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Running Title: Overview of the Hippo signaling pathway

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Abstract

How the organ size is adjusted to the proper size during development and how organs know that they reach the original size during regeneration remain long-standing questions. Based on studies using multiple model organisms and approaches for over 20 years, a consensus has been established that the Hippo pathway plays crucial roles in controlling organ size and maintaining tissue homeostasis. Given the significance of these processes, the dysregulation of the Hippo pathway has also implicated various diseases, such as tissue degeneration and cancer. By regulating the downstream transcriptional coactivators YAP and TAZ, the Hippo pathway coordinates cell proliferation and apoptosis in response to a variety of signals including cell contact inhibition, polarity, mechanical sensation and soluble factors. Since the core components and their functions of the Hippo pathway are evolutionarily conserved, this pathway serves as a global regulator of organ size control. Therefore, further investigation of the regulatory mechanisms will provide physiological insights to better understand tissue homeostasis. In this review, the historical developments and current understandings of the regulatory mechanism of Hippo signaling pathway are discussed.

Introduction

Both the growth to the right size and the patterning to an accurate shape are essential for an organ to ensure functionality during development and regeneration. These have been obviously curious topics in biology; however, the mechanism for how organ size is controlled remains poorly understood (1-3). Organ size control is tightly coordinated in response to physiological cues that represent intrinsic and extrinsic mechanisms. In 1931, Twitty and Schwind provided the first clue that an organ possesses an intrinsic mechanism to determine its final size. When embryonic tissues that would become a leg were hetero-transplanted between two species of salamanders that have legs of different sizes, the legs grew to the final size of the donor (4). Likewise, a surgically removed liver regenerates quickly and stops growing when it reaches its original mass, which indicates that the intrinsic information of the organ, not the external signals, determines its final size (5). In this regard, how a growing organ can sense its current size and remember its original size to regulate its growth is still a mystery. Metcalf demonstrated a striking observation that organ size can be determined by both autonomous and non-autonomous mechanisms in mammalian systems. He demonstrated that each of the multiple thymus grafts to a recipient animal grew to an adult size, which supports an autonomous mechanism; while spleen grafts exhibited restricted growth capacity (6, 7). Collectively, these previous reports suggest that both intrinsic and extrinsic mechanisms operate in the regulation of organ size determination.

Organ growth is a consequence of an increase in cell number and/or cell size, which is affected by mitogen or growth factor-mediated signal transduction. Extensive studies using multiple approaches have identified that several signaling pathways, including Wnt/β-catenin, Notch and mTOR, are also involved in the regulation of cell size and/or number in metazoans. A growing body of evidence suggests that the Hippo pathway functions as a central regulator of organ size determination by coordinating cell proliferation and apoptosis. Components of the Hippo pathway and its growth-

regulatory function are conserved in all metazoan animals as well as pre-metazoan origins (8, 9).

The Hippo pathway basically consists of a core kinase cascade containing the Ste-20 family of protein kinase MST1/2, the scaffolding protein Salvador, and large tumor suppressor kinase LATS1/2 to inhibit the transcriptional co-activators YAP and TAZ. A wide range of signals derived from cell contact, polarity, energy or mechanical stress and GPCR signaling activate the Hippo pathway, which eventually results in YAP/TAZ phosphorylation by LATS1/2 (10-12). As a result, 14-3-3 interacts and induces the cytoplasmic retention of phosphorylated YAP/TAZ, which leads to β-TrCP-mediated proteasomal degradation (13-15). However, the cytoskeleton rearrangement, genetic mutation or growth factors inhibit LATS1/2-mediated YAP/TAZ phosphorylation and enable YAP/TAZ to enter the nucleus to activate the transcriptional programs that are involved in cell proliferation and survival (12, 16). In addition, alternative models have shown that YAP/TAZ activity can be modulated by novel regulators such as deubiquitinase YOD1, MAP4 kinase or spatial organization of core components (17-20). The Hippo pathway has a significant impact on a variety of human diseases including tissue degeneration and cancer. Besides the primary functions of cell proliferation and organ size control, the reports recently published expand our understanding of the Hippo pathway into previously unrecognized cellular processes including miRNA biogenesis, innate immunity, autophagy, atherogenesis, and cell ploidy (21-29). Due to its importance in pathological and physiological contexts, the Hippo pathway has attracted much attention, and the numbers of reports are growing rapidly every year (Figure 1).

Long journey to elucidation of the Hippo pathway

Discovery of the core components

In 1995, a mosaic-based screening in a *Drosophila* imaginal disc to identify genes involved in growth control revealed *Warts (Wts)*, which encodes a kinase of the Nuclear Dbf2-related (NDR) family, as a tumor suppressor gene. The loss of *Wts* in flies resulted in robust overgrowth in multiple tissues without affecting cell fate determination (30, 31). The development of soft-tissue sarcomas and ovarian tumors in mice-deficient Lats1, a mammalian homologue of *Drosophila* Wts, further confirmed its tumor suppressive function in mammals (32). Although a cell cycle regulator CDC2 was identified as a binding partner (33), information regarding its regulators, substrates and additional binding partners remained largely unknown for several years since Wts was discovered. The identification of *Salvador* (*Sav*) by the two groups led to our interest in a novel growth-regulatory pathway (34, 35). Mutations in *Sav*, *WW45* in humans, which contains two WW-domains, resulted in a similar overgrowth elicited by a mutation in *Wts*. In particular, Hariharan and colleagues have addressed the physical interaction of Sav with Wts and its function that limits cell proliferation and stimulate apoptosis by regulating *Cyclin E* and *Diap* (*Drosophila* inhibitor of apoptosis) transcriptionally. Supporting its role in tumor suppression, mutation in the human homologue WW45 was observed in cancer cell lines (34). The next substantial advance in the size-control mechanism was the discovery of the *Hippo* (*Hpo*) tumor suppressor gene, which encodes Ste-20 family protein kinase and is the homolog of *MST1* and *MST2* in humans (36-40). Several independent groups have provided the evidence that Hpo genetically and physically interacts with Sav and Wts to restrict cell proliferation and function in a common pathway. Hpo-mediated phosphorylation of Sav and Wts is particularly significant because it provides the signaling module of a new growth-regulatory pathway (Hippo pathway) consisting of kinase cascade. Mats was incorporated as a bona fide regulator of the Hippo

pathway. The loss-of-function of Mats causes massive tissue growth, which is similar to the phenotype caused by the loss of Hpo, Sav or Wts. Further investigation revealed that Mats, *MOB1* in humans, phosphorylation by Hpo is required for Wts kinase activity (41, 42). Key findings on the Hippo signaling pathway are chronologically summarized in Figure 2.

