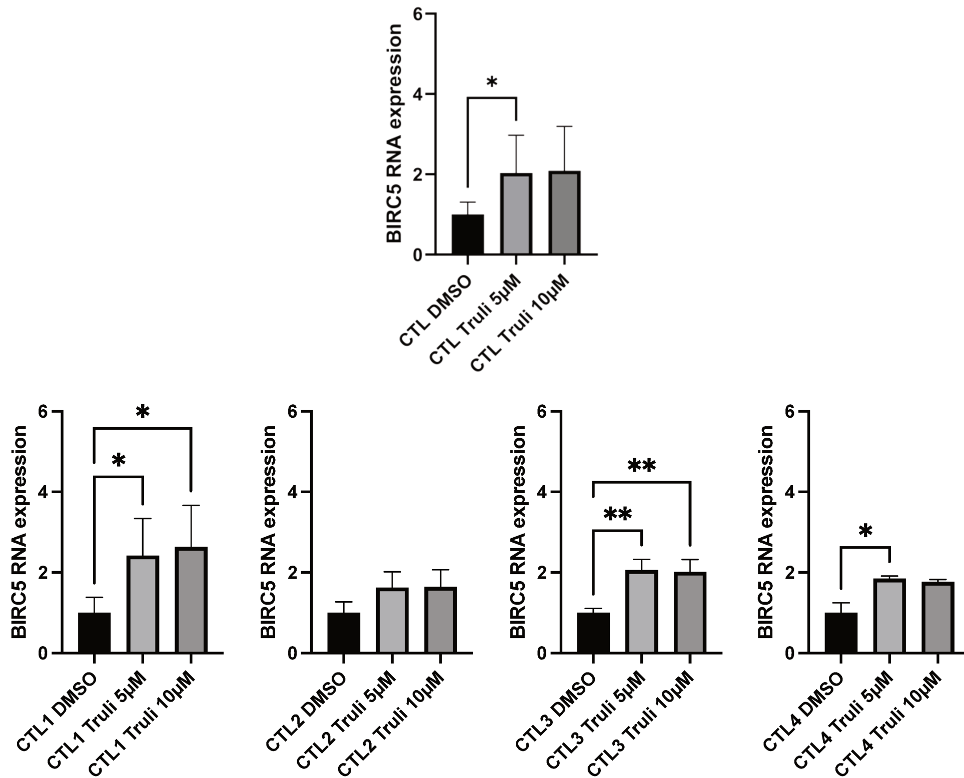
D
BIRC5 RNA expression
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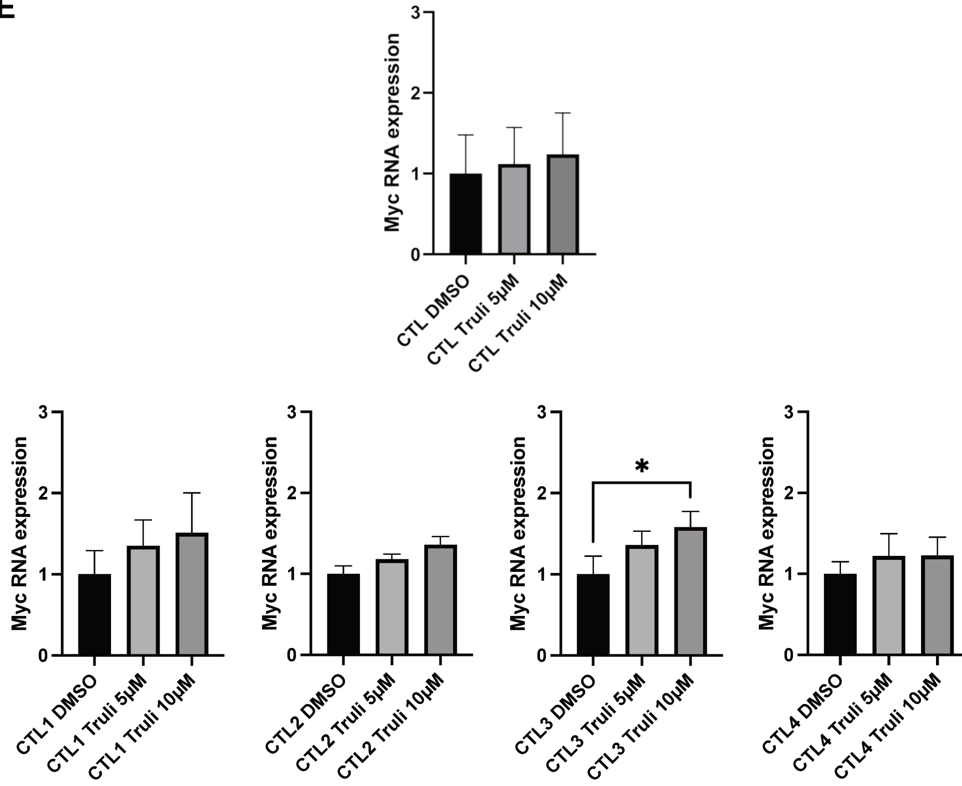

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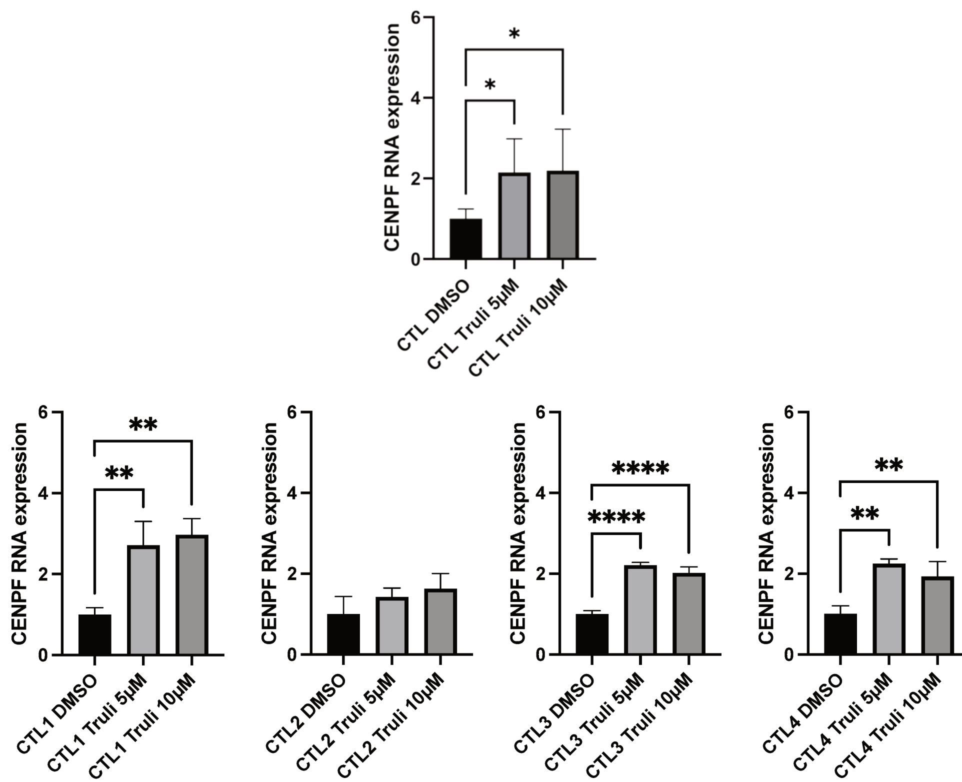

