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The Hippo Pathway and YAP/TAZ-TEAD Protein–Protein Interaction as Targets for Regenerative Medicine and Cancer Treatment

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Abstract: The Hippo pathway is an important organ size control signaling cascade, acting as the major regulatory mechanism of cell-contact inhibition. Yes Associated Protein (YAP) and Transcriptional co-Activator with PDZ-binding mot (TAZ) are its targets and terminal effectors: inhibition of the pathway promotes YAP/TAZ translocation to the nucleus, where they interact with Transcriptional Enhancer Associate Domain (TEAD) transcription factors family and co-activate the expression of target genes, promoting cell proliferation. Defects in the pathway can result in overgrowth phenotypes due to the modulation of tissue-specific stem-cell proliferation and apoptosis, and members of the pathway are directly involved in cancer development. The pharmacological regulation of the pathway might be beneficial in cancer prevention and treatment and useful for regenerative medicine applications; however, currently, there are very few compounds able to selectively modulate the pathway. In this review, we present an overview of the Hippo pathway, the sequence and structural analysis of YAP/TAZ, the known pharmacological modulators of the Hippo pathway and the design of new modulators to target the YAP/TAZ-TEAD interaction.

Keywords: Hippo pathway; stem cells; TAZ; YAP, TEADs; Verteporfin.

INTRODUCTION

Coordination of cell proliferation and death is essential not only to attain an appropriate organ size during development but also to maintain tissue homeostasis during postnatal life. The mechanism by which multicellular organisms orchestrate the growth of their individual cells and the size of their organs is a longstanding puzzle in developmental biology that still remains to be resolved. Signaling pathways converting extra- and intra-cellular events into gene transcription are pivotal in cell number regulation.

Recently, a newly discovered and evolutionarily and functionally conserved signaling kinase-cascade, the "Hippo pathway", has been shown to play a critical role in controlling organ size by regulating both cell proliferation and apoptosis [1-5]. Initially, this pathway was discovered in *Drosophila melanogaster* by mosaic genetic screens, which proved to be a powerful tool in the elucidation of this molecular signaling. Each member of this pathway in *Drosophila* has a correspondent counterpart in mammals sharing a high analogy- and homology-level with it. Several genetic and biochemical studies gradually demonstrated that the *Drosophila* mutants for any of the signaling pathway components exhibit an overgrowth phenotype (as a *hippo*potamus), leading to the current model in which the Hippo pathway is the major regulatory mechanism of cell-contact inhibition [2, 6]. The terminal effector-component of the Hippo pathway is a transcription co-activator, named Yorkie (Yki) in *Drosophila.* In mammals, Yes-Associated Protein (YAP) and its paralogue Transcriptional co-Activator with PDZ-binding motif (TAZ) have been identified as terminal effectors of the pathway [7]. The final effect of the Hippo pathway on the Yki/YAP/TAZ proteins involves phosphorylation on specific Serine residues, to confine them in the cytoplasm for subsequent degradation. Consequently, Yki/YAP/TAZ cannot translocate to the nucleus, where they would bind to other proteins (including TEADs in mammals) and act as transcription co-activators, triggering the expression of cell proliferation-promoting genes [1, 2, 8].

Hippo signaling alterations are increasingly being associated with cancer development [4, 9-11]. It was observed that a significant percentage of patients affected by certain cancers, such as cancer of the liver, breast or pharynx, harbor causative amplification or over-expression of the YAP gene; therefore, the use of YAP-inhibiting drugs could be tailored to the selected patients for optimal effects [12]. These clinical observations suggest that the pathway, particularly the YAP–TEAD complex, can be targeted to inhibit cancer or modulate proliferation. Because the YAP–TEAD complex corresponds to the final step of YAP activity, its targeted inhibition should diminish the potential side effects expected from targeting the upstream proteins of the pathway, which are more interconnected with other signaling networks. Therefore, the protein–protein interactions between

YAP and TEAD emerge as the best candidate target for modulating the Hippo pathway with small molecules.

Currently, there are no known compounds that can interfere in a consistent manner with the YAP–TEAD interaction. However, the YAP–TEAD complex structure has been resolved by X-ray crystallography, and mutagenesis studies have identified the amino acid residues crucial for forming the functional complex [13]. This structural information could allow the design of new compounds directed to the target protein. Consequently, modulating YAP activity could possibly help regulate the self-renewing, proliferative potential and the fate of stem cells and could hopefully direct stem cell differentiation towards a specific cell-lineage. From this perspective, the following points should be considered: (i) the Hippo pathway is involved in cross-talk with several growth regulatory signaling pathways; (ii) not all tissue-specific progenitors are regulated by this pathway and (iii) different sets of downstream target genes are regulated in a tissue-specific manner. Hence, the number of cross-linking signals could be very high, triggering a significant redundant and compensatory mechanism. Despite these limitations, a tissue-specific modulation of YAP activity could represent a powerful tool for future regenerative medicine applications. This review provides an overview of the Hippo signaling cascade, explaining the role of this pathway in differentiated cells and stem cells throughout organ development. An emphasis will be placed on the importance of the YAP–TEAD complex as a drug-target and on the possibility of using a medicinal chemistry approach to develop compounds that can interfere with stem cell fate, through this target, for future regenerative medicine applications.

Biology of the Hippo pathway

THE HIPPO SIGNALING PATHWAY AND YAP/TAZ REGULATION

The Hippo signaling pathway was initially described in *Drosophila*, where it gradually emerged as a major regulatory mechanism of contact-inhibition during the growth of the cell [1-5]. Numerous genetic and biochemical studies in *Drosophila* have led to a extensive characterization of the pathway: it consists of a series of serine/threonine phosphorylation events that lead to the inhibition of cell proliferation and the promotion of apoptosis via inhibition of the transcriptional co-activator Yki. The pathway is regulated by a variety of upstream modulators, including transmembrane

receptors belonging to the cadherin family (Ft, Ds) and components of tight-junctions (aPKC, Par3, Par6), adherens junctions (a-Catenin) or apical-basal polarity protein complexes (Crb, PatJ, Std, Lgl, Scrib, Dlg; see Tab. 1) [1, 2, 9]. Upon initiation of the pathway, a complex comprising Ex, Mer and Kibra proteins is formed, which in turn activates the core Hippo pathway components (Hpo, Sav, Mats and Wts). Consequently, the transcriptional co-activator Yki is phosphorylated on multiple sites, thereby creating a 14-3-3 binding site and resulting in cytoplasmic retention and inactivation of Yki. When the Hippo pathway is inactive, Yki bypasses the phosphorylation by Wts and enters the nucleus, where it binds and activates a transcription factor (Sd). This induces several downstream target genes, including promoters of cell-growth (Myc and Ban), promoters of cell-cycle progression (E2F1, cyclin A, B and E), inhibitors of apoptosis (Diap1) and components of other important signaling pathways [2, 8, 9].

The Hippo pathway is highly conserved in mammals. Ft, Ds, Ex, Mer and Kibra homologues (Ft1-4, Dchs1-2, FRMD6/Ex1, NF2 and WWC1/WWC2; see Tab. 1) do exist; however, their functional significance in regulating the pathway is just beginning to be delineated, and further investigation is needed to characterize the steps that initiate the pathway in mammalian cells [9, 14]. The core components and downstream effectors of the *Drosophila* pathway are highly conserved: Mst1/2 (Hpo homologues), Sav1 (Sav homologue), Lats1/2 (Wts homologues), MOBKL1A and MOBKL1B (collectively referred to as Mob1; homologues of Mats), and YAP and its paralogue TAZ (also called WWTR1; homologues of Yki) (Tab. 1) [4, 15]. Relationships among Hpo, Sav, Wts, and Mats are also conserved among mammalian Mst1/2, Sav1, Lats1/2, and Mob1 (Fig. 1). NF2, FRMD6 and WWC1/2 interact and promote activation of the Mst1/2-Sav1 complex by a mechanism that is not fully characterized [9]. As with the *Drosophila* Sav and Hpo proteins, the mammalian Sav1 protein interacts with and activates the serine-threonine kinases Mst1/2 through the SARAH domains present in both; however, the underlying mechanism is still unclear. The auto-activation of Mst1 and 2, which occurs by phosphorylation on threonine residues within their activation domain, triggers the phosphorylation and consequent activation of their direct substrates, Lats1/2 [16, 17]. The latter forms a complex with Mob1, which is phosphorylated by Mst1/2, resulting in an enhanced Lats1/2–Mob1 interaction. The activated Lats1/2-Mob1 complex in turn phosphorylates YAP (on Ser127) / TAZ (on Ser89), preventing their nuclear translocation via binding to cytoplasmic 14-3-3 proteins and thus playing the most critical role in regulating YAP/TAZ nuclear-cytoplasmic localization. Mutation of Ser127 in YAP and subsequent disruption

of the 14-3-3 binding site activates YAP, further confirming the inhibitory role of the phosphorylation on this specific residue [18, 19].

Although several upstream modulators of the pathway identified in *Drosophila* have structural and functional homologues in mammals, certain relevant differences have been highlighted. In contrast to dRASSF function in *Drosophila*, most mammalian RASSF homologues are scaffold proteins activating Mst1/2: RASSF1A, which has the highest homology with the fly protein dRASSF, activates the Mst1/2 kinases by preventing their dephosphorylation by PP2A [9, 20, 21]. Another Ras effector family member, RASSF6, binds to Mst2 and antagonizes Hippo signaling [22]. As in *Drosophila*, Ajuba (homologue of *Drosophila* Jub) physically interacts with Lats1/2 and Sav1, leading to the inhibition of the Hippo signaling activity [23]. The presence of conserved *Drosophila* orthologues in the mammalian Hippo pathway may serve as a mechanism of redundancy that protects the organism against cancer-causing mutations.