Yorkie/YAP as a transcriptional activator in the Hippo pathway

Since the expression of genes such as *Cyclin E* and *Diap* could be regulated by the Hippo pathway (36), the identification of the transcriptional regulator(s) that functionally link with upstream kinase cascade was required. In this regard, Huang *et al.* performed a yeast two hybrid screen with Wts as a bait to search for a transcriptional activator and finally identified *Yorkie* (Yki), *YAP* and its paralogue *TAZ* in mammals, as a downstream effector in growth regulation (10). Biochemical and genetic studies revealed that Yki is required for normal tissue growth and its activity is inhibited by Wts-mediated phosphorylation (10). In addition, the removal of *Yki* in *Drosophila* diminished the overgrowth phenotype caused by deleting upstream kinases *Wts* or *Hpo*, which indicated that Yki is a central transcription regulator of the Hippo signaling pathway. Guan and colleagues uncovered an underlying molecular mechanism by which Wts/LATS inhibits Yki/YAP activity. They observed YAP contains five LATS kinase consensus sequences (HxRxxS/T) and phosphorylation at S127 leads to cytoplasmic retention of YAP via the interaction with 14-3-3, thereby inhibiting transcriptional activity (43). The central mechanism of the Hippo signal transduction has been established by these remarkable findings and we are currently able to measure the output of the Hippo signaling by easily examining the level of phosphorylation on YAP-S127. In addition, the function of Yki as a critical effector in organ growth as demonstrated in flies was further confirmed in a genetic mouse model. The removal of *Yap* largely suppressed the phenotypes caused by the deletion of *Nf2*, a homolog of *Drosophila* Merlin that is an

upstream regulator of Hpo (44). Therefore, growth regulatory function by Hpo-Yki/YAP signaling is evolutionarily conserved in the mechanism of size control. Expecting its growth-promoting capability, YAP was suggested as a potential oncogene due to its association with gene amplification and epigenetic modulation in human cancers, and it is currently accepted as a critical "driver" gene (45-48). Taken together, the Hippo signaling plays a critical role in tumor suppression and its dysregulation is associated with human cancers. Further details about the role of Hippo signaling in different cancers are discussed in an accompanying paper (Kim and Myung, in this issue). The schematic diagram for the components of the Hippo signaling pathway described in this review is displayed in Figure 3.

Role of Yki/YAP in transcriptional activation

Unlike other transcriptional factors, Yki and YAP/TAZ do not have a DNA-binding ability; therefore, they require binding partner(s) that directly bind to DNA and mediate target gene expression. YAP was identified by Sudol as the Yes, tyrosine kinase, -associated protein (49). Therefore, before elucidation of its crucial role in organ size control, several DNA binding proteins, including p73, p53BP-2, SMAD7, ERBB4, PEBP2α, RUNX2 and TEAD, had been already reported as YAP-interacting transcriptional factors (50-56). The binding partners of YAP/TAZ are reviewed in detail in an accompanying paper (Kim *et al.*, in this issue). However, the physiological relevance of these interactions in organ size control remains unknown. Multiple lines of evidence revealed that *Scalloped* (Sd), TEAD1-4 in humans, is the major binding partner of Yki/Yap in regulating gene expression and tissue growth (57-60). The loss of TEAD as well as the TEAD-binding deficient mutant of YAP (S94A or S79A in humans or mice, respectively) can no longer activate target gene expression. Additionally, the knock-in (KI) of this TEAD binding mutant exhibits a similar phenotype as the YAP knockout (KO) in mice tissues (61, 62). The list of the genes induced by YAP generally overlapped with that activated

by TEAD or TEAD-VP16 (YAP-binding deficient but a constitutively active form of TEAD via a fusion of VP16 transactivation domain) (57, 60). Recent studies suggested that VGLL4 competes with YAP for binding TEAD and functions as a tumor suppressor (63). Tgi, a similarly functioning protein like VGLL4, was also observed in *Drosophila* (64). It is not known how YAP/TAZ is translocated into the nucleus and will be interesting to test whether those YAP/TAZ interacting partners are involved in that process.

Upstream signals of the Hippo pathway

Cell polarity and cell contact

The growth-regulatory pathway from MST1/2 (Hpo) to YAP/TAZ (Yki)-mediated transcription was established almost a decade after the first gene, *Wts,* was identified. However, it remains questionable which and how the physiological cues were delivered into the Hippo pathway in regulating organ size. The identification of the apical-basal and planar cell polarity machinery that regulate Hippo signaling initially provided the entry point to understanding these missing links. The FERM domain proteins Merlin (Mer) and Expanded (Ex) have known to co-localize at the adherens junctions to link transmembrane proteins to the cytoskeleton. In addition, the mutation in the *Neurofibromatosis type-2* (*Nf2*), a homolog of *Drosophila Merlin*, causes a familial cancer syndrome; but, its downstream mechanism remains elusive. Hamaratoglu *et al.* clearly demonstrated that Mer and Ex genetically function at the upstream of Hpo kinase and as tumor suppressors (65). Mer and Ex synergistically induce Wts phosphorylation and subsequent Yki inhibition. Moreover, it soon became apparent that atypical cadherin Fat could act at the upstream of Ex by promoting its stability and apical membrane localization (66-68). Also, the Sterile 20-like kinase Tao-1 has been reported as a mediator that connects upstream components Ex and Mer to the Hippo pathway by directly phosphorylating and

activating Hpo kinase, which leads to a Wts mediated repression of Yki (69, 70). Recently, it has been reported that Ex is required for apical localization of Schwannomin interacting protein 1 (Schip1) and Schip1 recruits Tao-1 kinase for the phosphorylation of Hpo kinase (71). Another major progress was made by the identification of Kibra (72-74). Kibra forms a complex with Ex and Mer to recruit the core components to the apical junction in the epithelial cell for Hippo signaling activation. Su *et al.* have further added another mode of regulation which shows that Kibra activates the Hippo pathway together with Mer and Sav, but independent of Ex, at the non-junctional site (medial apical cortex) (75). *Crumbs* (*Crb*) was identified as a putative surface receptor linking apical polarity with organ size determination. Interestingly, both overexpression and depletion of Crb, induces Yki activity, thereby driving tissue overgrowth; although, it has been reported as a tumor suppressor in human cancer (76-79). In addition to the apical component such as Ex, components of Crb polarity complex, including PALS1, PATJ and AMOT, are also able to strongly interact and induce cytoplasmic retention of YAP/TAZ when cells are in high density (80). These remarkable findings have established cell polarity as an intrinsic upstream signal of the Hippo pathway in controlling organ size and tumorigenesis.