G

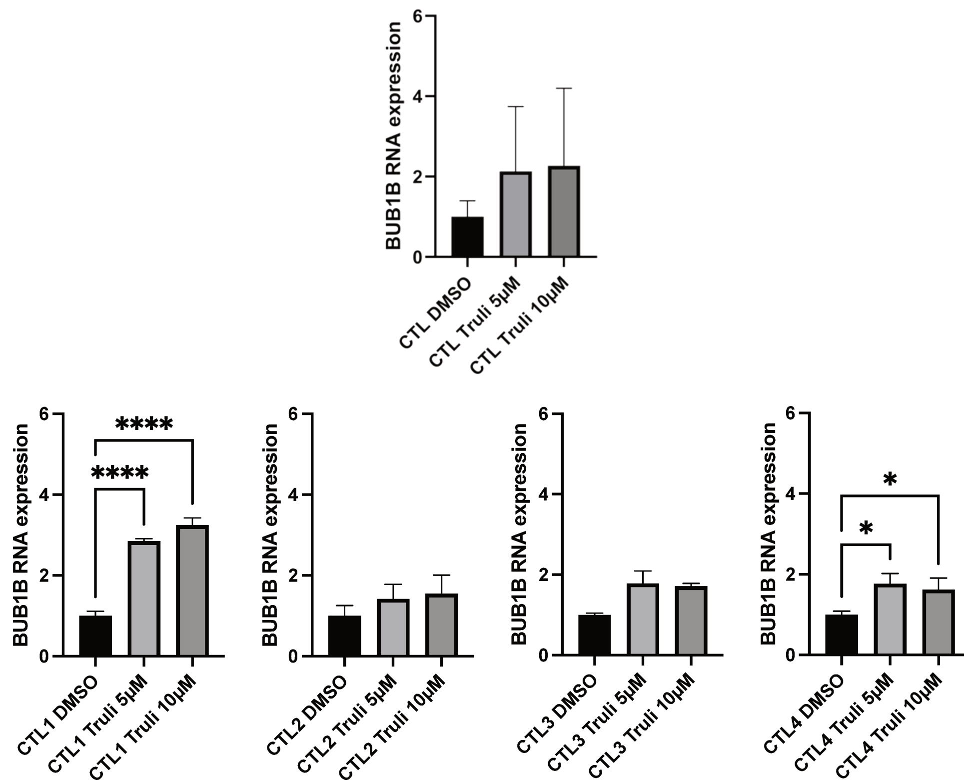

Figure 25. RNA expression of HP target genes (*CTGF*, *Cyr61*, *Axl*, *BIRC5*, *Myc*, *CENPF* and *BUB1B*) in control fibroblasts after 24 h of treatment with DMSO, TRULI 5 µM and TRULI 10 µM. In each 89anel the graph on top is representative of the 4 controls analysed together; while the graphs below represent the treatment in each sample individually. **A.** *CTGF* expression statistically decreases in CTL2 at both TRULI concentrations, and in CTL3 and CTL4 statistically decrease with TRULI 10 µM (CTL2 DMSO vs CTL2 TRULI 5 µM p=0.0047, CTL2 DMSO vs CTL2 TRULI 10 µM p=0.0029, Ordinary one-way ANOVA test; CTL3 DMSO vs CTL3 TRULI 10 µM p=0.0341, Kruskal-Wallis test; CTL4 DMSO vs CTL4 TRULI 10 µM p=0.0341, Kruskal-Wallis test). **B.** *Cyr61* expression statistically decreases in CTL2 and CTL4 at both TRULI concentrations, and in CTL3 statistically decrease with TRULI 5 µM (CTL2 DMSO vs CTL2 TRULI 5 µM p=0.0039, CTL2 DMSO vs CTL2 TRULI 10 µM p=0.0041, Ordinary one-way ANOVA test; CTL3 DMSO vs CTL3 TRULI 5 µM p=0.0341, Kruskal-Wallis test; CTL4 DMSO vs CTL4 TRULI 5 µM p=0.0083, CTL4 DMSO vs CTL4 TRULI 10 µM p=0.0197, Ordinary one-way ANOVA test) **C.** *Axl* expression does not change after TRULI treatment **D.** *BIRC5* expression statistically increases in all CTL TRULI 5 µM vs all CTL DMSO (CTL DMSO vs CTL TRULI 5 µM p= 0.0482, Kruskal-Wallis test). Specifically, in CTL1 and CTL3 the increase is observed at both TRULI concentrations, in CTL4 with only TRULI 5 µM (CTL1 DMSO vs CTL1 TRULI 5 µM p=0.0482, CTL1 DMSO vs CTL1 TRULI 10 µM p=0.0285, Kruskal-Wallis test; CTL3 DMSO vs CTL3 TRULI 5 µM

p=0.0031, CTL3 DMSO vs CTL3 TRULI 10 μM p=0.0037, Ordinary one-way ANOVA test; CTL4 DMSO vs CTL4 TRULI 5 μM p=0.0341, Kruskal-Wallis test). **E.** *Myc* expression statistically increase in CTL3 with TRULI 10 μM (CTL3 DMSO vs CTL3 TRULI 10 μM p=0.0341, Kruskal-Wallis test). **F.** *CENPF* expression statistically increases in all CTL TRULI 5 μM and 10 μM vs all CTL DMSO (CTL DMSO vs CTL TRULI 5 μM p= 0.0372, CTL DMSO vs CTL TRULI 10 μM p= 0.0372, Kruskal-Wallis test). Specifically, in CTL1, CTL3 and CTL4 TRULI increases *CENPF* RNA levels with both concentrations (CTL1 DMSO vs CTL1 TRULI 5 μM p=0.0047, CTL1 DMSO vs CTL1 TRULI 10 μM p=0.0023, CTL3 DMSO vs CTL3 TRULI 5 μM p<0.0001, CTL3 DMSO vs CTL3 TRULI 10 μM p<0.0001, CTL4 DMSO vs CTL4 TRULI 5 μM p=0.0017, CTL4 DMSO vs CTL4 TRULI 10 μM p=0.0075, Ordinary one-way ANOVA test). **G.** *BUB1B* expression statistically increases in CTL1 and CTL4 at both TRULI concentrations (CTL1 DMSO vs CTL1 TRULI 5 μM p<0.0001, CTL1 DMSO vs CTL1 TRULI 10 μM p<0.0001, CTL4 DMSO vs CTL4 TRULI 5 μM p=0.0104, CTL4 DMSO vs CTL4 TRULI 10 μM p=0.0264, Ordinary one-way ANOVA test). Data are normalized on housekeeping gene MAN2B1. Error bars show standard deviation of at least three independent experiments (*p<0.05; **p<0.01; ***p<0.001; ****p≤0.0001).

4.3.5 Conclusions

In this last part, we analysed 4 fibroblast samples taken from 4 different HD patients with different ages and gender and 4 fibroblast samples taken from healthy individuals as controls. In these cells we observed the basal growth of HD cells compared to control and showed that there were no substantial differences between control and patient cells. We also compared cell proliferation between controls and HD fibroblasts with BrdU assay and again we observed no significant differences. Unlike what has been observed in the literature (Aladdin et al., 2019; Jędrak et al., 2018), we therefore did not observe a substantial difference in cell growth and proliferation between HD fibroblasts and healthy fibroblasts.

Furthermore, we have interestingly shown how in HD fibroblasts the target genes of the Hippo pathway are not downregulated, as observed in the post-mortem brains of patients. We analysed 7 different HP target genes and none of them were found to be downregulated in HD cells. Hence, the deregulation of HP targets in HD seems to be brain specific; even thought HTT is expressed in all tissues its impact on HP can be variable between tissues.