At the nuclear level, YAP/TAZ associate with TEAD1-4 (homologues of *Drosophila* Sd) and stimulate the transcription of genes involved in the control of cell-proliferation, differentiation and development, such as Myc [24, 25], Gli2 [26], CTGF and Cyr61 [26-28]. The TEAD family transcription factors are the main YAP/TAZ partners in the regulation of gene expression. Knockdown of TEADs or disruption of the YAP–TEAD interaction abolishes YAP-dependent gene transcription and substantially diminishes YAP-induced cell proliferation and oncogenic transformation [4, 10, 29]. A mutation of TEAD1 Tyr421, which forms a hydrogen bond with YAP, results in loss of interaction with YAP and leads to the human genetic disease Sveinsson's chorioretinal atrophy [30]. Precise regulation of the YAP–TEAD interaction is therefore important in maintaining normal physiology. Despite a major role for TEADs in YAP/TAZ function, other transcription factors containing PPXY-motifs are known to interact with the WW-domains of YAP/TAZ, including Smad1, Smad2/3, RUNX, ErbB4 and p73 for YAP [4, 31], and RUNX, PPARγ, Pax3, TBX5 and TTF-1 for TAZ [8, 32-35]. The interaction of YAP with Smad1 is important for maintaining the pluripotency of mouse embryonic stem cells (mESCs), mediated by

the activation of the Bone Morphogenetic Protein (BMP) transduction pathway (Fig. 2A). Considering that BMP plays a role in mESC self-renewal and differentiation and that both BMP- and Hippo-pathways have the ability to control organ size, the regulated interaction of Smad1 and YAP could possibly mediate the cross-talk between the two signaling cascades [36]. Additionally, YAP and TAZ bind Smad2/3. In human embryonic stem cells (hESCs), Smad proteins are transcriptional modulators through which TGF-β family members regulate many developmental events (Fig. 2B). Particularly, the YAP–Smad2/3 interaction is believed to dictate the nuclear accumulation of Smad2/3 for subsequent transcription activation. In this manner, YAP regulates Smad nuclear localization and coupling to the transcriptional machinery [37]. YAP also interacts with p73, a p53 family pro-apoptotic transcription factor, to induce the expression of genes such as Bax, Puma and PML [38]. Another YAP interaction partner is RUNX2, which stimulates the osteoblastic differentiation of mesenchymal stem cells (MSCs), to promote chondrocyte hypertrophy and to contribute to endothelial cell migration and vascular invasion in bone development. YAP interacts with the full-length RUNX2 protein, as well as RUNX2-responsive promoter regions; however, the effects of YAP on the expression of the RUNX2 target genes appear to depend on the promoter, namely on the cohort of other DNA-binding proteins and co-factors brought to the gene by specific DNA sequences and protein–protein interactions [39, 40]. ErbB4 is a tyrosine-kinase receptor protein which is proteolytically processed by membrane proteases in response to the ligand, resulting in the translocation of its cytoplasmic COOH-terminal fragment (CTF) to the cell nucleus. YAP may associate with the cytoplasmic portion of the ErbB4 receptor and co-activate transcription mediated by CTF. Thus, the CTF of ErbB4 produced by γ-secretase cleavage, translocates, along with YAP, to the nucleus upon ligand stimulation, and YAP may act not only as a transcriptional co-activator for the CTF but also as a carrier protein for translocating the CTF from the membrane to the nucleus [41].

Phosphorylation of Yap at Ser127 promotes its binding to the 14-3-3 proteins and its subsequent cytoplasmic sequestration and inactivation; however, YAP phosphorylation can also induce its degradation: Lats1/2 phosphorylates YAP at Ser381, which primes YAP for subsequent phosphorylation by another kinase, possibly Casein Kinase 1 (CK1δ/ε). This might activate a phosphorylation-dependent degradation motif, termed phosphodegron. Subsequently, the E3-ubiquitin ligase SCFβ–TRCP is recruited to YAP, leading to its polyubiquitination and degradation. YAP/TAZ can also be inhibited through protein–protein interactions, in a phosphorylation-independent manner, resulting in their cytoplasmic sequestration [18]. Recently, YAP/TAZ and Angiomotin family proteins (AMOT) were shown to interact, resulting in YAP/TAZ inhibition through various mechanisms. AMOT was first identified as an "Angiostatin binding protein" promoting endothelial cell migration and angiogenesis, and due to its angiogenic function, it has been implicated in tumor growth. However, the angiostatin-responsive migration-promoting functions are observed in the AMOT p80 splicing variant that does not bind YAP, but not in the YAP-binding p130 variant. The AMOT family has three paralogues in humans and mice (AMOT, AMOTL1 and AMOTL2) and two isoforms generated by alternative splicing: the p80 isoform lacks 400 amino acids at the N-terminal present in the p130 isoform. YAP interacts with AMOT p130 alone because the p130 unique N-terminal region contains two PPXY motifs, which serve as binding partners for the YAP WW-domains. Mutation of the first PPXY motif, which is conserved in all three AMOT family members, significantly decreases the interaction with YAP. Mutation of the second PPXY motif, which is not conserved in AMOTL2, has little effect. The combined mutation of both PPXY motifs shows an effect similar to the mutation of the first PPXY motif. Therefore, the WW-domains of YAP and the first PPXY motif of AMOT play major roles in the YAP–AMOT interaction [42]. AMOT can inhibit YAP/TAZ by various mechanisms. Through physical interaction, AMOT recruits YAP/TAZ to various compartments such as tight-junctions and actin cytoskeleton, depending on the cellular location of YAP/TAZ': AMOT binds to Pals and PatJ, thus forming the tight junction-related Crumbs cell-polarity complex, which sequesters the

transcriptional co-activators YAP/TAZ at sites of cell–cell adhesion, preventing their nuclear localization [43-45]. In addition, AMOT is present in protein complexes along with ZO-1/2 and PTPN14, which mediate the cytoplasmic retention of YAP/TAZ, independent of their phosphorylation status. ZO-2 was reported to consistently inhibit TAZ-mediated transactivation and YAP2-dependent induction of proliferation [46, 47]. AMOT family proteins additionally inhibit YAP/TAZ by promoting their inhibitory phosphorylation; however, it is unclear whether induction of YAP phosphorylation indirectly depends on the modulation of its subcellular localization. Other known modulators of YAP/TAZ belong to adherens junctions: α-Catenin, an adherens junction component and a tumor suppressor, promotes YAP cytoplasmic localization and inhibits YAP activity by interacting with and stabilizing the YAP1/14-3-3 complex [48, 49]. Dlg and Scrib proteins are associated with baso-lateral polarity complexes and increase YAP/TAZ phosphorylation; however, the molecular mechanisms underlying this process in mammalian cells are still poorly understood [9, 50].

HIPPO SIGNALING PATHWAY AND STEM CELLS

Over the last decade there has been growing evidence that the Hippo pathway can affect tissue size by directly regulating stem cell (SCs) proliferation and maintenance. Numerous studies have investigated Hippo signaling in various stem cell populations and have revealed its crucial role in stem cell biology: in general, YAP overexpression or inactivation of any Hippo signaling pathway component promotes proliferation and prevents differentiation of different tissue-specific SCs. However, not all tissue-specific progenitors are regulated by this pathway, and its manipulation leads to different effects depending on the specific cell-type considered. Consequently, the manipulation of Hippo signaling regulation may represent a possibility to influence stem cell commitment and differentiation towards a specific and unique cell-lineage. Thus, the manipulation

of the Hippo regulation mechanism may represent a useful tool in clinical applications and regenerative medicine.

EMBRYONIC STEM CELLS (ESCs)

Embryonic stem cells are isolated from the Inner Cell Mass (ICM) of blastocysts. They are pluripotent stem cells because they have the ability to give rise to all cells and embryo tissues, except placental extra-embryo tissue. Therefore, they represent the source of all tissues comprising the developing embryo, the fetus and ultimately the adult organism. ESCs depend on various signals for self-renewal: mouse-ESCs rely on the cytokine Leukemia Inhibitory Factor (LIF) and signals from Bone Morphogenic Proteins (BMPs), which reinforce the pluripotency network and block progression to lineage-commitment (Fig. 2A), whereas human-ESCs rely on Fibroblast Growth Factor (FGF) signaling and a balance between Transforming Growth Factor-β (TGF-β) and BMP-signaling (Fig. 2B) [3, 14, 51]. YAP and TAZ are important for regulating ESC self-renewal and differentiation. The importance of YAP/TAZ during embryogenesis was uncovered when the transcription factor TEAD4 was found to be critical for the induction of Cdx2, which is a transcription factor required for the development of the trophectoderm-lineage, the outer cells of the blastocyst stage embryo [52, 53]. However, TEAD4 expression is not restricted to the outer cells of the blastocyst. YAP is found only in the nucleus in the outer cells; it is cytoplasmic in the ICM cells and nuclear in the outer cells of the embryo, thus allowing TEAD4-mediated Cdx2 transcription in these cells [54]. Although YAP is found in the cytoplasm in the ICM of mouse blastocyst, it is found in the nucleus of ESCs, which are derived from the ICM. As ESCs differentiate, YAP nuclear localization and protein levels diminish, and this is accompanied by increased Hippo pathway activity [55]. The conditional knockout of TAZ in hESCs (but not YAP) and loss of YAP or TAZ in mESCs result in loss of self-renewal and ESC pluripotency; overexpression of YAP prevents differentiation in mESCs and results in increased reprogramming of fibroblasts to Induced

Pluripotent Stem Cells (IPSCs) [37, 55]. The latter are differentiated cells reprogrammed to an ESC-like state by inducing the activity of four transcription factors (Oct-4, Sox-2, Klf-4 and cMyc) [55, 56]. IPSCs can self-renew, they are indistinguishable from ESCs in several ways; therefore, they are considered a valuable resource for cell or tissue replacement therapy. However, these data suggest that YAP activity enhances the reprogramming process, in conjunction with Oct4, Sox2 and Klf4. A possible mechanism may be via the Yes-Kinase-mediated phosphorylation of YAP, which induces a YAP/TEAD2-gene expression program. YAP/TEAD2 directly bind to promoters and activate the transcription of important stemness genes, such as Sox-2, Oct-4 and Nanog [55]. YAP/TAZ contribute to maintaining mESC pluripotency *in vitro* by mediating the BMP-induced transcriptional program: the BMP signaling pathway leads to the activation of Smad1 transcription factor and the recruitment of YAP to Smad1 on target gene promoters, enhancing Smad1 activity [36]. In hESCs, TAZ binds Smad2/3-Smad4 heterodimers in response to TGF-β stimulation; TAZ depletion impairs Smad2/3-Smad4 accumulation in the nucleus and transactivation activity, and the cells tend to differentiate [37, 50].

SKIN STEM CELLS (SSCs)

To regenerate continuously and maintain its structural and functional integrity, the skin relies on the self-renewing abilities of epidermal SCs residing in the basal layer of epidermis. Asymmetric divisions in this SC compartment produce short-lived progenitor cells that stratify, leave the basal layer, and move up through the suprabasal layers to the outer surface of the skin, as they terminally differentiate. YAP plays an important role in epidermal development and SC homeostasis [3, 51]. Recent studies have highlighted that YAP overexpression causes a severe thickening of the epidermal layer and that this hyperplasia is driven by the expansion of undifferentiated interfollicular SCs and progenitor cells; the knockout of YAP leads to an epidermal hypoplasia, and this phenotype has been attributed to the gradual loss of epidermal stem/progenitor cells and their

limited capacity to self-renew [3, 27]. In the skin, YAP is not regulated by the canonical Hippo kinases, but rather by α-Catenin, a component of Adherens Junctions (AJs), which is an upstream negative regulator of YAP. These AJs could act as "molecular biosensors" of cell density and positioning according to the "crowd control molecular model": sensing increased cell density leads to inhibition of SC expansion by inactivating YAP, whereas low basal cell density translates into nuclear YAP localization and cell proliferation [48, 49].