Cell-adhesion molecules are regulators of the Hippo pathway and function as tumor suppressors. α-catenin, an adaptor protein between the membrane and actin cytoskeleton for adherens junction (AJ) formation, was discovered as a molecular linker between the AJ and Hippo pathway (81, 82). Through molecular and genetic analysis of the mouse model, it was reported that α-catenin functions as a tumor suppressor in skin epidermis by preventing nuclear localization and transcriptional activity of YAP (61, 83). A component of cell adhesion junction Echinoid was also identified as a tumor suppressor and upstream modulator of the Hippo pathway. Echinoid physically interacts with and stabilizes Sav, thereby inactivating the Yki activity in *Drosophila* (84, 85). Thus, cell junction formation exerts as an intrinsic cue to regulate the Hippo signaling. A detailed discussion on the upstream paths for Hippo signaling in *Drosophila* organ development is reviewed in an accompanying paper (Choi, in this issue).

Physical signals

Cells are exposed to physical constraints or mechanical cues during organ growth and regeneration and then activate the Hippo pathway to limit tissue overgrowth by restricting cell proliferation and survival. Cell contact inhibition is a well-established growth-inhibitory signal and acts as a determinant of YAP inactivation in the cell culture system as well as *in vivo*. At high cell density, LATS1/2 kinase is activated and phosphorylates YAP and TAZ at S127 and S89, respectively, which generate a docking site for the 14-3-3 interaction. 14-3-3 retains YAP/TAZ at the cytoplasm and consequently induces their degradation via the β-TrCP E3 ligase-mediated ubiquitin proteasome system. The levels of many Hippo signaling components are controlled by ubiquitin modification. Further discussion on the role of ubiquitin modification in diverse cellular contexts is described in an accompanying paper (Kim and Jho, in this issue). On the other hand, YAP is predominantly present in the nucleus at low cell density, when LATS1/2 kinase activity is low. A physiological relevance to cell density-dependent YAP regulation was further elucidated in the early fate decision of trophectoderm and inner cell mass in mice embryos. YAP primarily localizes in the nuclei of outer cells, where cells are not fully enclosed by other cells, and it mimics low cell density. Whereas, YAP is phosphorylated and present in the cytoplasm of inner cells that are surrounded by other cells which mimics high cell density. The differential localization patterns of YAP between the inner and outer cells is crucial for cell fate determination (57, 86). In addition to LATS1/2 kinase, the non-receptor tyrosine phosphatase PTPN14 has emerged as a cell-density dependent regulator of YAP regardless of its enzymatic activity. The direct interaction of PTPN14 with YAP promotes the cytoplasmic sequestration of YAP from the nucleus in response to cell contact inhibition (87-89). More recently, Nemo-like kinase (NLK) regulates YAP localization and transcriptional activity in a cell-density dependent manner. At low cell density, NLK directly phosphorylates YAP at S128, which blocks LATS1/2-mediated phosphorylation

at S127 and the 14-3-3 interaction. However, NLK is unable to activate YAP at a high cell density due to its cytoplasmic localization and degradation (90).

Many types of mechanical signals, including fluid shear stress, cell geometry and tensional force, have an effect on the complex architectures of organs and a growing body of evidence has placed YAP/TAZ as the critical mechanosensitive transducers that translate mechanical inputs into gene expression (91-93). These roles of YAP/TAZ in mechanotransduction are important in the physiological and pathological relevance to better understand related diseases, including atherosclerosis and cardiac hypertrophy, and mesenchymal stem cell differentiation (93). Physical alteration by ECM stiffness and cell shape regulates YAP/TAZ nuclear localization via the cytoskeleton rearrangement which is determined by Rho-GTPase, FAK-Src or LATS1/2 dependent pathways (94, 95). Recent report suggests that extracellular stiffness applied from the cytoskeleton into the nucleus can stretch the nuclear pore and consequently increase the nuclear entry of YAP (96). Further details about the regulation of Hippo signaling by actin remodeling are reviewed in an accompanying paper (Seo and Kim, in this issue).

Cellular stress

Because YAP/TAZ are multifunctional transcription activators involved in a variety of cellular responses and a tight coordination between metabolism and the signaling pathway involved in cell proliferation is eventually required for organ growth, it has been expected that several stress signals such as nutrient starvation, energy stress and hypoxia may have effect on the Hippo signaling pathway (11, 97). The first link of YAP/TAZ to cellular metabolism emerged from the mevalonate pathway that has crucial roles in multiple cellular processes by synthesizing essential bioactive molecules such as cholesterol, bile acid and dolichol (98, 99). Pharmacological inhibitors of the mevalonate pathway

efficiently block YAP/TAZ activity though RHO-GTPase (99). Serum or glucose deprivation rapidly induces LATS1/2 activation and subsequent YAP/TAZ inactivation for energy homeostasis. Moreover, the kinase AMPK, as a sensor of cellular energy stress, directly phosphorylates YAP at S94, which is a residue essential for the interaction with TEAD, to disrupt the interaction with TEAD (100, 101). Recent reports further provided interesting finding that YAP is O-GlcNAcylated by OGT in response to metabolic nutrients. YAP O-GlcNAcylation inhibits LATS1 mediated phosphorylation of YAP and represses YAP activity in tumorigenesis (102, 103).

Hypoxia represents a low level of oxygen at the tissue level and is a common feature in a majority of malignant tumors. HIF-1α functions as an oxygen sensor and is highly increased under hypoxia. Recent reports suggest that hypoxic stress deactivates the Hippo pathway via SIAH2-meidated LATS1/2 degradation. Augmented YAP interacts with HIF-1α which enhances YAP stabilization and function in tumors (104). Likewise, HIF-2α promotes cancer cell growth by inducing YAP expression and activity (105). In addition to YAP, HIF-1α can activate the *WWTR* gene that encodes the TAZ protein and serves as a co-activator (106). Further investigation provided details regarding the molecular mechanism for SIAH2-mediated LATS degradation under hypoxia. TGF-β signaling and expression of its target gene *Zyxin* are significantly elevated under hypoxia. As a result, Zyxin forms a functional ternary complex with LATS2 and SIAH2 and facilitates LATS2 degradation (107). Osmotic stress is recently reported to modulate the Hippo signaling; although, temporal duration exposed to osmotic stress differently affects YAP activity (108, 109). Acute osmotic stress promotes YAP nuclear localization and transcriptional activity by NLK-mediated phosphorylation (108). However, at later stages, osmotic stress promotes cytoplasmic translocation of TEAD via direct interaction with p38, which leads to the inhibition of YAP activity (109).