As no differences in cell growth and proliferation, nor deregulation of HP target genes, were detected in HD fibroblasts compared to healthy fibroblasts, we focused on testing

whether TRULI is as efficient as in HEK293T and smNPCs in increasing YAP/TAZ-TEAD activity in fibroblasts. Therefore, we only analysed the healthy samples.

Surprisingly, treatment with TRULI does not increase cell growth and proliferation in these 4 samples analysed together. However, looking at each individual sample we observed that the compound is able to increase cell growth in two out of four samples, while proliferation increase only in one sample under TRULI treatment.

Finally, by measuring the expression levels of the target genes after TRULI treatment, we showed how surprisingly the expression of the two YAP/TAZ-TEAD target genes *CTGF* and *Cyr61* drops drastically in the majority of the samples, suggesting other mechanisms involved in this regulation or possible off-target effects of TRULI. Among all HP target genes analysed, Axl and Myc show no significant changes after treatment with TRULI. In contrast, the RNA levels of *BIRC5*, *CENPF* and *BUB1B* increased with TRULI treatment.

In conclusion, we have seen how the effect of TRULI in fibroblasts does not mirror that observed in HEK293T and smNPCs. In fact, we observed great variability between different fibroblast samples, suggesting a tissue-specific effect and also variability between individuals.

5 Discussion and Future Directions

Our initial goal was to identify the best molecules capable of increasing YAP/TAZ-TEAD interaction and inducing its activity. To do this, we selected several compounds (available at the beginning of this study) that acted at different steps of the Hippo pathway cascade, as previously described. We selected XMU-MP1, one of the best-known activators in the literature, which inhibits MST1/2, leading to a decrease in YAP/TAZ phosphorylation (Fan et al., 2016). We selected TRULI, which is another activator known to inhibit LATS1/2, and thus similarly decreases phosphorylation of YAP/TAZ (N. Kastan et al., 2021). In addition, we selected Q2 and B22 which act further downstream directly on YAP/TAZ-TEAD binding (Pobbati et al., 2019) and included Amb1, a ligand of TEAD discovered by us (Gibault et al., 2018). Finally, we selected ML349, which potentially could affect TEAD activity by inhibiting its depalmitoylation (N. G. Kim & Gumbiner, 2019b; Won et al., 2016). By Luciferase assay, using a synthetic TEAD luciferase reporter, we tested the ability of these compounds to activate YAP/TAZ-TEAD binding. For all the molecules, we tested different concentrations to determine which was the best at activating the binding, and

surprisingly among all the molecules tested, only TRULI significantly increased TEAD activity. The best effect was obtained using TRULI 5 µM and TRULI 10 µM, with an increase in the order of 10 to 15-fold compared to the control. Unlike TRULI, molecules such as Amb1, Q2 and ML349 have no effect on TEAD activity. Surprisingly, B22 and XMU-MP1 not only did not increase TEAD activity but actually decreased it. Specifically, XMU-MP1 decreased TEAD activity in a dose-dependent manner. This is surprising because it has been observed in the literature that this compound is able to promote YAP activation, increase its nuclear localisation and in turn increase its target genes *CTGF* and *Cyr61* (Fan et al., 2016). However, XMU-MP1 is controversial, indeed, it is able to decrease the phosphorylation of YAP and increase its activity, but on the other hand, there are also evidences in the literature that it does not always lead to YAP activation. For example, Kastan and colleagues observed that this compound reduces the amount of phosphorylated YAP, but it also significantly reduces the amount of total YAP, thus leading to an actual increase in the ratio of phosphorylated YAP to total YAP; furthermore, they did not observe an increase in proliferation following treatment with this molecule, contrary to what has been observed previously (N. Kastan et al., 2021). Furthermore, it was shown that XMU-MP1, in human mini-organs, not only does not increase active YAP and proliferation, but even induces growth arrest and disrupts the cell cycle (Mitchell et al., 2020). These conflicting results observed in literature may be explained by potential off-target effects, with XMU-MP1 being able to strongly inhibit 21 other kinases in addition to MST1/2 (Fan et al., 2016); or the discrepancy in observed effects could be due to the different tissues in which the molecule was tested and thus could have a tissue-specific effect. This could also explain why the other molecules did not induce TEAD activity in our model. From the group of compounds we tested, one winner clearly emerged, namely TRULI, the LATS1/2 kinase inhibitor. As shown by Kastan and colleagues, this molecule is able to decrease the phosphorylation of YAP, increase its nuclear localisation and induce cell proliferation (N. Kastan et al., 2021). Consistent with this study, our dose response luciferase experiments showed that the concentration that most increases TEAD activity is 10 µM. We therefore selected this activator for the continuation of our experiments. In addition to 10 µM, we decided to use also 5 µM that increased TEAD activity up to 10-fold compared to the control. With a view to future use of this compound as a drug, it is in fact preferable to use a reduced concentration of the molecule in order to minimise side or off-target effects but still have a considerable pharmacological response. Interestingly, the results of TRULI luciferase assay are very similar to those recently obtained by Shalhout and colleagues with

the compound PY60 (Shalhout et al., 2021). Using a luciferase assay similar to ours in HEK293 cells, this team have shown that PY60 increases TEAD activity in a dose-dependent manner; this result is comparable to the result obtained in our study using TRULI in the same cell line. This molecule acts on a different target than the compounds we tested, opening up the range of potential pharmaceutical targets of the Hippo pathway even further. In fact, PY60, acts on annexin A2, a protein that interacts with YAP, probably guiding it close to the cytoplasmic membrane and allowing phosphorylation by HP cascade kinases. The compound thus releases ANXA2 from the membrane, preventing phosphorylation of YAP and leading to its subsequent activation. Thus, this study has highlighted ANXA2 as a new player in the complex regulation of the Hippo pathway and underlines how new potential HP targets are recently emerging. When we selected the possible activators of YAP/TAZ-TEAD, this study had not yet been published, but it would be interesting to compare our results with the effects of this other promising molecule. This does, however, corroborate our results obtained with TRULI luciferase assay, as it shows how two different activators of YAP, acting on two different targets lead to similar activation of TEAD. However, in addition to the molecules used in this project, compounds that were not yet available at the beginning of this study and that also look promising should be tested in the future. These include other LATS inhibitors such as TDI-011536 (a new compound derived from TRULI), VT02956 and GA-017 (Aihara et al., 2022; N. R. Kastan et al., 2022; S. Ma et al., 2022) or the eicosapeptide 4E, a VGLL4 inhibitor (Adihou et al., 2020).

Having shown that under our experimental conditions TRULI is the best compound in activating YAP/TAZ-TEAD, we tested the effect that treatment with this molecule has on cell growth in HEK293T. Consistent with what was observed in the luciferase experiments, treatment with TRULI 5 μM and 10 μM increased cell growth, with the maximum peak reached after 24 h of treatment, the same treatment time as indicated by Kastan and colleagues. Furthermore, in addition to what the authors saw that TRULI treatment increased proliferation in mice utricles cells, cardiomyocytes and Muller cells in organoids, we showed that the molecule also induces cell growth in HEK293T, as well as activating YAP/TAZ-TEAD. This allowed us to use this very easy-to-handle and efficient cell model for our subsequent studies on the analysis of RNA and protein levels of the YAP/TAZ-TEAD targets.