SMALL INTESTINE STEM CELLS (ISCs)

The intestinal epithelium is one of the most rapidly regenerating tissues in the body, turning over completely every 4–5 days through the continual proliferation of intestinal stem cells located at the base of the crypt (Crypt Base Columnar (CBC) cells, also known as Lgr5(+) cells) and at the "+4 position" relative to the crypt bottom [51]. The major factor promoting the self-renewing capacity of Lgr5+ ISCs in mammals is the Wnt-pathway. The driving force behind Wnt-signaling is β-Catenin. Generally, in a Wnt-unstimulated cell, Glycogen Synthase Kinase-3 (GSK-3) phosphorylates the cytoplasmic pool of β-Catenin and promotes its degradation through an ubiquitin-mediated proteasome pathway (Fig. 2C, left). In a Wnt-stimulated cell, the Wnt receptor Frizzled activates Disheveled (Dsh), which in turn inhibits GSK-3 activity. On its inhibition, GSK-3 no longer phosphorylates β-Catenin, which accumulates in the cytosol. Stable β-Catenin subsequently enters the nucleus, forms a transcriptional complex with members of the Lef/Tcf family of DNA-binding proteins and regulates downstream target genes (Fig. 2C, right) [57]. Recently, the Hippo pathway together with its final effector YAP, have been found to be critical in regulating ISC self-renewal and differentiation. YAP is found in the nucleus of ISCs and some other crypt cells but is primarily cytoplasmic in the upper crypt and villi, where it is likely that Hippo targets YAP for phosphorylation and consequent inhibition [3, 51]. The phosphorylation of

YAP/TAZ and their cytoplasmic localization counteracts Wnt-activity. Overexpression of active YAP or conditional knockout of Mst/Sav-1 expands progenitor-like cells and blocks differentiation [58, 59]. The aberrant proliferation induced by unphosphorylated YAP in ISCs is in part or wholly due to the hyperactivation of Wnt-signaling because of enhanced β-Catenin transcriptional activity. Specifically, when YAP and TAZ are phosphorylated by the Hippo pathway and sequestered in the cytoplasm, phosphorylated YAP/TAZ interact with Dsh and β-catenin (Fig. 2C, right). Cytoplasmic YAP inhibits the nuclear translocation of Dsh and β-catenin (through a mechanism which is distinct from the degradation pathway), whereas cytoplasmic TAZ inhibits the activity of the degradation-complex member Casein Kinase 1 (CK1), blocking Dsh phosphorylation. In the Wnt-ON state, if phosphorylated YAP/TAZ is lost, or Hippo signaling is ablated, cells undergo hyperactivation of Wnt-signaling owing to increased Dsh phosphorylation and/or nuclear accumulation as well as additional nuclear β-catenin (Fig. 2D). Once in the nucleus, Dsh acts as a transcriptional co-factor to induce β-catenin target genes in conjunction with another transcriptional co-factor cJUN [56]. In addition, YAP interacts with β-catenin and TEAD in the nucleus to activate the expression of proliferation-related genes. In conclusion, when the Wnt pathway is predominant, compared with phosphorylated YAP, Wnt produced by Paneth cells (components of the intestinal stem cell niche) and other sources is detected by ISCs in intestinal crypts: the ISCs divide and cells progress upward out of the crypt and begin to differentiate. If YAP becomes overabundant in the cytoplasm of the crypt cells, Wnt signaling is repressed, and the ISC niche is disrupted. This causes aberrant upward migration of Paneth cells and loss of ISCs. Because of the ISC loss, the intestinal epithelium degenerates.

NEURAL, LIVER AND CARDIAC MUSCLE STEM CELLS

Neural progenitor cells reside along the sub-ventricular zone in the developing vertebrate neural tube and are responsible for generating the myriad of cell types comprising the mature central

nervous system. The conditional knockout of Mst/Lats or YAP-activation expands neural progenitor cells in the neural tube [3, 51, 60]. In the cerebellum, endogenous YAP is highly expressed in Cerebellar Granule Neural Precursors (CGNPs). YAP overexpression expands CGNPs in the cerebellum and leads to medulloblastoma. The CGNPs rely on Sonic-Hedgehog (Shh)-signaling to expand and Shh-signaling induces the expression and nuclear localization of YAP, which then drives the proliferation of these cells [61].

The adult liver has a distinctive ability to rapidly regenerate following acute injury. The regeneration of the organ is dependent on the ability of hepatocytes and cholangiocytes (bile duct cells) to proliferate and on heterogeneous populations of transit-amplifying bipotential progenitor cells known as “oval cells” in rodents [62]. YAP overexpression leads to a dramatic but reversible liver hyperplasia, caused by an exacerbated proliferation of mature hepatocytes [58]. Conversely, conditional loss of YAP function leads to impaired liver function, primarily because of accelerated hepatocyte turnover due to enhanced apoptosis. The conditional knockout of Mst1-2 leads to liver overgrowth, with mixed hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) phenotypes, whereas conditional knockout of Sav-1 leads to a similar liver overgrowth and development of HCC/CC mixed tumors, but shows increased number of oval cells without concomitant hepatocyte-expansion [62-64].

In contrast to that in tissues such as the liver, the role of Hippo signaling in the heart is just beginning to be delineated. Sav1/Lats2/Mst1-2 conditional knockout or YAP overexpression promotes cardiomyocyte proliferation, resulting in embryos displaying a cardiomegaly phenotype, whereas YAP conditional knockout leads to myocardial hypoplasia [51, 65]. YAP interacts with β-Catenin in the nucleus to promote Wnt-signaling (a well-known promoter of cell-stemness and proliferation in the heart), thus enhancing neonatal cardiomyocyte proliferation [65]. Moreover, YAP indirectly promotes Wnt-signaling through the activation of the Insulin-like Growth Factor (IGF) pathway (a potent signaling system that stimulates growth and blocks apoptosis in many

different cell types), resulting in the inactivation of GSK3β and consequently the Wnt degradation complex [66].

Structural and inhibition studies

YAP/TAZ/Yki DOMAINS ORGANIZATION

The human YAP gene is located at 11q13; it can be transcribed into at least 4 isoforms (YAP1-4), which are generated by differential splicing of short exons located within the transcriptional activation domain of YAP: isoforms 1, 2, 3 and 4 have 504, 450, 488 and 326 residues in length, respectively (Fig. 3, Table 3) [67-70]. YAP was originally identified in chicken as an interacting protein of Yes protein tyrosine kinase. The interaction was shown to be mediated by the SH3-domain of the Yes protein and the (Pro)-rich region (PVKQPPPLAP) of YAP (SH3 binding domain, Fig. 3). Due to its size of 65 kDa, the chicken protein was referred to as YAP65 (Yes-associated protein of 65 kDa). The human and mouse homologues were identified by using the YAP65 cDNA to probe human and mouse cDNA libraries. During the course of sequence analysis by comparing YAP with other proteins, a conserved module was observed in several proteins of various species. This was named the WW-domain to reflect the sequence motif containing two conserved and consistently positioned tryptophan (W) residues (Fig. 3). Two consecutive WW-domains are present in all isoforms except isoform 2, which has only one WW domain. Isoform 3 containing 488 residues and two WW domains is the most thoroughly studied isoform. These WW-domains are important for YAP to interact with transcription factors containing the PPXY-motif. The N-terminal region harbors the TEAD-binding domain, containing HXRXXS-motifs (14-3-3 binding domain, Fig. 3). Phosphorylation of S127 within this motif creates a binding site for 14-3-3 proteins and results in YAP-cytoplasmic sequestration and inactivation. The C-terminus of Yap proteins exhibits strong transactivation property (Transcriptional activation domain, Fig. 3), and it

contains a PDZ-binding motif (FLTWL, Fig. 3) critical for nuclear translocation and binding to the PDZ-domain of other regulator-proteins such as ZO2. YAP proteins also contain a coiled-coil domain within the transcriptional binding domain (Fig. 3) [69].

The TAZ gene can be transcribed into three variants, all of which have the same coding region for a protein having 400 amino acids in length (Fig. 3, Table 3). TAZ, also referred to as WWTR1 (WW-domain containing transcription regulator 1), is homologous to YAP3 with 42% amino acid sequence identity and displaying similar domain organization but having only one WW-domain. Biochemically, TAZ displays transcriptional co-activator function via interaction with PPXY-containing transcriptional factors through its WW-domain. The C-terminal region is responsible for the transcriptional co-activation property. Similar to YAP, TAZ has a C-terminus with a PDZ-binding motif (FLTWL) and a N-terminal region, which harbors the TEAD-binding domain containing the HXRXXS-motifs with the S89, whose phosphorylation creates a binding site for the 14-3-3 proteins, resulting in TAZ inactivation.

Both YAP and TAZ are homologous to fly Yki (Yorkie). Similar to YAP, Yki contains two WW-domains. The N-terminal region of Yki shows the highest homology to YAP and TAZ, presenting the Scalloped (Sd)-binding domain with S111 homologous to YAP-S127 and TAZ-S89.

YAP–TEAD COMPLEX: THE MAIN MEDIATOR OF YAP/TAZ/Yki TRANSCRIPTIONAL FUNCTION

TEADs in mammals and Sd in *Drosophila* are the major transcriptional factors mediating the biological outcome of YAP/TAZ and Yki, respectively. YAP was identified as a tight binding and major interacting protein for TEAD2 and was proposed to function as a general transcriptional co-activator for the TEADs transcriptional factors. There are four related family members (TEAD1–4) in mammals (Fig. 4, Table 4) [70]. The N-terminal regions of TEADs and Sd contain a conserved

TEA domain involved in recognizing DNA elements such as GGAATG in the promoter region of target genes. The NMR structure of the TEA domain (PDB ID: 2HZD) revealed a three-helix bundle fold with the helix 3 containing a bipartite Nuclear Localization Signal (NLS) [71]. The C-terminal regions of TEADs and Sd interact with YAP/TAZ and Yki, respectively. Mutation of specific YAP residues abolishes most, if not all, activity of YAP in promoting cellular transformation and mediating the transcriptional outcome, suggesting that interaction with TEADs is the major functional pathway of YAP. A similar functional relationship between TEAD and TAZ has been demonstrated: the TAZ residues essential for interacting with TEAD are also essential to induce transformation and are well conserved in YAP and Yki. The putative TEAD2-YAP Binding Domain (YBD) in a crystal structure of TEAD (PDB ID: 3L15) adopts an immunoglobulin IgG-like fold with two β-sheets packing against each other to form a β-sandwich [69]. One β-sheet contains five antiparallel strands, including β1, β2, β5, β8, and β9, whereas the other contains seven parallel and antiparallel strands, including β3, β4, β6, β7, and β10–12. In addition to the two major β-sheets, TEAD2-YBD contains two helix-turn-helix motifs that are absent in the IgG-fold. One helix-turn-helix motif consists of αA and αB and connects β3 and β4. This motif along with the β2-β3 loop encircles the C-terminal β12 strand, forming an unusual pseudoknot structure. The second helix-turn-helix motif consists of αC and αD and connects β9 and β10. This motif caps the opening at one end of the β-sandwich. There are several surface-exposed residues that are identical in TEAD-YBD from all species. All these conserved residues form a contiguous surface on one face of TEAD2-YBD that contains β7, αC, αD, and on the back of the strands β4, β11, and β12. The other face of TEAD2-YBD contains few conserved residues. Conserved residues are essential to shape the YAP binding pocket: the strands β4, β11, and β12 form the base of this pocket, whereas β3, αA, and αD form the walls. The residues in the center of the pocket are hydrophobic, whereas several residues lining the periphery of the pocket are polar or charged, including E267, K277, K301, E404, N405, and E429. Therefore, despite having an extensive YAP-binding surface, this pocket on TEAD2-YBD has both hydrophobic and hydrophilic characters, and it represents a major anchoring point