MST1/2 is originally isolated as a stress-responsive kinase activated by extreme stress, such as heat shock, okadaic acid or sodium arsenite (110), and mediates cellular responses to oxidative stress and

longevity through the FOXO transcriptional factor (111). Recent work revealed that the YAP directly forms a functional complex with FOXO1 to activate the expression of antioxidant genes and consequently exert protective effects against oxidative stress and ischemia/reperfusion (I/R)-caused injury in the heart (112). Further identification and functional analysis of new players that link stress and nutrients to Hippo signaling will provide potential therapeutic intervention points for treating cancer and metabolic diseases.

Hippo pathway is a hub for integration of other signaling pathways

Multiple signaling pathways converge and integrate with other pathways in tightly regulating numerous cellular responses, development and pathogenesis. In this review, we discuss the cross-talks between Hippo and other signaling pathways such as Wnt, Notch, GPCR and TGFβ pathways (Figure 3).

Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway is known for its crucial role in embryonic development and tissue homeostasis in adults (113). In the absence of Wnt stimulation, a key transcriptional activator β-catenin is constantly degraded by a destruction complex that includes Axin, APC and GSK-3β, thereby maintaining a low level of β-catenin in the cytoplasm. Conversely, cytoplasmic β-catenin is accumulated in the presence of Wnt stimulation and enters the nucleus to activate the Wnt-responsive transcriptional program (113, 114). Multiple studies suggested that the Hippo pathway and Wnt/β-catenin signaling are integrated for the fine regulation of its own signaling in a physiological context. The first evidence for crosstalk between the Hippo and Wnt/β-catenin pathway was provided by

Attisano and colleagues (115). The activation of the Hippo pathway increases the level of cytoplasmic TAZ to repress Wnt/β-catenin signaling by inhibiting CK1δ/ε-mediated phosphorylation of Dishevelled (DVL). The *Taz*-KO mouse is known to develop a severe polycystic kidney (PKD) that is similar to the phenotype caused by aberrant Wnt/β-catenin signaling activation and it exhibits elevated transcriptional activity of β-catenin in kidney cysts. Interestingly, the levels of Armadillo, β-catenin homolog in *Drosophila*, and expression of its target genes are increased in the *hpo* and *wts* mutant clone of *Drosophila* (115). Similarly, YAP/TAZ physically binds and sequestrates β-catenin in the cytoplasm, thereby suppressing Wnt signaling (116). Furthermore, cytoplasmic YAP displayed a tumor suppressive function by restricting the nuclear translocation of DVL without modulating Axin-APC-GSK3β complex, and subsequently dampening Wnt signaling activation during intestinal regeneration (117). Alternatively, the Hippo pathway regulates the subcellular distribution of SHP2 tyrosine phosphatase that is known to be a potent activator of Wnt/β-catenin signaling at the nucleus in a cell-density dependent manner. In high cell density, SHP2 is cytoplasmic via an interaction with phosphorylated YAP/TAZ, which suggests that the activation of Hippo signaling suppresses the Wnt signaling activity. Conversely, unphosphorylated YAP/TAZ promotes SHP2 nuclear localization and ultimately activates Wnt signaling in low cell density (118). Taken together, there are at least four different ways that the Hippo signaling inhibits Wnt/β-catenin signaling and the main mechanism is sequestration of the activators of Wnt/β-catenin signaling in the cytoplasm by interacting with cytoplasmic YAP/TAZ.

It is likely that the *in vivo* reciprocal relationship between Wnt/β-catenin and the Hippo pathway depends on the tissue context. Deletion of the Hippo components, *Sav* and *Mst1/2,* in neonatal heart displayed induced cardiomyocyte proliferation and cardiomegaly together with the elevated Wnt/β-catenin signaling signature (119). YAP and β-catenin forms a complex in the promoter of the progenitor genes, such as *Sox2* and *Snai2/Slug,* and enhances gene expression. Heterozygotic removal of *β-*

catenin in *Sav*-KO suppressed cardiomyocyte proliferation and heart overgrowth, which suggests that Wnt/β-catenin signaling is required for the phenotypes in Hippo mutants (119). Likewise, the ablation of the Hippo signaling by genetically deleting *Mst1* and *Mst2* (*Mst1/2*-DKO) resulted in the elevation of YAP/TAZ as well as β-catenin signaling activity. However, in contrast to its effect on the heart, additional loss of *β-catenin* in the *Mst1/2*-DKO mouse livers caused very severe hepatomegaly and accelerated tumor formation than those observed in *Mst1/2*-DKO (120). In line with this, the clinical investigation reveals that the YAP activation signature exhibits a negative correlation with β-catenin activation signature in patients with hepatocellular carcinoma (121). These reports suggest that YAP/TAZ can act as oncogene or tumor suppressor in a context dependent manner.

A large body of evidence has suggested that YAP/TAZ are intrinsic regulators of Wnt signal transduction and orchestrates Wnt-mediated biological responses. Similar to the β-catenin regulation, YAP and TAZ were integral components and sequestrated by the β-catenin destruction complex in the cytoplasm. β-catenin mediates TAZ, but not YAP, degradation in the absence of Wnt. YAP/TAZ also inhibit β-catenin activity via the recruitment of β-TrCP into the destruction complex. However, in the presence of Wnt, YAP and TAZ are released from the destruction complex and enter into the nucleus to enhance target gene expression (122, 123). These provocative findings suggest that the expression of Wnt target genes is regulated by the well-known β-catenin-TCF/LEF as well as YAP/TAZ-TEAD complexes. However, Pan and colleagues demonstrated that YAP is not regulated by the β-catenin destruction complex, but rather the APC protein complex that facilitates the phosphorylation of YAP/TAZ through the Hippo kinase cascade (124). It is not clear whether this discrepancy is due to difference in the cellular context. Further work is required to resolve this discrepancy.

G-protein-coupled receptors (GPCRs)