Having selected the best activator, the best concentrations and the best treatment time, we analysed the RNA and protein levels of the YAP/TAZ-TEAD targets, CTGF and Cyr61. We confirmed that the increase in the binding, indeed, leads to an increase in these classical

and well-known targets and that these are consistent with the observed increase in cell growth. Indeed, we have shown that treatment with TRULI increases *CTGF* and *Cyr61* RNA levels. The increase in expression is remarkable, with TRULI 10 µM leading to an approximately 3-fold increase in both genes, and TRULI 5 µM leading to an approximately 2-fold increase of *Cyr61*. This is important because we have confirmed that 10 µM concentration increases the expression of these target genes to a greater extent but additionally we have shown that half of the concentration (5 µM) leads to a significant increase in the expression of *Cyr61*; this mirrors what we observed with luciferase and cell growth assay. The increased expression of these target genes also in HEK293T, due to treatment with TRULI, confirms the data shown by Kastan where the authors had observed an increase in utricular supporting cells (N. Kastan et al., 2021). Then analysing the protein expression of the same targets in HEK293T we showed that for Cyr61 the increase in protein after TRULI 10 µM treatment closely mirrors the increase in RNA observed previously. However, we were not able to detect CTGF protein by Western blot, and also for Cyr61, even if we succeeded, the protein levels were barely detectable. This is probably due to the fact that these two targets are very poorly expressed in HEK293T, especially CTGF. It is important to emphasise the different gene expression in different cellular models, since as we have shown in our study, treatment with the molecule can also lead to different gene expression in different cellular models. In HEK293T, we have therefore shown that TRULI is the compound that best activates YAP/TAZ-TEAD, significantly increases the expression of their target genes and this probably leads to the increased cell growth observed following treatment with this molecule. Therefore, regarding the different gene expression and the different effect the molecule could have on cells from different tissues, our next step was to observe the effect of TRULI in a model more similar to neural cells, as a potential use of this compound in neurodegenerative diseases such as Huntington's disease or Parkinson's disease. We used smNPCs as a cell model, which as previously described is an excellent model for studying neurodegenerative diseases (Reinhardt et al., 2013; Strauß et al., 2021). We tested both smNPCs wt and smNPCs carrying a triplication of the SNCA gene, which is implicated in Parkinson's disease (Srinivasan et al., 2021). In this way, we were able to test the effects of TRULI on healthy neural-like cells and cells from patients suffering from a neurodegenerative disease in parallel. In smNPCs, under the same experimental conditions used for HEK293T (same TRULI concentrations and same treatment time) we observed a different effect on the expression of the two target genes. Indeed, the increase caused by TRULI on the expression

of the two genes *Cyr61* and *CTGF* is much more remarkable than that seen in HEK293T in both lines of smNPC (wt and tripl). In fact, in HEK293T the increase due to TRULI treatment is in order of 2 to 3-fold for both genes, while in smNPCs the increase is in order of 5-fold for *Cyr61* and of 5 to 10-fold for *CTGF*. Moreover, TRULI increases CTGF mRNA levels in a dose dependent manner in both smNPC wt and tripl. These data highlight the importance of knowing the different effect that treatment with the molecule can cause in different cell lines. Furthermore, when analysing the protein levels of the two targets following treatment with TRULI in the two smNPC lines, we observed that Cyr61, and also CTGF in this case, increase. Thus, in the smNPCs we were able to detect CTGF protein and this is probably because the basal expression of this protein is already higher than in HEK293T and therefore by increasing with TRULI the production of the protein we were able to detect it by Western blot.

Unlike HEK293T, for smNPCs it was not possible to analyse cell growth by quantifying cell numbers individually using Incucyte, because as mentioned above these cells grow by aggregating and the software is unable to distinguish individual cells. Therefore, for these cell lines, we quantified confluence and in agreement with previous data, we showed that treatment with TRULI induces an increase in confluence and thus cells grow more than the control by occupying more surface area of the well. We observed an increase in confluence in both smNPC lines, but with a difference between the two lines. In fact, for smNPC wt, the increase in confluence is obtained only with TRULI 10 μM, whereas for smNPC tripl, surprisingly, confluency increases with both concentrations of the molecule and the effect is comparable, showing that same effect can be reached with half of the concentration. In these models (HEK293T and smNPC), under the experimental conditions used, we demonstrated that TRULI significantly increases YAP/TAZ-TEAD, induces an increase in both the RNA and protein levels of the two targets CTGF and Cyr61, and leads to an increase in cell growth. However, it emerged that the effect of TRULI is not the same in all models and varies from one cell line to the other, and although in all of them both the expression of YAP/TAZ-TEAD targets and cell growth increase, the values of these increases vary, emphasising that the effect of the compound on the different cell lines must be taken into account. We also observed that in many cases the use of TRULI 5 μM is sufficient to cause an increase in target expression and cell growth. This is important for a possible future pharmacological use of this compound, as with half the concentration the same effect can be achieved, reducing the dangers of possible side effects as well as toxicity. Furthermore, we have seen that the compound works equally well in both wt and tripl

smNPCs, and this finding is very promising again from the point of view of a possible pharmacological use of the molecule because we have seen the positive effects in a cell line closer to a model of neurodegenerative disease.

For the last part of the project, we used HD fibroblasts given the difficulty in finding neural cells from post-mortem brains of HD patients. Fibroblast lines are primary cells derived directly from HD patients, unlike artificially constructed models, and as seen in the literature, fibroblasts from HD patients have reduced cell proliferation (Aladdin et al., 2019; Jędrak et al., 2018). We used fibroblasts taken from 4 different patients of different ages and from 4 healthy people. We first checked whether HD fibroblasts actually proliferate less than controls and if the HP target genes are deregulated as observed in HD post mortem brains (Mueller et al., 2018). However, analysing the cell growth of our pool of 4 patients and comparing them with 4 controls, we observed that there was no significant difference in cell growth between patients and controls. Thus, at least for this small pool of patients examined, we did not notice a substantial difference in cell growth compared to healthy individuals.

According to data obtained from cell growth, analysing all the HD fibroblasts together and comparing them to all the controls, even for cell proliferation we did not observe a reduction in HD fibroblasts. Our results were unexpected and surprising as they contrast with what has been observed in the literature, even though the samples we analysed were taken from patients with the same age range and same range of CAG repeat numbers as those taken by Jędrak and colleagues (Jędrak et al., 2018). However, concerning proliferation in HD fibroblasts, there are conflicting data in the literature. As we have seen, there are studies showing that HD fibroblasts have less proliferation than healthy fibroblasts, but there are also studies where the opposite has been seen (Goetz et al., 1981; Hung et al., 2018). Interestingly, Hung and colleagues immortalised fibroblasts taken from HD patients through human telomerase reverse transcriptase (hTERT) to avoid senescence problems in fibroblast culture, and showed that these HD fibroblasts, called TruHD, proliferate more than the control, however these are still more susceptible to cell death and have energy deficits. Given these contradictory data concerning the proliferation of HD fibroblasts, it is necessary to expand the number of patients to be analysed to check whether there is a substantial difference in proliferation between the fibroblasts of HD patients and the fibroblasts of healthy individuals.