for YAP. A conserved tyrosine in human TEAD1-YDB (Y421, corresponding to Y442 in TEAD2) is mutated to histidine in patients with a rare eye disorder called Sveinsson's chorioretinal atrophy [30]. This mutation disrupts the YAP–TEAD interaction, thus hindering YAP-dependent induction of proliferation. The crystal structure of the complex between human TEAD1 and YAP (PDB ID: 3KYS) confirmed the structure of the putative YBD and shed light on the structural features of the YAP–TEAD interaction [13]. In this structure, YAP sequesters TEAD in a manner akin to a pair of forceps, with three major interaction interfaces. A β strand in YAP (residues 52 to 57) represents the first structural motif interacting with TEAD1 β7, through a series of H-bonds. The conserved hydrophobic residues (61 to 73) of helix α1 in YAP represent the second contact area. An unusual twisted-coil region in YAP (residues 86–100), also referred to as Ω-loop motif, fits snugly within a hydrophobic site on the TEAD1 surface [13]. The last two regions are connected, in YAP, through a linker characterized by a PXXΦP motif (residues 81–85). Similar to YBD in TEADs proteins, YAP, TAZ and Yki conserve, in different species, the residues necessary for interaction with TEAD and therefore for the growth promoting activity of the YAP oncogene. In the crystal structure of mouse TEAD4-YAP (PDB ID: 3JUA), the major interactions of the last two interfaces are conserved, whereas the β strand has not been resolved, and the Ω-loop is referred to as a second alpha helix (α2) [72]. It appears that all three sites of interaction act in concert to mediate the YAP–TEAD complex; β strand, helices α1 and Ω-loop/α2 in YAP appear to contribute most significantly to the interaction with TEAD, whereas the PXXΦP-containing loop appears to play a minor role. The second interaction interface involves the helix α1 of YAP and the helices α3 and α4 of TEAD, where the residues L65, L68 and F69 of YAP form the LXXLF motif interacting with the hydrophobic groove formed by the residues F329, Y361, F365, K368, L369, L372, V381 and F385 of TEAD; all residues involved in this contact area are highly conserved in YAP and TEADs (Fig. 5, left panel). The third interaction site is primarily mediated by the Ω-loop of YAP, which fits in the pocket formed by β4, β11, β12, α1 and α4 of TEAD1. The interaction of the YAP Ω-loop with TEAD1 is chiefly mediated by hydrophobic interactions: M86, L91, F95 of YAP establish Van der

Waals contacts with I262, V257, L297 and V406. This interaction is then strengthened by the polar contacts of K89 and S94 of YAP with D264 and E254 and Y421 of TEAD, respectively (Fig. 5, right panel). Although the sequence of TAZ lacks the PXXΦP motif, a computational model of its structure has suggested that its residues 24-56 can form both a α-helix and a □W-loop that can adopt a conformation reproducing the spatial arrangement of the α1 and W□-loop of YAP, respectively [70, 72].

Recently, the crystal structures of murine Vgll1 and Vgll4 complexed with TEAD4 have been elucidated, uncovering the important role of Vestigial-like proteins 1-4 (Vg in *Drosophila*) as co-transcriptional factors. Similar to YAP and TAZ, Vgll proteins exert their function by binding the C-terminal region of TEAD proteins through the Tondu domain(s) (TDU). Mouse Vgll1-TEAD4 crystal structure (PDB ID: 4EAZ) [73] revealed that Vgll1 interacts with TEAD through two structural elements: the first interaction interface is composed by hydrogen bonds formed by a β strand of Vgll1 (β2), interacting with β7 of TEAD; the second interface is mediated by hydrophobic interactions between Vgll1 helix α1 (TDU domain) and TEAD helices α3 and α4. As revealed by the superposition of the respective crystallographic complexes, mVgll1 and YAP bind to overlapping regions of TEAD, highlighting similarities and differences between the binding modes of the proteins. The β2 strand of mVgll1 and the β1 of YAP occupy the same region, whereas α1 of mVgll1 is superposed to hYAP α1, sharing a short hydrophobic motif (V41, F45, A48 in mVgll1 and L65, F69, V72 in hYAP) that interacts with the groove formed by TEAD α3 and α4 (Fig. 6). In contrast, mVgll1 lacks the Ω-loop, which is fundamental for YAP/TAZ binding to TEAD-YBD surface [13]. Mutagenesis and TR-FRET experiments to describe the interaction modes of Vgll1 fragments and hTEAD4 [74] have revealed that the peptide fragment formed by β2 and α1 is fundamental for mVgll1 binding, showing nanomolar affinity for hTEAD4. This was unexpected, as YAP fragments missing the □-loop lack most of their affinity for TEAD [13]. TR-FRET studies additionally showed that mVgll1-derived peptides can compete with hYAP, which supports the

hypothesis that YAP and Vgll have mutually exclusive effects on TEAD-dependent gene transcription. This hypothesis was further supported by a recent study, which clarified the function of Vgll4 and reported the crystallization of the mVgll4-TEAD4 complex (PDB ID: 4LN0) [75]. This crystal structure highlights striking differences between mVgll1 and mVgll4.

INHIBITION STRATEGIES OF YAP/TAZ/Yki

Considering the strict correlation between YAP hyperactivation and the outbreak of several malignancies, the modulation of its functions represents an innovative and promising approach to prevent and treat human cancers. Although the lack of information about the complete YAP structure prevents the application of a structure-based approach to discover novel compounds binding to YAP, the available structural data indicate that the YAP/TEAD complex is a suitable target for developing new cancer therapeutics. The TEAD surface should be druggable through the fragment-based strategy, and a battery of diverse compounds can be envisioned, which could dock into one or any combination of the three binding sites to disrupt or weaken the YAP–TEAD complex. Even if the design of small-molecule inhibitors of protein–protein interactions have traditionally represented a significant challenge, the availability of crystal coordinates for the YAP–TEAD complexes along with recent successes in structure-based design of protein–protein inhibitors allow to envision a more optimistic scenario for future accessibility to novel pharmacological tools interfering with YAP-mediated TEAD activation.

To identify small molecules that modulate YAP-dependent transcription by computer-aided drug design, Sudol et al. evaluated the possibility of targeting the WW domain, which recognizes proline rich motifs (PPXY), fundamental for YAP interaction with LATS and for its transcriptional activity [67]. Previously, the same group had predicted by virtual models that the cardiac glycoside digitoxin (Fig. 7D) could be considered as a putative ligand at the WW domain of dystrophin [76].

Digitoxin is used to treat cardiac arrhythmias, where its apparent mechanism of action involves modulating the activity of the sodium-potassium ATPase transporter pump. The potential affinity of digitoxin for the WW-domain has emerged by a docking strategy developed to dock 287 FDA-approved small molecule drugs with 35 peptide-binding proteins, including 15 true positives. The selection of peptide binding domains included twenty co-crystal structures selected from the Protein Data Bank (PDB), representing a subset of eukaryotic linear motif (ELM) peptide binding domains complexed with peptides. Regarding the true positives selection, fourteen co-crystal structures and one NMR model were selected from the PDB, with the requirements that they have a protein and a small molecule mimicking a natural peptide in the complex. A combined ligand and target normalization procedure was performed to improve the ability to rank true positives. The docking energy score was combined with a score based on the number of similar interactions formed between the compound and the native ligand. The 20 top ranking hits included 6 true positives, including digitoxin. Considering the similarities between YAP and dystrophin, Sudol et al. built a homology model of YAP WW domain and hypothesized that digitoxin may bind the portion recognizing the PPXY motif [67]. Thus, digitoxin was docked to the canonical hydrophobic groove within the WW-domain of YAP. Digitoxin could engage in an extensive network of intermolecular Van der Waals and hydrogen bonding contacts with an array of residues, such as Y188, L190, T197 and W199, lining the hydrophobic groove within the WW domain. Moreover, these residues are critical for the binding of PPXY ligands. However, other residues such as H192 and Q195 within the WW-domain, which also play a key role in the binding of PPXY ligands, do not appear to be important for binding digitoxin. This suggests that digitoxin is unlikely to target all WW-domains indiscriminately and that potential opportunities exist for the chemical modification of digitoxin to enhance its specificity toward a small group of WW-domains involved in regulating a specific signaling cascade such as the Hippo pathway. Currently, this is only a speculative hypothesis and no experimental data regarding the affinity of digitoxin for YAP are available.

Recent studies have highlighted the capability of G-protein coupled receptor (GPCR) signaling to regulate the Hippo pathway in a manner that is dependent on the specific G-protein coupled to the receptor [11, 77]. This discovery arose from the observation that in multiple cell lines, YAP is highly phosphorylated under serum starvation, whereas the addition of serum results in a rapid decrease in YAP phosphorylation. This suggested the presence of a serum component that activates YAP by inducing its dephosphorylation and nuclear localization. Further studies demonstrated that the active ingredient in serum was an amphiphilic molecule with an acidic group, such as lysophosphatidic acid (LPA) or sphingosine-1-phosphate (S1P). LPA and S1P act via activation of G12/13- or Gq/11-coupled receptor signaling, which leads to inhibition of Lats1/2 kinases and subsequent activation of YAP [77, 78]. In contrast, treatment of the same cell lines with epinephrine and glucagon, which are known Protein-Kinase A (PKA) activators, increases YAP phosphorylation, via activation of the Gs-coupled receptors, which stimulate adenylyl cyclase (AC). This results in the accumulation of cAMP, an important second messenger with diverse physiological functions, including cell proliferation and differentiation. Thus, cAMP acts through protein kinase A (PKA) to stimulate Lats kinase activity and inhibit YAP/TAZ. Altogether these data underline the possibility to modulate YAP/TAZ by a wide range of extracellular signals via GPCRs.

In a recently registered patent, Kung-Lian Guan et al. report the results of a HTS strategy based on a reporter assay in a mammalian cell culture system, consisting of a luciferase reporter and a Gal4-fused TEAD transcription factor [79]. A collection of small molecules was screened to search for potential compounds that regulate YAP, thus leading to changes in the TEAD-dependent expression of the luciferase-reporter. A particular chemical compound (CI08) was identified, which is an oxime derivative of 9H-Fluoren-9-one bearing two piperidinyl-sulfonyl groups (Fig. 7C). This compound potently inhibits YAP by promoting its ubiquitination and subsequent proteasome-mediated degradation. It also inhibits cell proliferation and retards the migration of multiple cancer cell lines *in vitro*, demonstrating its capability to inhibit YAP activity by decreasing the YAP protein levels in

a cell-type-independent manner. Anti-tumor potential of CI08 has also been evaluated in a xenograft mouse model, showing that CI08 blocks melanoma and lung adenocarcinoma tumor growth and induces apoptosis in cancer cells.