G-protein-coupled receptors (GPCRs) contain seven transmembrane domains and are the largest family of the plasma membrane receptors that mediate signals from the outside to the inside of the cell. A major conceptual advance was the identification of GPCRs as a bona fide upstream regulator of the Hippo signaling pathway (125). GPCR signaling differentially regulates the Hippo pathway, which depends on the types of heterotrimeric G-protein that are induced by the different ligands. Guan and colleagues examined the effect of serum on YAP/TAZ phosphorylation and activation to search for upstream regulators of the Hippo pathway. Serum induces the dephosphorylation and nuclear translocation of YAP, and further investigation identified bioactive phospholipids in serum, such as lysophosphatidic acid (LPA) or sphingosine 1-phosphate (S1P), as the YAP/TAZ activating components. LPA or S1P treatment was sufficient to promote YAP/TAZ activation through $G_{12/13}$-coupled receptor signaling. Further investigation indicated GPCRs that activate $G_{12/13}$, $G_{q/11}$ and $G_{i/o}$ signaling inhibit YAP/TAZ phosphorylation and degradation. In particular, the YAP/TAZ regulation by GPCR is closely associated with a variety of cancers. For example, $G_{q/11}$-active mutation is observed in approximately 80% of uveal melanoma where YAP has the essential role in tumorigenesis. Mechanistically, these GPCRs inhibit LATS1/2 activity through Rho-GTPase, thereby inducing YAP/TAZ dephosphorylation and nuclear localization. In contrast, ephinephrine, glucagon or dopamine that activates Gs-coupled receptor elevated LATS1/2 kinase activity through protein kinase A and consequently inhibits YAP/TAZ activity. LATS1/2 served as the key downstream mediators in the regulation of YAP/TAZ by GPCRs; but, it remains controversial since Miller *et al*. demonstrated that LATS1/2 was not involved in S1P-mediated YAP dephosphorylation (126). Frizzled (Fz) is a primary receptor for Wnt ligands and belongs to the GPCR family. A recent study reported that the noncanonical Wnt ligands, such as Wnt5a/b, activate YAP/TAZ via Fz-$G_{12/13}$-RhoGTPase-LATS1/2 signaling axis that is independent from the canonical Wnt/β-catenin pathway (127). It would be of importance to identify more GPCRs which transduce the extracellular signal to the Hippo signaling

pathway in different cellular contexts, since these GPCRs can be used as specific therapeutic targets by using currently available agonists or antagonists.

Notch signaling pathway

Notch signaling functions in diverse developmental and homeostatic processes and is activated by cell-cell contact that induce the direct interaction between the Notch receptors and their cognate ligands (Jagged and Delta-like). These bindings induce sequential proteolytic cleavage of the Notch receptor and ultimately generate the Notch intracellular domain (NICD) to activate the expression of target genes (128-130). Although the Hippo pathway was initially reported to be involved in the Notch-dependent differentiation in *Drosophila* (131, 132), a direct link between the two signaling pathways was extensively studied in mammalian development and tissue homeostasis. Genetic manipulation revealed that YAP was required for intestinal expansion, smooth muscle differentiation and controlling liver cell fate via the up-regulation of the Notch signaling (133-136). During embryonic development, the Notch pathway is activated in the trophoectoderm (TE), which give rise to the placenta, where NICD and YAP cooperatively activate *Cdx2* transcription for TE lineage specification (137). A series of studies provided the mechanistic insight that Notch receptors and its ligand *Jagged1* were direct target genes of YAP and NICD enhanced YAP/TAZ activity by at least promoting stabilization, which creates a positive feedback loop during tumorigenesis (120, 134, 138).

TGF-β signaling

The transforming growth factor-β (TGF-β) superfamily participates in the regulation of numerous biological processes, including cell growth, organogenesis and stem cell function (139). The TGFβ

ligands bind to their specific Ser/Thr kinase receptors to induce the activation of SMAD transcription factors and its target gene expression. Recent studies have unveiled a cooperative interaction between the Hippo and TGFβ-SMAD signaling in many cellular responses (140-142). In human embryonic stem cells (hESC), the Hippo pathway cooperates with the TGFβ signaling to maintain pluripotency (143). Upon TGFβ stimulation, TAZ physically binds and promotes SMAD2/3 nuclear localization to induce the transcription of genes that are involved in the maintenance of hESC pluripotency (143). Conversely, in response to cell density, the Hippo pathway suppresses the TGFβ signaling via YAP/TAZ-mediated SMAD cytoplasmic sequestration (80). However, YAP/TAZ-dependent SMAD localization needs to be further clarified since the controversial result was reported that the TGFβ signaling and SMAD nuclear localization was not affected by cell density in most cell types (144). In addition to its role in embryonic stem cells, YAP controls patterning of the developing lung and induces specific transcriptional programs to generate airways (145, 146). YAP also induces the differentiation of hepatoblasts into biliary epithelial cells by cooperating with the TGFβ signaling (147). YAP/TAZ and TGFβ signaling can form a positive feedback loop in tumorigenesis. TGFβ ligands, including BMP and TGFβ2/3, can be transcriptionally induced by the inactivation of the Hippo pathway (148, 149). A specific loss of Mob1a/b, which is a negative regulator of YAP/TAZ, in the liver results in the increase of *Tgfβ2/3* expression. Indeed, the genetic ablation of the *Tgfβ receptor* (*Tgfbr2*) in *Mob1a/b*-deficient mice livers partially alleviated liver enlargement, fibrosis and tumorigenesis (149).

Multiple molecular layers between the Hippo pathway and TGFβ signaling have been reported. The Hippo pathway scaffold RASFF1A was degraded by the ITCH E3 ligase mediated proteasome pathway in response to TGFβ signaling, thereby permitting the YAP-SMAD2 complex formation and nuclear localization for transcriptional activation of the TGFβ-responsive targets (150). SnoN, which is a target gene of TGFβ signaling and maintains the repressed state of TGFβ-responsive targets in the absence of ligands as a negative feedback mechanism (151), promotes TAZ stabilization via the

inhibition of LATS-mediated phosphorylation and is required for oncogenic function of TAZ (147). Interestingly, the expression and localization of SnoN can be regulated by cell density-dependent Hippo pathway activation (152). Overall, those reports clearly suggest that there are extensive cross-talks between the components of the Hippo and TGFβ signaling, and thus we need to be aware that modulation of one signaling may have elicit unexpected effects by regulating any other signaling.

Concluding Remarks

Owing to vast efforts of many researchers, the Hippo pathway has been firmly established as a master regulator of organ size control. Also, the molecular mechanism of the Hippo pathway that transduces from the extracellular stimuli to transcription has been well elucidated. Apart from its primary functions in organ size determination, development and tumor suppression, recent studies revealed the unexpected roles of Hippo signaling in diverse cellular processes such as angiogenesis (Park and Kwon, in this issue), miRNA biogenesis, regulation of innate immunity and mechanotransduction. Given these various cellular functions, it is not surprising that the Hippo pathway senses multiple stimuli and translates it to intracellular responses by cooperating with other signaling pathways. Despite the rapid and extensive progress in the Hippo pathway, further provocative findings are expected in coming years to answer fundamental questions in this field and to provide clinical insights for curing human diseases.