After comparing cell growth and proliferation between HD fibroblasts and control fibroblasts, the aim was to analyse HP target genes to see if, as observed in post-mortem

brains, they are also deregulated in HD fibroblasts. RTqPCR data showed, however, that the two classical YAP/TAZ-TEAD targets, CTGF and Cyr61, are not downregulated in HD fibroblasts. Furthermore, our analysis showed that the expression level of these two HP target genes is highly variable. This is not surprising in primary cells such as skin fibroblasts, as it is known that Cyr61 and CTGF in these particular cells are influenced not only by age but also by the exposure to sunlight (and thus ultraviolet radiation) of the individuals from which they were taken (T. Quan et al., 2002, 2010; T. H. Quan et al., 2006). It is clear that these two HP targets can be influenced by various factors (such as age and exposure to sunlight) as well as dysregulation due to disease; therefore, we shifted our attention to other HP target genes: *Axl*, *BIRC5*, *Myc*, *CENPF* and *BUB1B*. Even for these new targets, however, we observed that they were not downregulated in HD. In general, therefore, unlike in post-mortem brains, we did not observe downregulation of HP target genes in HD compared to controls. This suggests either that the dysregulation of HP observed in brains is tissue-specific, therefore in neural cells HP is deregulated while in peripheral cells it is not; or at the stage of the disease at which the fibroblasts were taken dysregulation of HP is not yet observed as is seen in neural cells of dead patients. One hypothesis could be that in the early stage therefore HP is not yet deregulated, whereas as the disease progresses this dysregulation may occur.

Due to unexpected results obtained with HD fibroblasts, we focused our attention on assess the efficiency of TRULI in fibroblasts and see the effect that the molecule has on cell growth, proliferation and expression of HP target genes even in this cell type.

Analysing the HP target genes upon TRULI treatment, we observed that, surprisingly, the two classical HP target genes, *CTGF* and *Cyr61*, decreased dramatically in the majority of fibroblasts analysed. This was an unexpected result, since they are the main and the most studied target genes of YAP/TAZ-TEAD and since in the previously tested models, HEK293T and smNPCs, the expression of these genes increased significantly with TRULI treatment. This therefore suggests that these two targets are differently regulated in fibroblasts. One of the possible causes of their downregulation following treatment with the compound is the possible off-target effects of TRULI. In fact, the authors who developed TRULI showed that there are 34 kinases that have more affinity for the molecule than LATS1/2, and some of these are significantly affected by the compound (N. Kastan et al., 2021). Therefore, it is important to take into account the possible off-target effects of the molecule and the functions of these other potential targets of the compound. Indeed, by examining the other potential targets of TRULI, surprisingly we found ROCK1, that is

implicated in the regulation of YAP and could explain the downregulation of CTGF and Cyr61 in fibroblasts due to TRULI treatment. Indeed, it has been observed that CTGF is a downstream factor of RhoA/ROCK in renal and lung fibroblasts, and it is upregulated through activation of RhoA signalling in keloid fibroblasts (Heusinger-Ribeiro et al., 2001; Z. Huang et al., 2006; Mun et al., 2014). These data are also supported by *in vivo* studies. Indeed, it was observed that CTGF expression is significantly decreased in the cardiac tissue of ROCK$^{-/-}$ and ROCK$^{+/-}$ mice (Rikitake et al., 2005; Y. Zhang et al., 2006). Interestingly, it was observed that YAP/TAZ are activated by Rho-ROCK1 signalling pathway and that high expression of YAP/TAZ correlates with high expression of ROCK1. In particular, it was shown that pharmacological inhibition of ROCK1 leads to an increase in YAP phosphorylation, reduced YAP/TAZ nuclear localization and the significant decrease in *CTGF* and *Cyr61* RNA levels in intestinal fibroblasts (Ou et al., 2021). This is consistent with what we observed in skin fibroblasts with TRULI treatment. Furthermore, these data suggest that the 2 targets, CTGF and Cyr61, may be differently regulated in fibroblasts than in HEK293T and smNPCs. Indeed, it appears that in fibroblasts, the two targets are regulated by the ROCK1/YAP axis and TRULI could also have an effect on this kinase by inhibiting it and leading to a decrease in the expression of CTGF and Cyr61. ROCK1 was only one of the possible alternative targets of TRULI, so further studies will be needed to thoroughly investigate all potential off-target effects of the compound, including at the tissue-specific level.

Regarding the other HP target genes, however, we showed that *BIRC5*, *CENPF* and *BUB1B* increase with TRULI treatment in the majority of the fibroblasts. Even for these genes, the effect of the two concentrations of TRULI on RNA level showed individual variability.

Overall, TRULI increases YAP/TAZ-TEAD activity in the majority of the samples tested (CTL1, CTL3 and CTL4), as shown by the increase of the targets analysed. However, only in CTL1 is visible a phenotypic effect (increase in cell growth and proliferation) and it is also the only one where *CTGF* and *Cyr61* are not downregulated. This suggest that in this sample the phenotypic effect is mainly due to the effect of TRULI on HP. On the contrary in the remaining 2 samples (CTL3 and CTL4) there is no phenotypic effect and additionally *CTGF* and *Cyr61* are downregulated. This implies that TRULI is acting not only on the HP but could have off-target effect that could counteract YAP/TAZ-TEAD activation via LATS inhibition, resulting in no visible impact on cell growth or proliferation. An exception is CTL2 where a decrease in *CTGF* and *Cyr61* is visible and no other HP targets are increased; therefore, it is reasonable to assume that in this sample the increase in cell growth is

probably not due to YAP. This could probably also be due to another off-target effect of TRULI.

From all these results in fibroblasts, we can deduce that the effect of the molecule may vary from individual to individual. Therefore, it seems necessary to study the effect of TRULI in fibroblasts in depth, possibly expanding the number of samples to be analysed to check the observed variability and whether the effects given by the molecule are consistent with those obtained in this first study on fibroblasts taken from a small number of individuals.

On the other hand, we showed how well TRULI works in the other two cellular models analysed (HEK293T and smNPCs), activating YAP/TAZ-TEAD, increasing RNA and protein level of Cyr61 and CTGF and increasing cell growth. It appears that the compound may have a tissue-specific effect. It therefore follows that a tissue-specific analysis is necessary in order to fully understand the effect of this molecule and the regulation it has in different cells. Specifically, it is clear that extensive off-target studies of TRULI are required, given the difference in the effect the molecule has in fibroblasts compared to HEK293T or smNPCs.

In conclusion, we showed for the first time a comparison of different YAP/TAZ-TEAD activators, identifying TRULI as the best molecule in increasing their activity. This compound is able to increase the expression of several YAP/TAZ-TEAD target genes and increase cell growth in different cell models, including smNPCs derived from PD patients, suggesting that TRULI may have a beneficial effect in neuronal models of neurodegenerative diseases. This suggests that TRULI could have a potentially positive effect in neuronal models of Huntington's disease, as it was observed in HD that HP is deregulated and that YAP-TEAD binding and their activity are decreased as well as the expression of their target genes (Mao et al., 2016; Mueller et al., 2018; Yamanishi et al., 2017). Therefore, in the future, it would be of interest to observe neural cell models of Huntington's disease, possibly analysing cells from post-mortem brains of HD patients and *in vivo* using mouse models of HD, to see first whether the Hippo pathway is deregulated and then whether by treatment with TRULI the deregulation can be reversed. It would be useful to assess whether treatment with TRULI induces increased cell survival.

In the future, it would also be interesting to test this molecule in other areas where increased cell proliferation or growth would be beneficial, such as in wound healing and cardiac cell regeneration taking into account the potential off-target effects of TRULI and the tissue-specific effect it appears to have.