Liu-Chittenden et al. set up a luciferase reporter assay to test the YAP-dependent transcriptional activity of a Gal4-TEAD4 assembly and screened the Johns Hopkins Drug Library to identify molecules that inhibit the YAP–TEAD interaction. Verteporfin (VP, trade name: Visudyne by Novartis) and protoporphyrin IX (PPIX) have been identified as top hits of the screening (Fig. 7A). VP and PPIX are compounds belonging to the porphyrin family, which are aromatic heterocyclic molecules composed of four modified pyrrole units, interconnected at their α-carbon atoms via methine bridges [29]. Co-immunoprecipitation assays revealed that both PPIX and VP inhibit the YAP–TEAD complex formation at 10 μM, whereas VP showed >50% inhibition at 2.5 μM, showing a higher potency than PPIX. VP is used clinically as a photosensitizer in the photodynamic therapy of neovascular macular degeneration, where it is activated by laser light to generate reactive oxygen radicals that eliminate the abnormal blood vessels. As an inhibitor of the YAP–TEAD interactions, however, it does not require light activation. It was determined that VP selectively binds YAP, thus altering YAP conformation and abrogating its interaction with TEAD *in vitro*. Moreover, it was also demonstrated that VP inhibits the oncogenic activity of YAP *in vivo*: VP suppresses the liver overgrowth resulting from either YAP overexpression or activation of endogenous YAP [29]. Recently, the effects of VP without light activation have been evaluated on human retinoblastoma cell lines [80]. This study shows that VP determines inhibition of cell growth and viability and that it interferes with the YAP–TEAD proto-oncogene pathway in human retinoblastoma cells. These results are encouraging because VP is a clinically applied drug with few side effects. Moreover, considering that *in vivo* results were obtained using an aqueous preparation in which VP bioavailability is sub-optimal, compared with the lipid-based formulation used in

Visudyne, this strategy may represent an excellent therapeutic approach with minimal adverse effects.

Very recently, Zhang et al. made an important headway in the study of the YAP–TEAD complex, discovering a potent cyclic peptide, which inhibits the protein–protein complex by mimicking the Ω loop of YAP [81]. Truncation studies and an alanine scan performed on the TEAD-binding domain of YAP provided information about the optimal length of the synthetic peptide and advantageous mutations to obtain active parent peptides of YAP. Moreover, the authors optimized the peptide by applying conformational constraints to the structure. As observed in the YAP–TEAD co-crystal structure, 3KYS, R87 and F96 of YAP are kept in close contact, within the Ω loop, by a cation-pi interaction. The alanine scan study indicated that they are important for forming the complex, even if these residues are not involved in a direct interaction with TEAD surface. This suggested that R87 and F96 have a critical role in maintaining YAP in its active conformation. The authors thus applied a macrocyclization strategy, replacing R87 with homocysteine and F96 with cysteine and inducing the formation of a disulfide bond between the two new residues. The inhibitory activity of the peptide was improved through several beneficial mutations: replacement of M86 with 3-Cl-phenylalanine, mutation of L91 to norleucine and of D93 to alanine resulted in a cyclic peptide with IC_{50} equal to 0.025 μM, nearly 1500-fold more potent than the parent $YAP^{84–100}$ peptide fragment, which corresponds to the Ω loop (Fig. 8). A computational model provided a rational explanation of the high potency of the synthetic peptide, showing favorable Van der Waals interactions between the 3-Cl-phenylalanine substituent and hydrophobic residues on TEAD surface. GST pull-down and functional assays revealed that the synthetic cyclic peptide competes with the endogenous YAP protein, showing a high affinity for TEAD. Altogether, this study reveals not only a potent inhibitor of the YAP–TEAD interaction but also an exhaustive structure-activity relationship landscape for the YAP Ω loop. Additionally, an enhancement of the hydrophobic properties at M86, L91 and F95 of YAP represents a suitable strategy to improve the affinity of YAP-derived peptides for TEAD,

and polar interactions between S94 of YAP and Y421 and E255 of TEAD are fundamental. This information can be advantageous for the design of small molecules inhibiting the YAP–TEAD complex.

CONCLUSIONS

Over the last few years, the Hippo pathway has emerged as a promising anticancer target, as revealed by several experimental lines of evidence, which indicate that targeting the Hippo pathway represents an effective strategy against oncogenic progression. It is becoming increasingly clear that the Hippo pathway can regulate SC proliferation and maintenance; therefore, its modulation may be therapeutically useful for tissue repair and regeneration following injury. Moreover, the complex network of regulatory components within the Hippo pathway is being elucidated, and robust assays that can measure the activity of this pathway have been established. Altogether, these findings offer new possibilities for discovering useful pharmacological tools to further understand the precise role of the Hippo pathway components and simultaneously design novel small molecules that modulate the Hippo pathway. Although small molecules interfering with the Hippo pathway have been reported, the molecular information is scarce and incomplete. For example, sphingosine-1-phosphate (S1P) has been reported as a potent activator of YAP, promoting YAP nuclear localization [78]. As revealed by siRNA transfection experiments, S1P determines YAP activation through the Rho GTPase-mediated pathway, which is a well-known positive modulator of YAP/TAZ. A recently published patent reports the results of a HTS campaign that led to the discovery of compounds, including a compound in Fig. 7C, which promotes a GPCR-mediated activation of the Hippo pathway and the consequent YAP phosphorylation and ubiquitination [79]. Recently, a small molecule (C19, 4-(4(3,4-dichlorophenyl)-1,2,5-thiadiazol-3-yloxy)butanol, Fig. 7B) has been observed to inhibit the Hippo pathway by activating Mst/Lats kinase and promoting TAZ (but not YAP) phosphorylation and inactivation [82]. Furthermore, C19 markedly inhibited

tumor growth *in vivo*. However, the molecular site of action for these compounds is not precisely characterized, which hampers structure-based discovery of new small molecules and their chemical optimization. Currently, YAP/TAZ could be considered a promising target for this aim, considering the availability of some structural information about their complex with TEAD. Although a potent cyclopeptide that disrupts the YAP–TEAD complex formation has been recently discovered, the only small molecule reported so far to directly inhibit the protein–protein interaction is verteporfin. Despite the interest regarding this compound, which is already being used as a drug, detailed structural information about its interaction with YAP or TEAD is still lacking, which signifies a pivotal role to (virtual) screening campaigns and/or fragment-based approaches for the discovery of new small-molecule inhibitors of YAP activation.

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Table **1**: Hippo signaling pathway core components and modulators in *D. melanogaster* and mammals

D. melanogaster **Modulators**	**Mammals**	**Role in the pathway**	**Effect on Yki/YAP**
Ft (Fat)	Ft1-4	In *Drosophila*, Ft initiates signaling upon binding to Ds	-
Ds (Dachsous)	Dchs1-2	In *Drosophila*, Ds binds to and activates Ft	-
Ex (Expanded)	FRMD6 (Ex1)	In *Drosophila*, the Ex-Mer-Kibra complex activates Hpo	-
Mer (Merlin)	NF2	In *Drosophila*, the Ex-Mer-Kibra complex activates Hpo	-
Kibra	WWC1-2	In *Drosophila*, the Ex-Mer-Kibra complex activates Hpo	-
Crb (Crumbs)	Crb1-3	Apical polarity complex with PatJ and Std	-
PatJ	PatJ, MUPP1	Apical polarity complex with Crb and Std	
Std (Stardust)	Pals1	Apical polarity complex with Crb and PatJ	
aPKC	aPKC	Apical polarity complex with Par3 and Par6 (in *Drosophila*)	+
Par3	Par3	Apical polarity complex with Par6 and aPKC (in *Drosophila*)	
Par6	Par6	Apical polarity complex with Par3 and aPKC (in *Drosophila*)	
Lgl	Lgl1-2	Baso-lateral polarity complex	-
Scrib (Scribble)	Scrib	Baso-lateral polarity complex	
Dlg (Disc Large)	Dlg1-4	Baso-lateral polarity complex	
dRASSF		In *Drosophila*, dRASSF competes with Sav in binding to Hpo	+
	RASSF1-6	In mammals, RASSF activates Mst1-2 (except RASSF6)	-
dSTRIPAK	PP2A	Dephosphorylates Hpo/Mst1-2	+
Jub	Ajuba	Interacts with Sav/Sav1 and Wts/Lats1-2	+
Dco		In *Drosophila*, Dco enhances Ft activation	
	CK1δ/ε	In mammals, CK1δ/ε primes YAP for degradation	-
	AMOT, AMOTL1, AMOTL2	Modulate YAP subcellular localization and/or phosphorylation	-
ZO-2	ZO1-2	Sequesters YAP/TAZ in the cytoplasm	-
	PTPN14	Sequesters YAP/TAZ in the cytoplasm	-
α-Catenin	α-Catenin	Stabilizes YAP-14.3.3 complex	-

Core components			
Hpo (Hippo)	Mst1-2	Phosphorylates and binds to Sav/Sav1; phosphorylates Mats/Mob1	-
Sav (Salvador)	Sav1	Forms a kinase complex with Hpo/Mst1-2; the complex phosphorylates Wts/Lats1-2	-
Wts (Warts)	Lats1-2	Forms a kinase complex with Mats/Mob1	-
Mats	MOBKL1A-B (Mob1)	Forms a kinase complex with Wts/Lats1-2; the complex phosphorylates Yki/YAP/TAZ	-
Yki (Yorkie)	YAP, TAZ	Transcriptional co-activator	
Sd (Scalloped)	TEAD1-4	Transcription factor	

Table **2**: Known effects and mechanisms of action of YAP in different stem cell types

Stem Cell Type	YAP Activation	YAP Inactivation	Mechanism of Action
Embryonic SCs	Pluripotency support; Hindering of differentiation; Reprogramming process enhancement	Loss of self-renewal and pluripotency	Direct induction of important stemness-genes (i.e., Oct4, Sox2, Nanog)
Skin SCs	Thickening of epidermal layer; Hyper-keratinization; Tumor formation	Epidermal hypoplasia;	YAP overexpression drives the expansion of undifferentiated interfollicular SCs and progenitor cells; YAP inactivation drives the gradual loss of epidermal stem/progenitor cells and their limited capacity to self-renewal
Intestinal SCs	ISCs proliferation and expansion of progenitor-like cells	Loss of ISCs and degeneration of the intestinal epithelium	Nuclear YAP enhances Wnt-signaling Cytoplasmic YAP represses Wnt-signaling
Neural SCs	Neural progenitor cells expansion; Expansion of cerebellar granule neural precursors; Medulloblastoma		Shh-signaling induces the expression and nuclear localization of YAP in cerebellar granule neural precursors
Liver SCs	Liver hyperplasia; hepatocyte hyperproliferation	Impaired liver function; hepatocyte apoptosis	YAP overexpression enhances proliferation of mature hepatocytes; YAP inactivation induces enhanced hepatocyte apoptosis
Cardiac Muscle SCs	Cardiomyocyte proliferation	Myocardial hypoplasia	Nuclear YAP enhances Wnt-signaling (both directly and via IGF-pathway);

Table **3**: Percentage of identity among the four isoforms of hYAP and hTAZ

% identity	hYAP2	hYAP3	hYAP4	hTAZ
hYAP1	89	96	64	38
hYAP2		92	53	42
hYAP3			61	39
hYAP4				28

Table **4**: Percentage of identity among the four isoforms of hTEAD

% identity	hTEAD2	hTEAD3	hTEAD4
hTEAD1	63	69	73
hTEAD2		61	61
hTEAD3			68

Figure legends:

Figure 1. The Hippo signaling pathway in mammals: the Hippo pathway is a kinase cascade consisting of serine/threonine phosphorylation events leading to cell proliferation inhibition and the promotion of apoptosis via inhibition of the transcriptional co-activators YAP/TAZ. The picture is a schematic representation of several interactors and regulators that play an important role in the mammalian Hippo pathway; pointed arrowheads represent activation, blunted arrowheads represent inhibition; dashed lines represent regulation mechanisms that are not fully elucidated (see text for detailed description).