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Conflict of interest

The authors declare no competing financial interests

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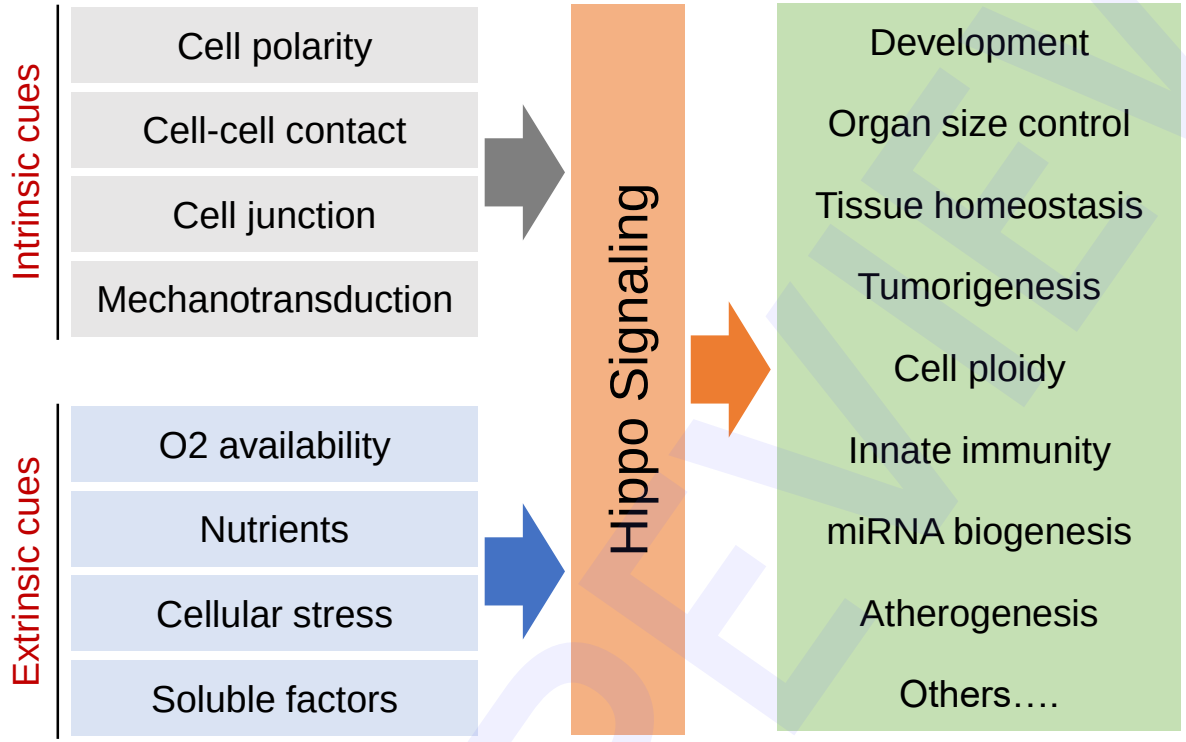
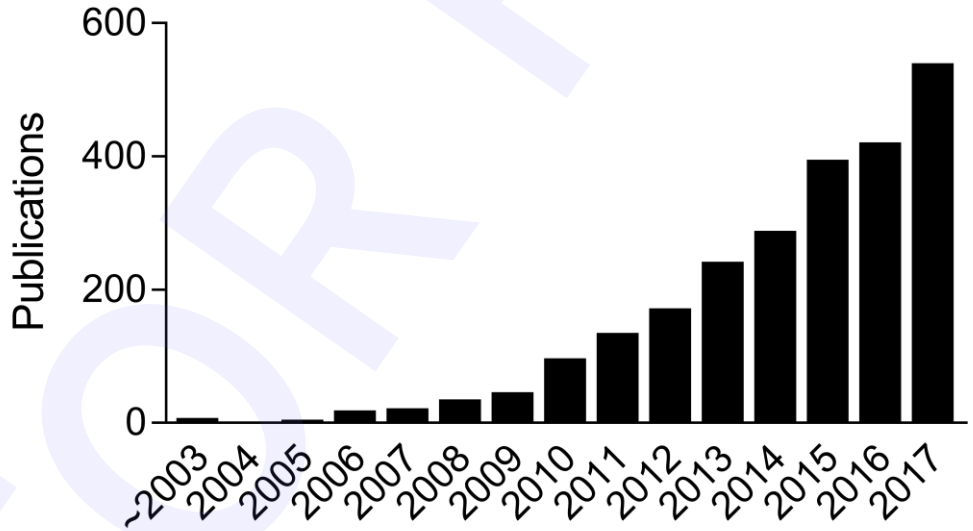
Figure Legends

Figure 1. The pathophysiological function and importance of the Hippo pathway. Both intrinsic and extrinsic signals regulate the Hippo pathway that is crucial for proper developmental processes and tissue homeostasis (upper panel). As the interest and importance of the Hippo pathway is growing, so does the number of publications (bottom panel).

Figure 2. Timeline of the major advances and discoveries in the Hippo pathway

Figure 3. Schematic model of the Hippo pathway and cross-talk with other signaling pathways in mammals. The Hippo pathway is a kinase cascade that consists of MST1/2-LATS1/2 and can be activated by diverse stimuli including cell density, polarity and mechanical cues to suppress YAP/TAZ transcriptional activity. Many modulators in the Hippo pathway have been added via multiple approaches (left panel). The crosstalk of the Hippo pathway with other signaling pathways such as Wnt/β-catenin, Notch and TGF-β to regulate YAP/TAZ activity (right panel).


Intrinsic cues
Cell polarity
Cell-cell contact
Cell junction
Mechanotransduction
Extrinsic cues
O2 availability
Nutrients
Cellular stress
Soluble factors
Hippo Signaling
Development
Organ size control
Tissue homeostasis
Tumorigenesis
Cell ploidy
Innate immunity
miRNA biogenesis
Atherogenesis
Others….