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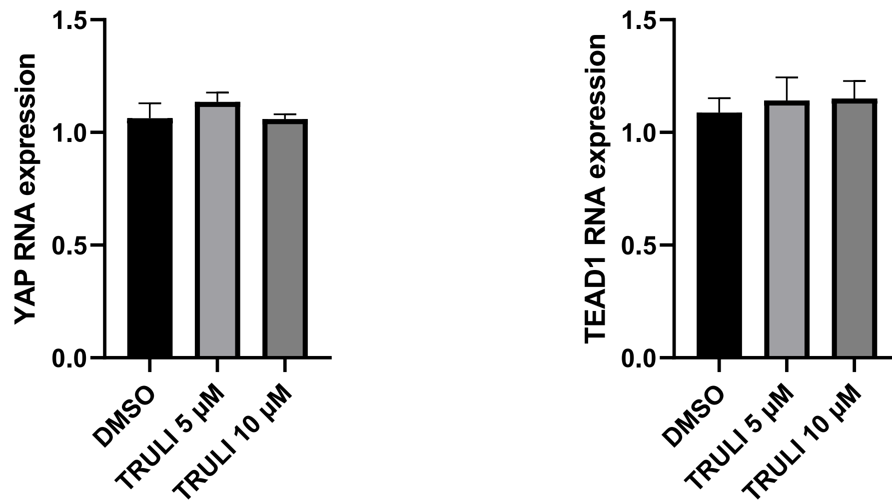
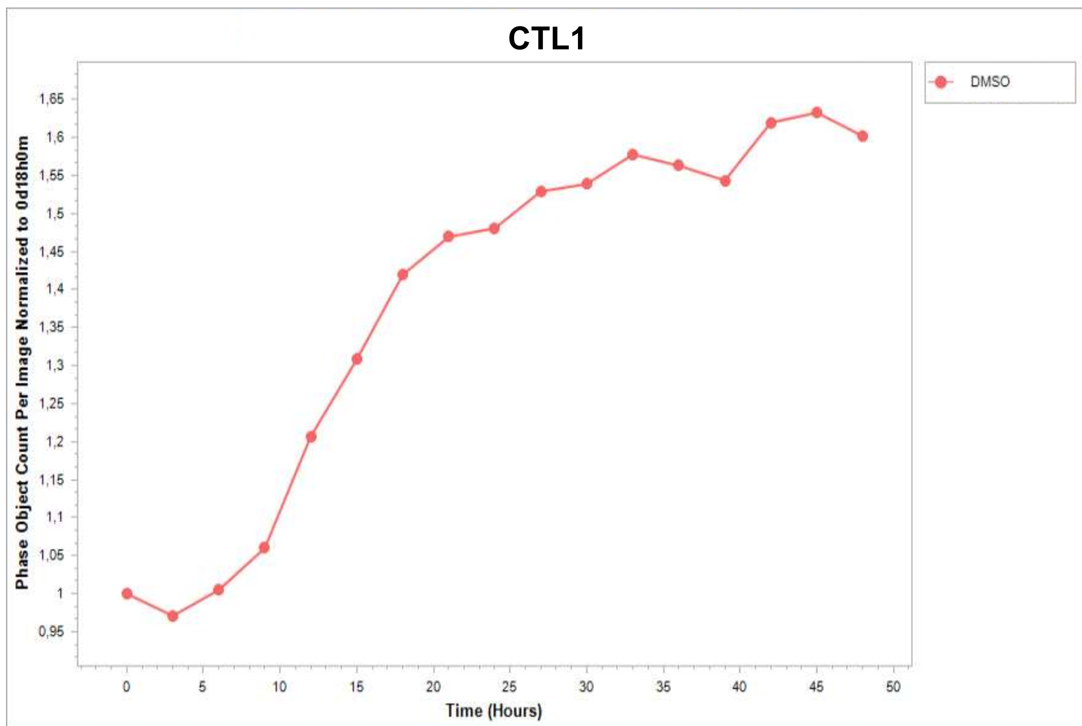
7 Supplementary data