Figure 2: (A) Self-renewal and differentiation regulation in mouse-ESCs: mESCs depend on the cytokine Leukemia Inhibitory Factor (LIF), signals from Bone Morphogenic Proteins (BMPs) and Wnt-signaling to reinforce the pluripotency network and block differentiation and progression to lineage-commitment. The LIF-pathway leads to the activation of JAK-STAT signaling, with STAT3 homodimers acting as transcription factors for major pluripotency and self-renewal genes; BMP-signaling determines the activation of Smad1/5-Smad4 transcription factors, targeting promoters for major stemness genes. (B) Self-renewal and differentiation regulation in human-ESCs: the self-renewal and differentiation of hESCs depend on Fibroblast Growth Factor (FGF) signaling and a balance between Transforming Growth Factor-β (TGF-β) and BMP-signaling. FGF-signaling supports the self-renewal via Protein Kinase C (PKC) and Phosphatidyl-Inositol-3-Kinase (PI3K). TGF-β signaling leads to Smad2/3 activation and the formation of Smad2/3-Smad4 heterodimer, which act as transcription factors promoting self-renewal and blocking differentiation. BMP-signaling leads to Smad1/5 activation and subsequent Smad1/5-Smad4 heterodimer formation, promoting differentiation and inhibiting self-renewal: hESCs fate depends on the balance between the two pathways. (C) Wnt-signaling in intestinal-SCs: Wnt-OFF state (left), Wnt-ON state (right); (D) Hippo/Wnt-signaling effects on intestinal crypt destiny: active YAP or Hippo ablation enhances Wnt-signaling, increasing Dsh phosphorylation and consequent β-catenin nuclear accumulation. Once in the nucleus, YAP interacts with β-catenin and TEAD to activate the expression of proliferation-related genes, and Dsh acts as a transcriptional co-factor to induce β-catenin target genes.

Figure 3: Alignment of the four isoforms of human YAP and human TAZ. Identical residues are highlighted as follows: red if identical among 3 out of 5 sequences, yellow if identical among 4 out of 5 sequences, and green if identical among all the 5 sequences. Protein domains are indicated as follows: TEAD-binding domain in magenta, 14-3-3-binding domain in red, WW domains in

blue, SH3-binding domain in green, Transcriptional activation domain in orange, Coiled-coil domain in brown, PDZ-binding domain in purple.

Figure 4: Alignment of the four isoforms of human TEAD. Identical residues are highlighted as follows: red if identical among 3 out of 5 sequences, yellow if identical among 4 out of 5 sequences, and green if identical among all the 5 sequences. Protein domains are indicated as follows: DNA-binding TEA domain in orange, YAP-binding domain in magenta.

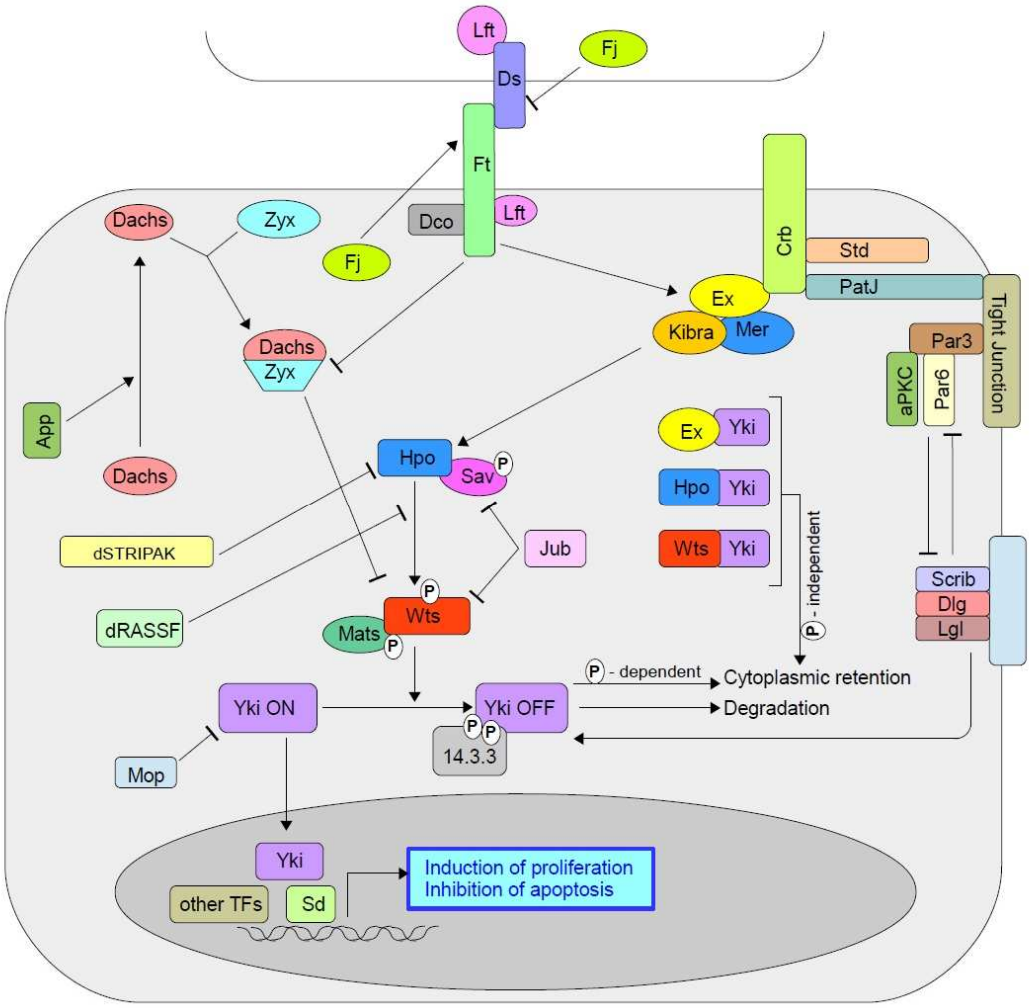
Figure 5: Protein–protein interactions between human YAP (orange ribbons; residues 50–100 from crystal structure 3KYS) and human TEAD (white ribbons; residues 200–426 from crystal structure 3KYS). Left panel: lipophilic residues on α-helix 1 (orange carbons) of YAP interact with the hydrophobic groove formed by the α-helix 3 and 4 of TEAD (blue ribbons). Right panel (resulting from 90° rotation around y axis): the interaction between the YAP Ω-loop and TEAD is mediated through Van der Waals interactions between hydrophobic residues and through hydrogen bonds highlighted by yellow dots (YAP residues: orange carbons; TEAD residues: white carbons).

Figure 6: Comparison of the interacting conformations of human YAP (orange ribbon; residues 50–100 from crystal structure 3KYS) and mouse Vgll1 (cyan ribbons; residues 19–50 from crystal structure 4EAZ) with human TEAD (white surface; residues 200–426 from crystal structure 3KYS).

Figure 7: Chemical structures of small molecules interacting with Hippo pathway components are shown; (a) verteporfin (trade name Visudyne by Novartis) consists of a mixture of equally active regioisomers; verteporfin is a light-activated drug, used in photodynamic therapy for the treatment of macular degeneration. (b) C19 (4-(4(3,4-dichlorophenyl)-1,2,5-thiadiazol-3-yloxy)butanol). (c) 2,7-bis(piperidin-l-yl-sulfonyl)-9H-fluoren-9-one oxime. (d) Cardio-active glycoside digitoxin.

Figure 8: Chemical structure of a potent cyclopeptide inhibiting the YAP–TEAD interaction. The peptide mimics the YAP Ω loop (residues 84–100); R87 and F96 were mutated to cysteine and homocysteine, respectively, to allow the formation of an internal disulfide bond; further modifications were performed to improve binding affinity to TEAD. α-carbons of mutated residues are marked by an asterisk.

Figure 1: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Induction of proliferation
Inhibition of apoptosis