Publications
600
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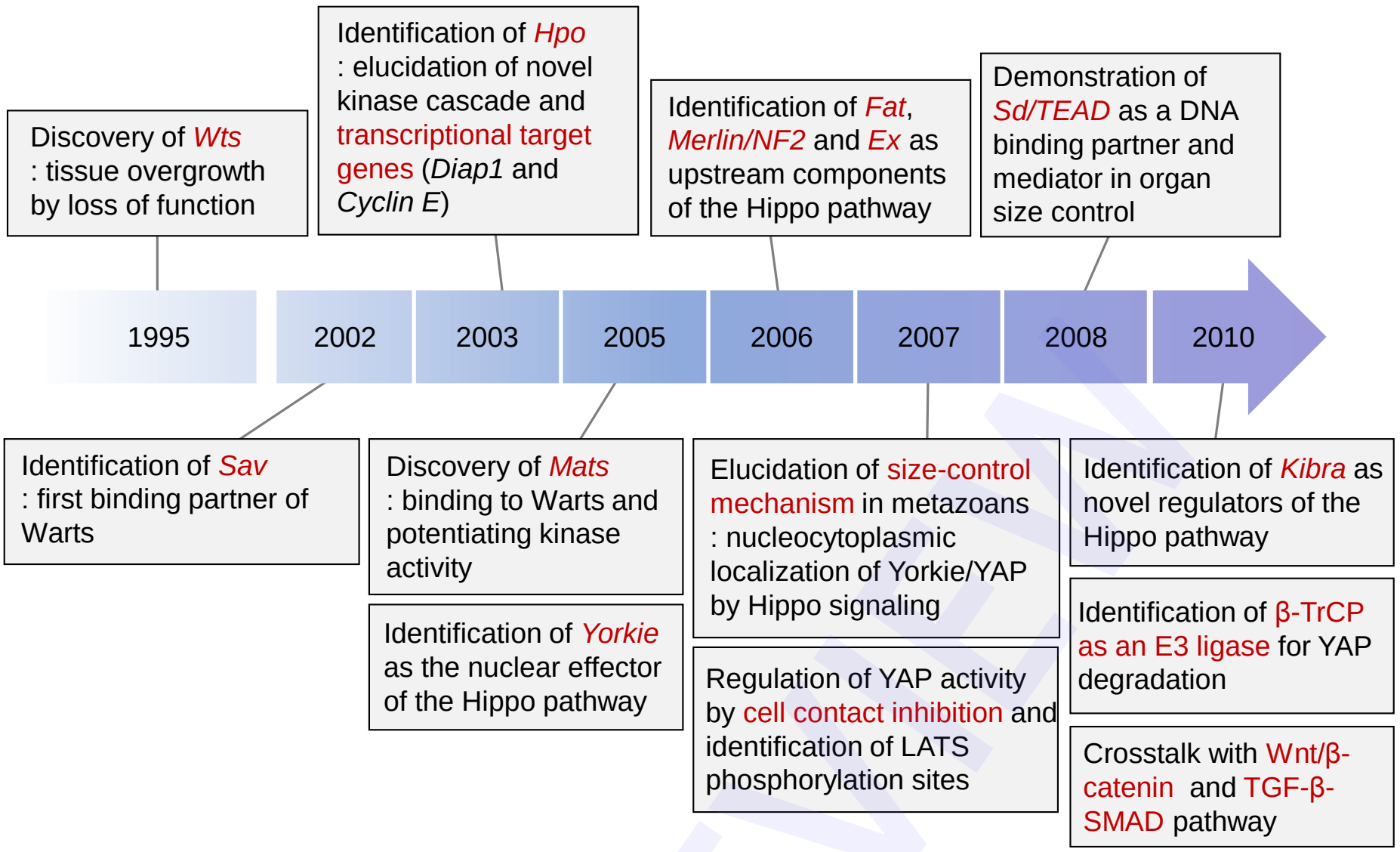
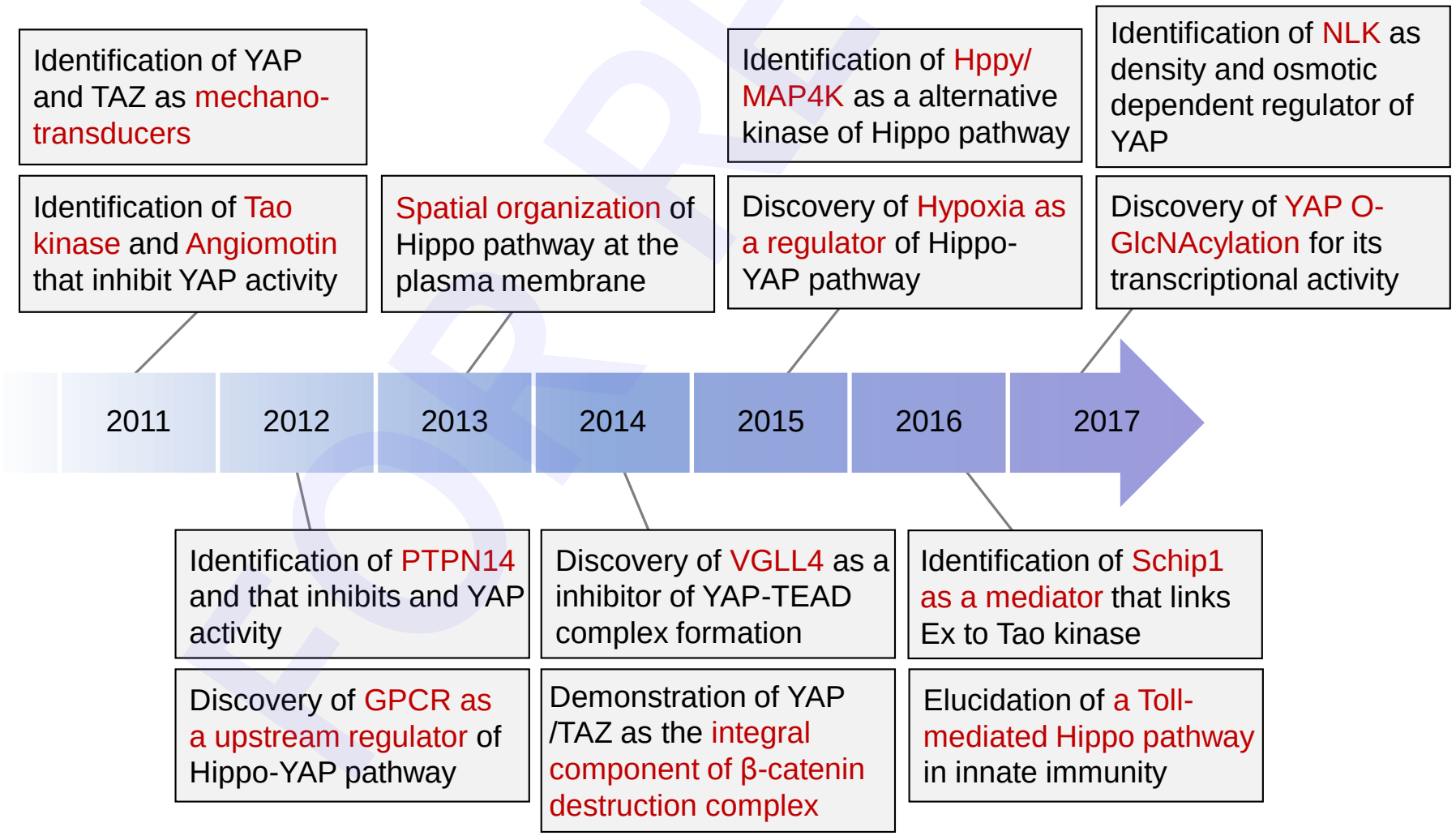
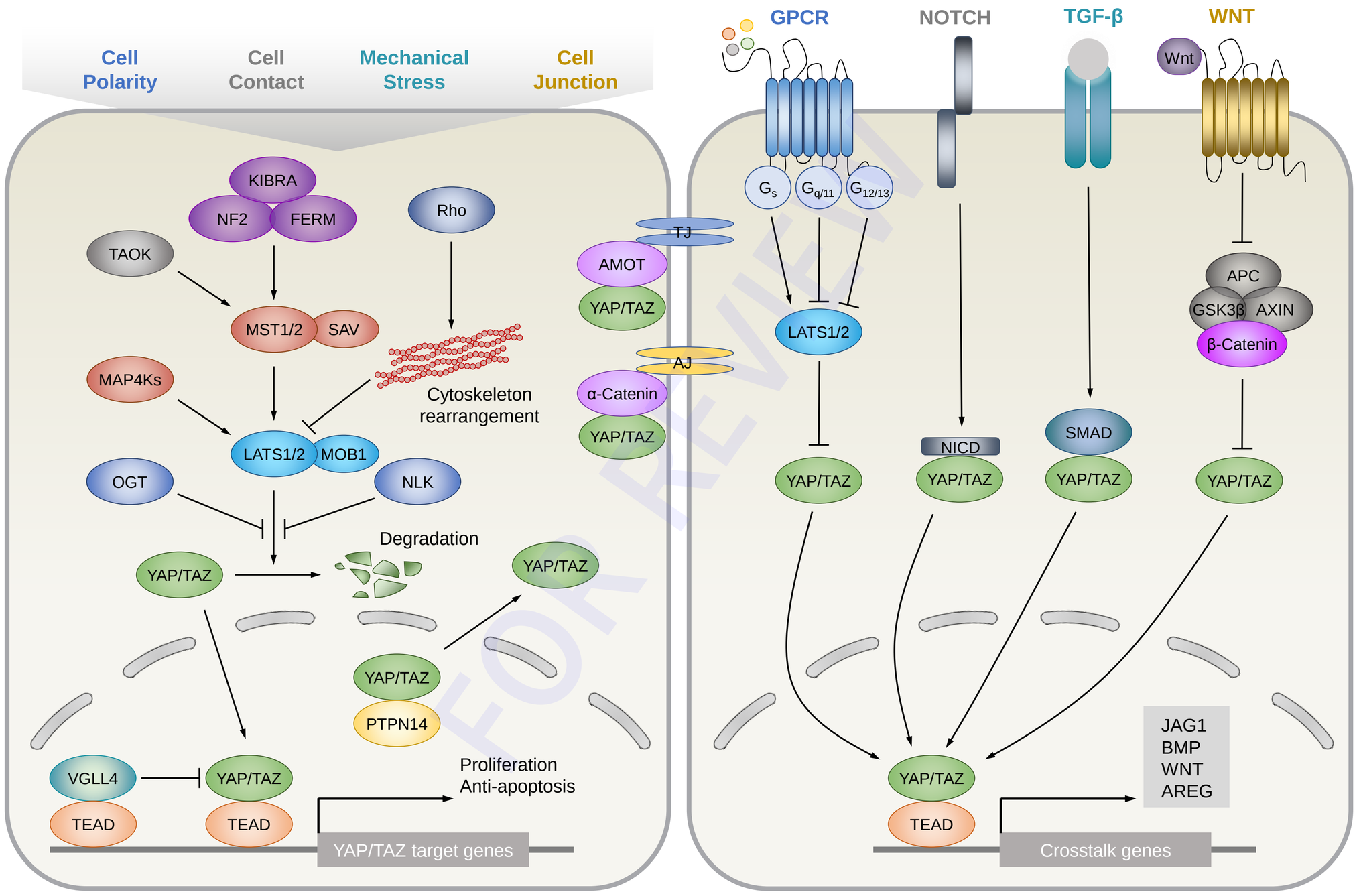