Supplementary Figure 1. RNA expression of YAP (graph on the left) and TEAD1 (graph on the right) in CTL1 fibroblast line after 24 h of treatment with DMSO for the control and TRULI 5 µM and TRULI 10 µM. YAP and TEAD1 are expressed in fibroblasts and treatment with TRULI does not change their expression. Data are normalized on housekeeping gene MAN2B1. Error bars show standard deviation of at least three independent experiments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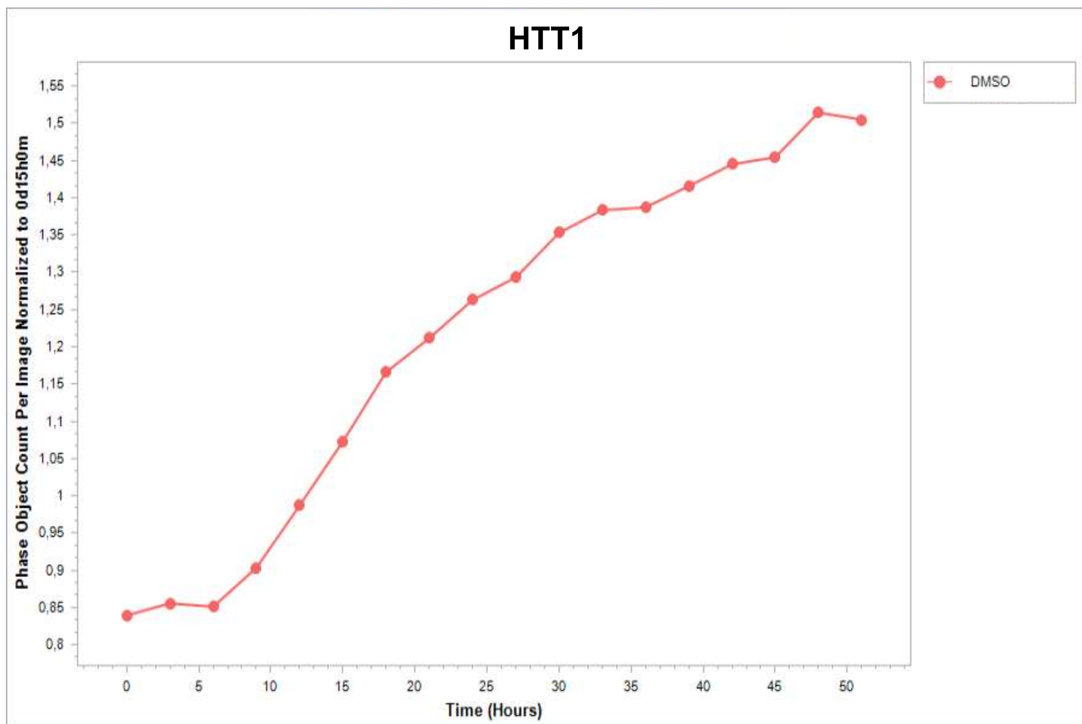
1,4
1,35
1,3
1,25
1,2
1,15
1,1
1,05
1
0,95
0
5
10
15
20
25
30
35
40
45
50
Time (Hours)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DMSO
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1,4
1,35
1,3
1,25
1,2
1,15
1,1
1,05
1
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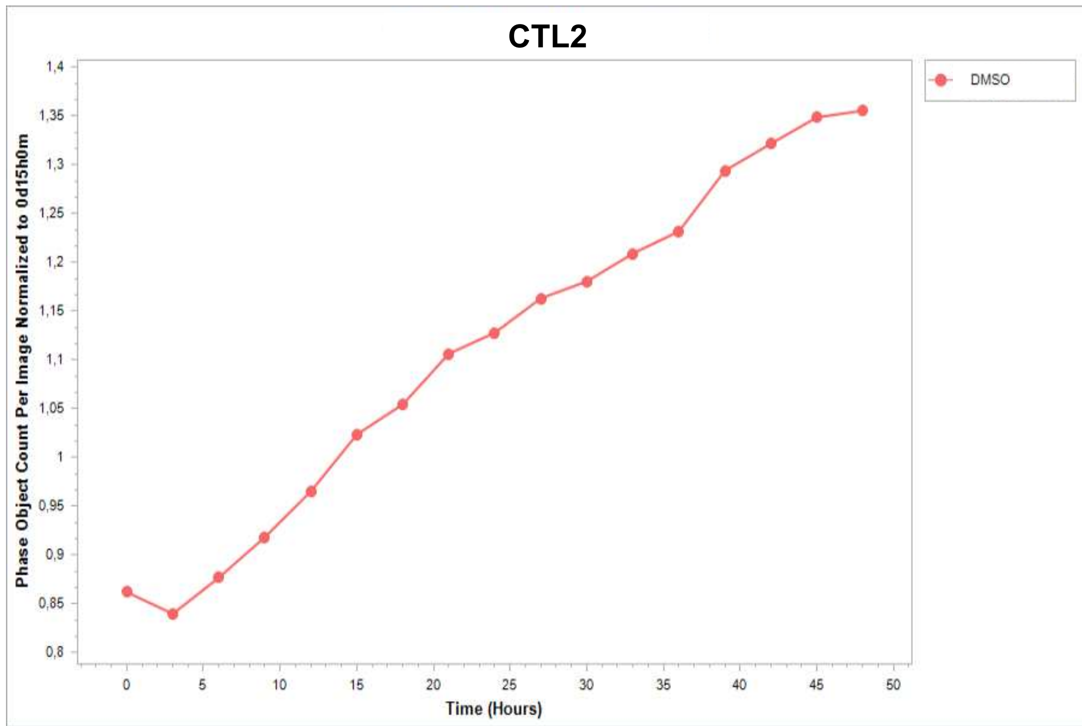
0,9
0,85
0,8
0
5
10
15
20
25
30
35
40
45
50