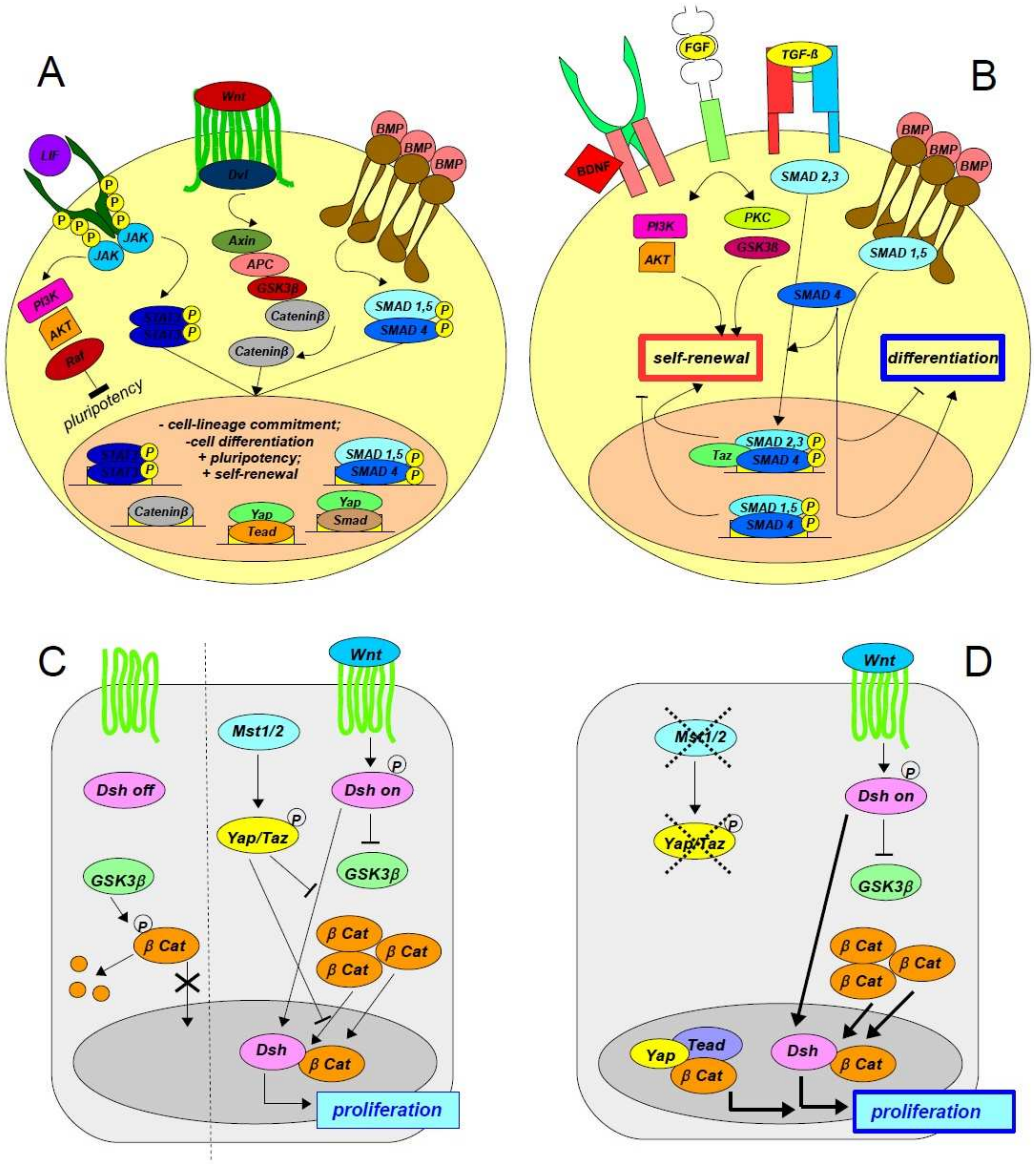
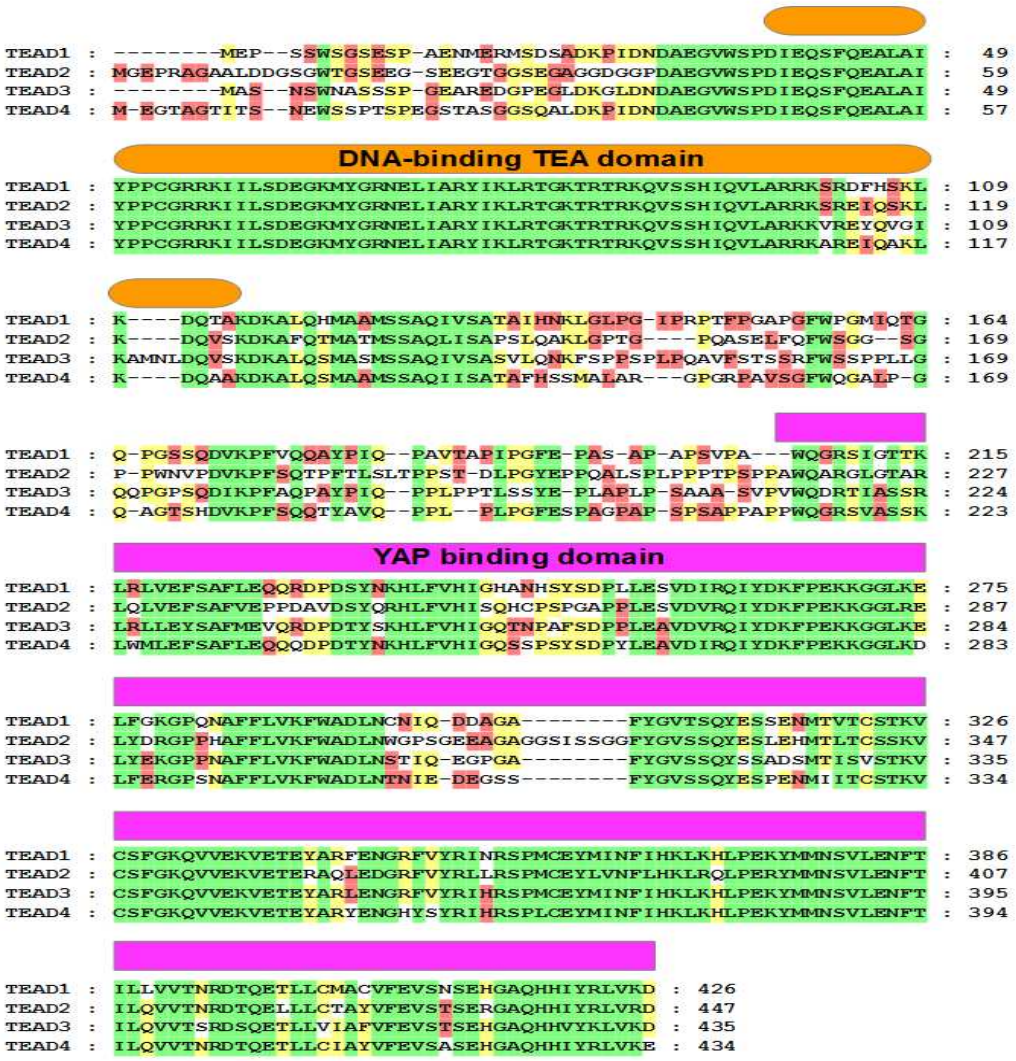

A
Wnt
Dvl
LIF
JAK
JAK
PI3K
AKT
Raf
pluripotency
Axin
APC
GSK3β
Cateninβ
Cateninβ
BMP
BMP
BMP
SMAD 1,5
SMAD 4
- cell-lineage commitment;
-cell differentiation
+ pluripotency;
+ self-renewal
Cateninβ
Yap
Tead
Yap
Smad
B
FGF
TGF-ß
BDNF
SMAD 2,3
PI3K
AKT
PKC
GSK3ß
SMAD 1,5
SMAD 4
self-renewal
differentiation
Taz
SMAD 2,3
SMAD 4
SMAD 1,5
SMAD 4
C
Wnt
Mst1/2
Dsh off
Dsh on
Yap/Taz
GSK3β
GSK3β
β Cat
β Cat
β Cat
β Cat
Dsh
β Cat
proliferation
D
Wnt
Mst1/2
Yap/Taz
Dsh on
GSK3β
β Cat
β Cat
β Cat
Yap
Tead
β Cat
Dsh
β Cat
proliferation

Figure 2

Figure 3:


TEAD binding domain
14-3-3 bd
WW domain
WW domain
SH3 bd
Coiled-coil domain
Transcriptional activation domain
PDZ bd

Figure 4:



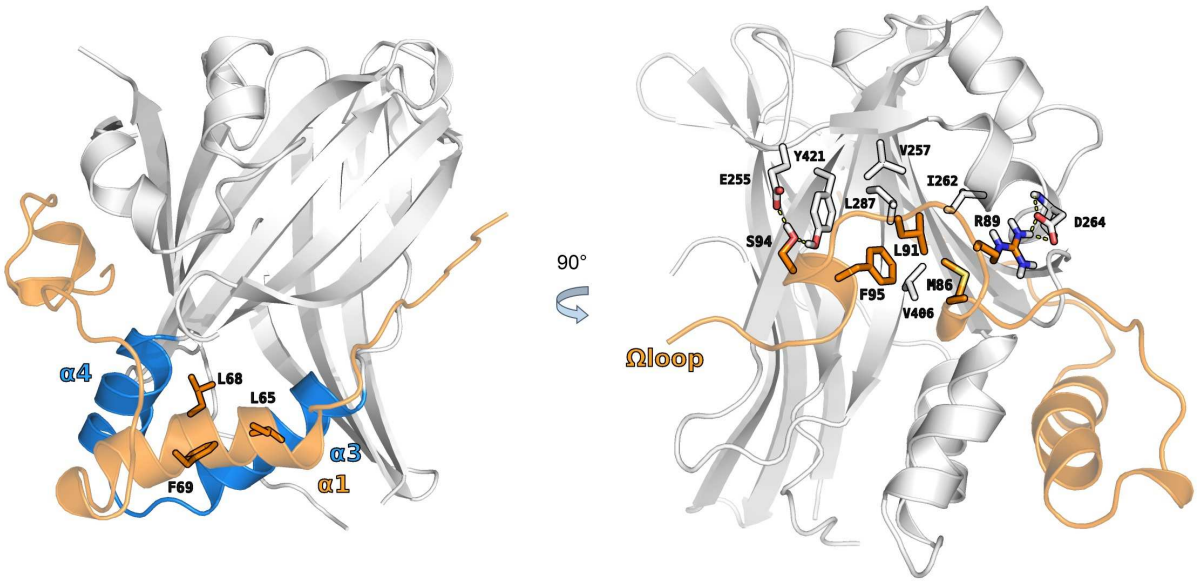

α4
L68
L65
α3
α1
F69
90°
Y421
V257
E255
I262
L287
R89
D264
S94
L91
F95
M86
V406
Ωloop

Figure 5:

Figure 6:

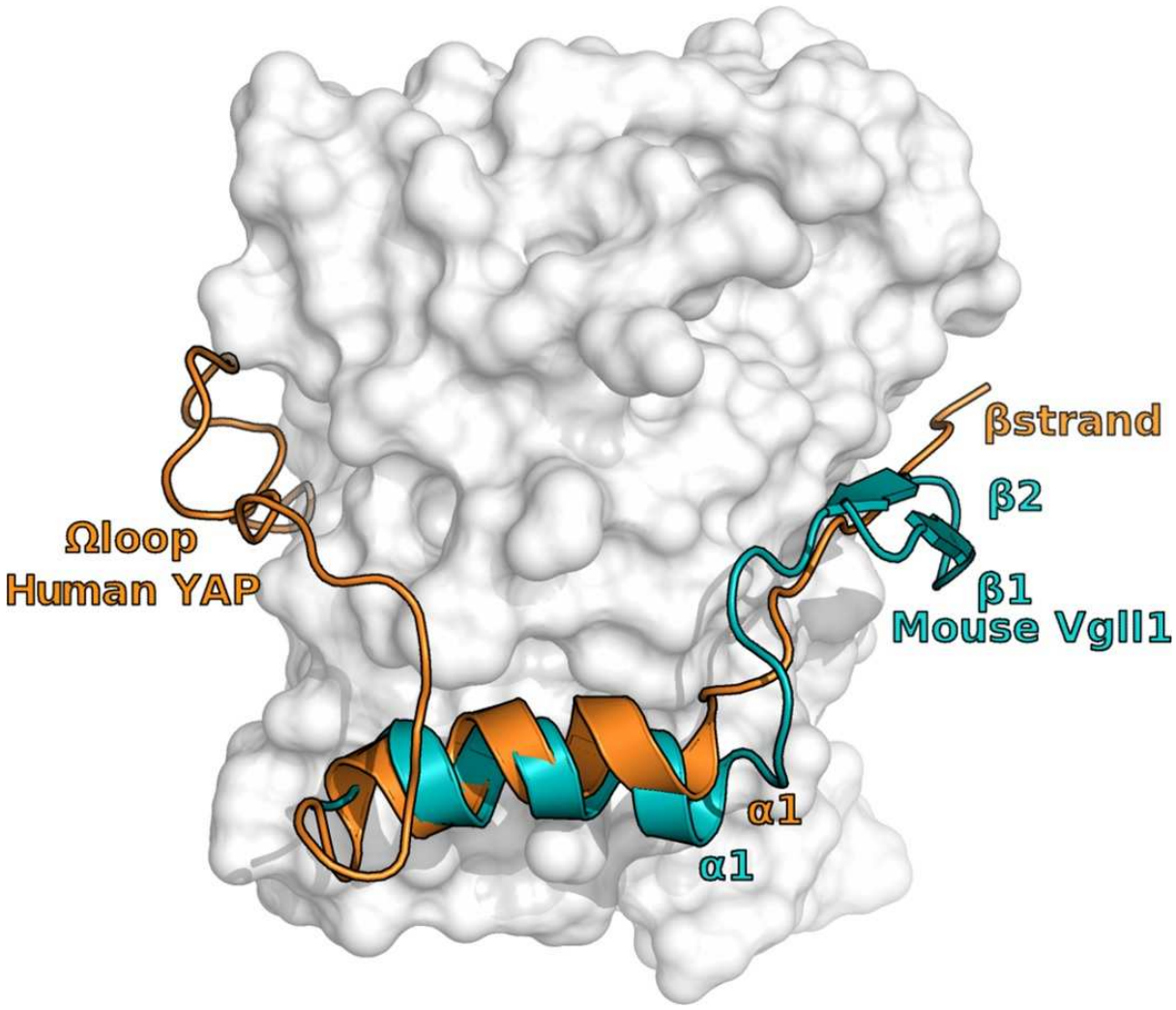
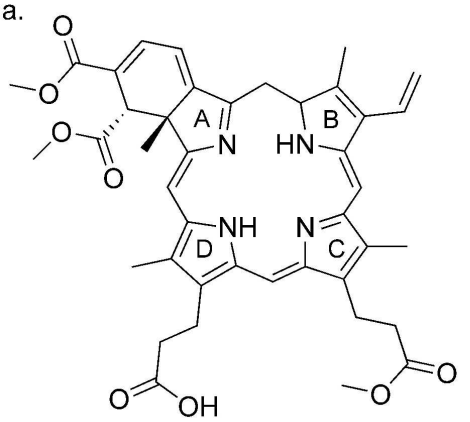
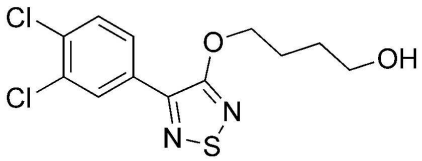