1995
Discovery of *Wts* : tissue overgrowth by loss of function
2002
Identification of *Sav* : first binding partner of Warts
2003
Identification of *Hpo* : elucidation of novel kinase cascade and transcriptional target genes (*Diap1* and *Cyclin E*)
2005
Discovery of *Mats* : binding to Warts and potentiating kinase activity
Identification of *Yorkie* as the nuclear effector of the Hippo pathway
2006
Identification of *Fat*, *Merlin/NF2* and *Ex* as upstream components of the Hippo pathway
2007
Elucidation of size-control mechanism in metazoans : nucleocytoplasmic localization of Yorkie/YAP by Hippo signaling
Regulation of YAP activity by cell contact inhibition and identification of LATS phosphorylation sites
2008
Demonstration of *Sd/TEAD* as a DNA binding partner and mediator in organ size control
2010
Identification of *Kibra* as novel regulators of the Hippo pathway
Identification of β-TrCP as an E3 ligase for YAP degradation
Crosstalk with Wnt/β-catenin and TGF-β-SMAD pathway


2011
Identification of YAP and TAZ as mechano-transducers
Identification of Tao kinase and Angiomotin that inhibit YAP activity
2012
Identification of PTPN14 and that inhibits and YAP activity
Discovery of GPCR as a upstream regulator of Hippo-YAP pathway
2013
Spatial organization of Hippo pathway at the plasma membrane
2014
Discovery of VGLL4 as a inhibitor of YAP-TEAD complex formation
Demonstration of YAP /TAZ as the integral component of β-catenin destruction complex
2015
Identification of Hppy/ MAP4K as a alternative kinase of Hippo pathway
Discovery of Hypoxia as a regulator of Hippo-YAP pathway
2016
Identification of Schip1 as a mediator that links Ex to Tao kinase
Elucidation of a Toll-mediated Hippo pathway in innate immunity
2017
Identification of NLK as density and osmotic dependent regulator of YAP
Discovery of YAP O-GlcNAcylation for its transcriptional activity


Cell Polarity
Cell Contact
Mechanical Stress
Cell Junction
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NF2
FERM
TAOK
MST1/2
SAV
MAP4Ks
LATS1/2
MOB1
OGT
NLK
Rho
Cytoskeleton rearrangement
TJ
AMOT
YAP/TAZ
AJ
α-Catenin
YAP/TAZ
Degradation
YAP/TAZ
YAP/TAZ
YAP/TAZ
PTPN14
VGLL4
YAP/TAZ
TEAD
TEAD
Proliferation
Anti-apoptosis
YAP/TAZ target genes
GPCR
NOTCH
TGF-β
WNT
Wnt
G_s
$G_{q/11}$
$G_{12/13}$
LATS1/2
APC
GSK3β
AXIN
β-Catenin
YAP/TAZ
NICD
YAP/TAZ
SMAD
YAP/TAZ
YAP/TAZ
YAP/TAZ
TEAD
JAG1
BMP
WNT
AREG
Crosstalk genes