Time (Hours)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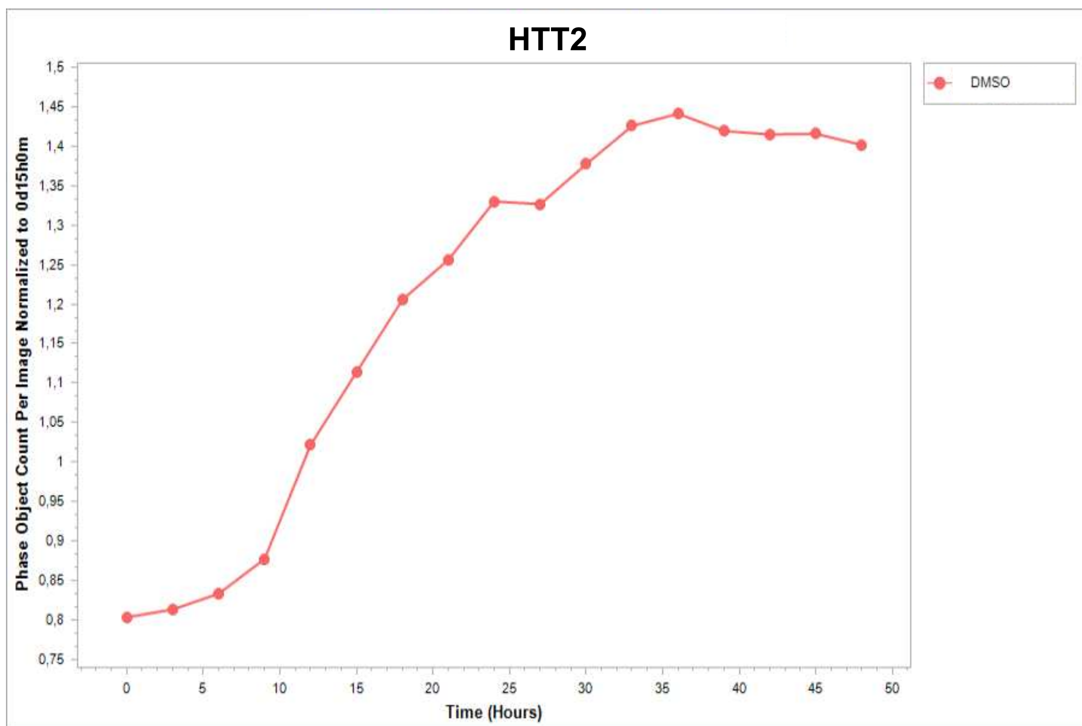
Time (Hours)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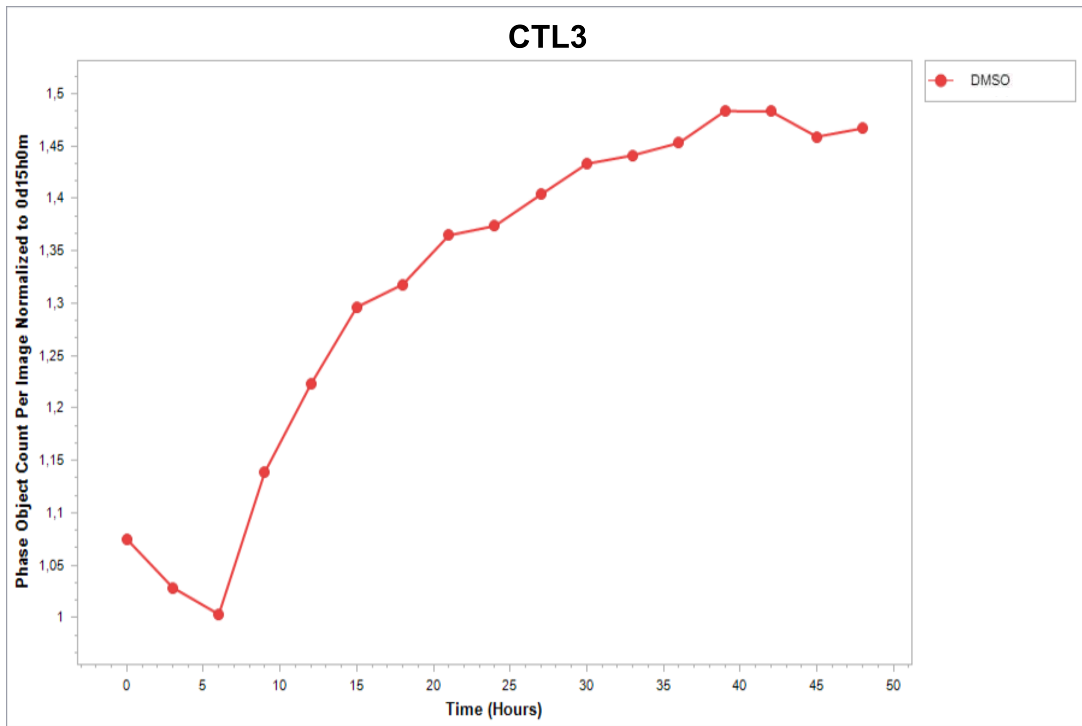
Time (Hours)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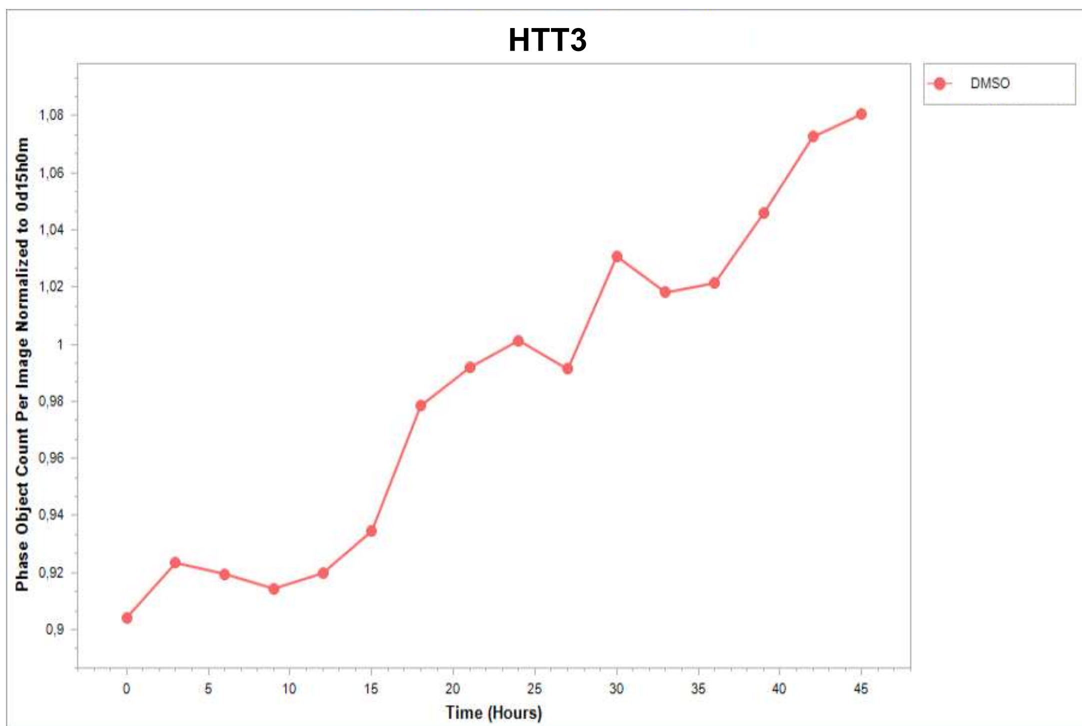
1
0
5
10
15
20
25
30
35
40
45
50
Time (Hours)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DMSO
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0
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10
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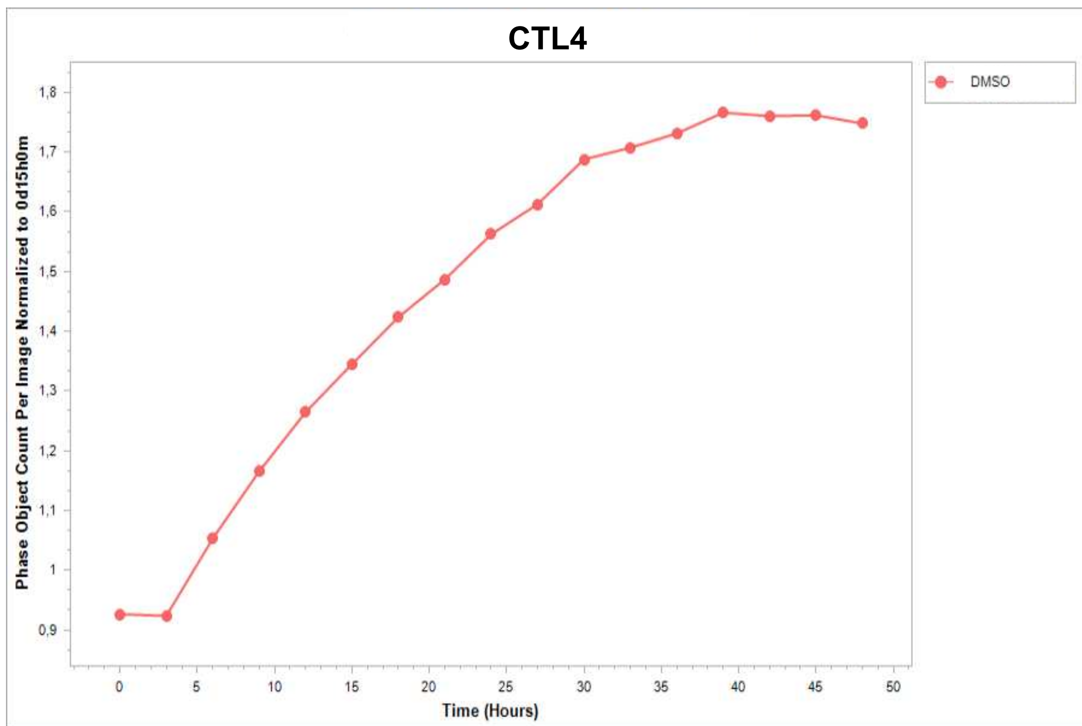
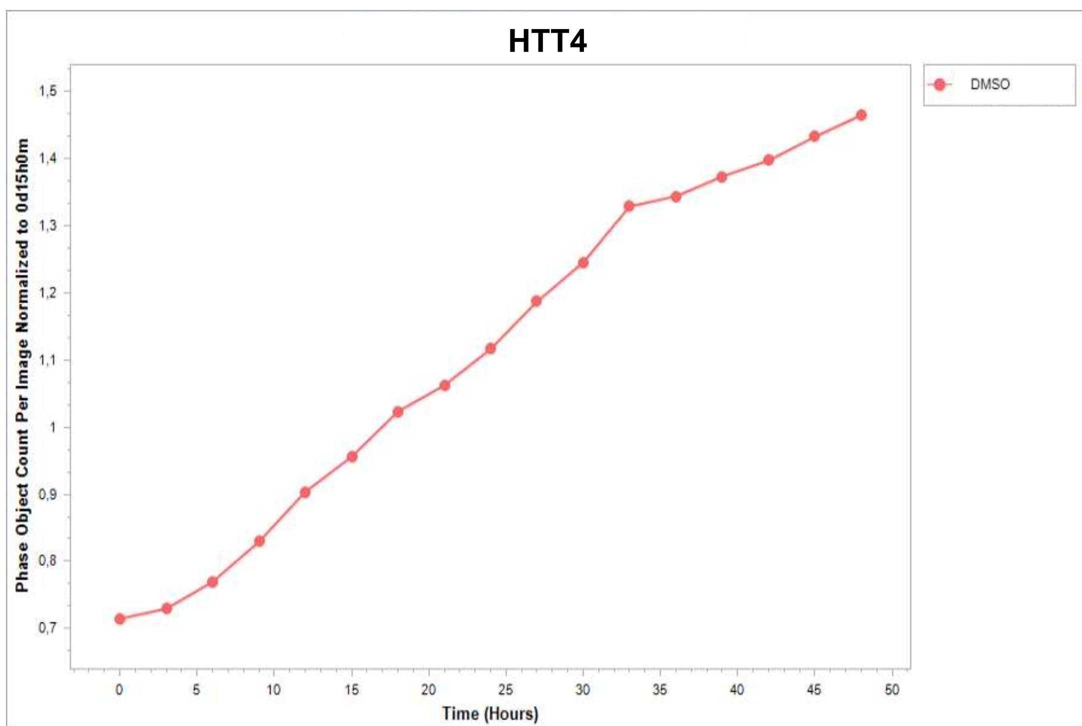
20
25
30
35
40
45
Time (Hours)





Supplementary Figure 2. Cell growth curve generated after software analysis of photos acquired every 3 hours via Incucyte. The figure shows 8 graphs representing the cell growth of the 4 controls (CTL1, CTL2, CTL3, CTL4) and the 4 patients (HTT1, HTT2, HTT3, HTT4).