Figure 7:

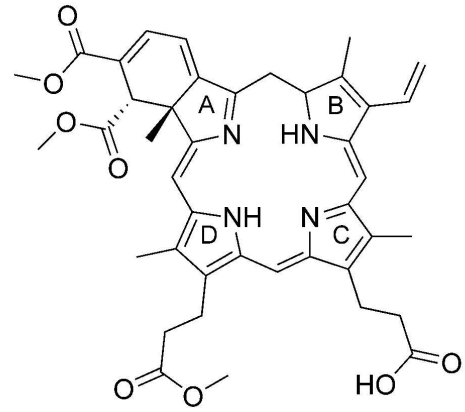
a.

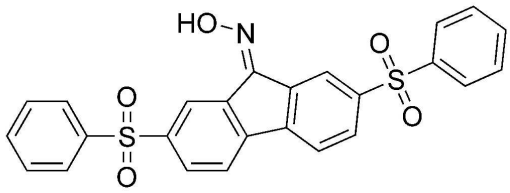


b.



c.





d.

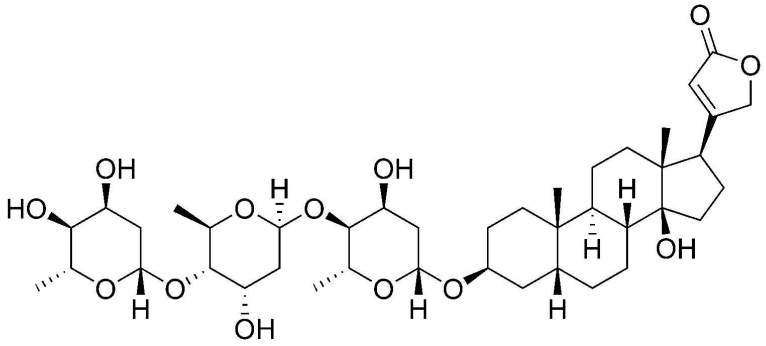
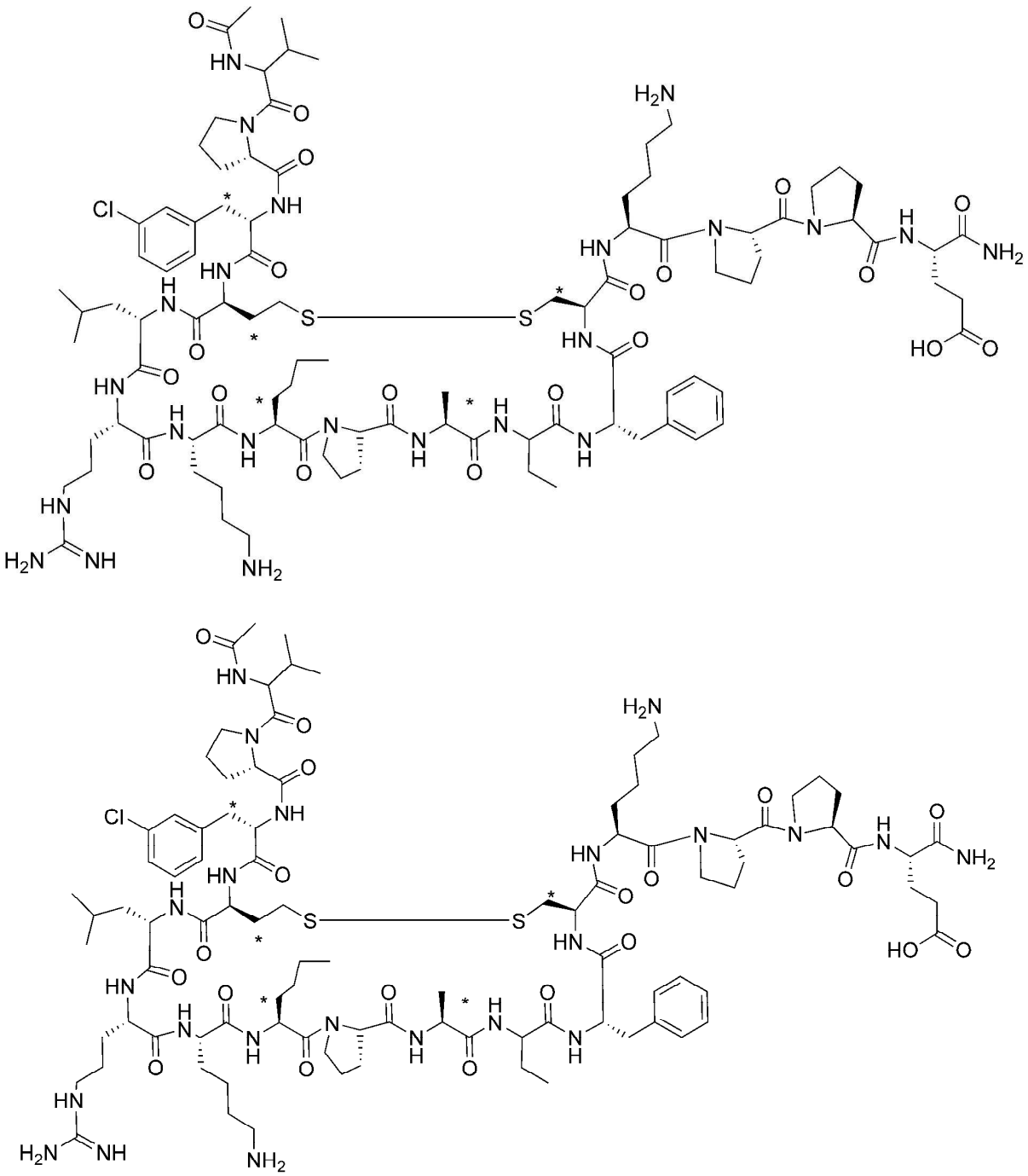


Figure 8:



Biographycal sketch of each author.

Matteo Santucci received his master degree in Pharmaceutical Biotechnologies at the University of Modena and Reggio in 2012. He is currently a student of the PhD course "Science and Technologies of Health Products" at the University of Modena, in the laboratory of Prof. Maria Paola Costi. His main research interests concern different protein-enzyme purification; drug-target interaction studies between small peptides/bioactive compounds and target enzyme and enzymatic studies in medium-high throughput screening in drug-preclinical development, including optical, fluorimetric and calorimetric techniques (UV-Vis and fluorescence spectroscopy, isothermal titration calorimetry).

Tatiana Vignudelli studied Medical Biotechnolgies at the University of Modena and Reggio Emilia, where she graduated in 2002 after a visiting fellowship at Thomas Jefferson University in Philadelphia (USA). In 2008 she received her PhD in Biotechnology and Molecular Medicine at the University of Modena and Reggio Emilia and she is currently collaborating with Prof. Costi's Lab. Her research has mainly been focused on the molecular mechanisms underlying signal transduction pathways regulating differentiation and proliferation of hematopoietic stem cells.

Stefania Ferrari graduated in Pharmaceutical Chemistry and Technology in 1998 and received her doctorate in the Sciences of Drugs in 2002 at the University of Modena and Reggio Emilia. Since 2002 she has been collaborating with Prof. Costi in many projects focused in desing and synthesis of new compounds, computation and biophysical studies of drug-ligand or protein-protein interactions, proteomics and translational research. From 2014 she holds a three-years temporary researcher position at University of Modena and Reggio Emilia.

Marco Mor received his Laurea in Pharmaceutical Chemistry and Technology at the University of Parma (Italy) in 1990. He had collaborated with the Chemoinformatics and Drug Design group at Glaxo Research Center in Verona for three years. In 1993 he became Lecturer at the University of Parma, where he is now Full Professor of Medicinal Chemistry and coordinator of the PhD course in Drugs, Biomolecules and Health Products, at the University of Parma. His research is mainly focused on computer-aided design and SAR analysis of GPCR ligands, tyrosine kinase inhibitors and modulators of the endocannabinoid system.

Laura Scalvini received her master's degree in Pharmaceutical Chemistry and Technology from the University of Parma in 2012. She is currently a student of the PhD course "Design and Synthesis of Biologically Active Compounds" at the University of Parma. Her main research interest is computer-aided design of modulators of endocannabinod system and small molecules for the modulation of stem cell fate.

Maria Laura Bolognesi received her PhD in Pharmaceutical Sciences in 1996, studying under Carlo Melchiorre at Bologna University. After postdoctoral studies at the University of Minnesota with Philip S. Portoghese, she returned to Bologna University in 1998 as an Assistant Professor and became an Associate Professor in 2005. In 2009 she was awarded a position of Distinguished Visiting Professor at Universidad Complutense de Madrid and in 2014 a position of Special Visiting Researcher (Pesquisador Visitante Especial) at University of Brasilia. Her research focuses on the design and synthesis of small molecules as probes for the investigation of biological processes or as drug candidates

Elisa Uliassi received her master's degree in Pharmaceutical Chemistry and Technology in 2012 from the University of Bologna. She is currently a doctoral student in the Bolognesi Lab. Her research work focuses on the development of small molecules for stem cell fate modulation.

Maria Paola Costi received a degree in Chemistry and Pharmaceutical Science, and in Pharmacy at the University of Modena. She obtained her PhD in Medicinal Chemistry in Pharmaceutical Science in 1989. She is professor in Medicinal Chemistry and her expertise is in medicinal chemistry and translational research. She is actively working in three main research area on the identification and synthesis of leads in the topic of Thymidylate synthase enzymes structure, function, inhibition and network-pathways in cancer; folate related enzymes involved in parasitic disease and beta-lactamase structure, function and inhibition. She has coordinated international projects in the area of ovarian cancer and in infectious diseases.

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