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Signal integration in TGF-β, WNT, and Hippo pathways

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Abstract

Complete sequences of animal genomes have revealed a remarkably small and conserved toolbox of signalling pathways, such as TGF-β and WNT that account for all biological diversity. This raises the question as to how such a limited set of cues elaborates so many diverse cell fates and behaviours. It is now clear that components of signalling pathways are physically assembled into higher order networks that ultimately dictate the biological output of pathway activity. Intertwining of pathways is thus emerging as a key feature of a large, integrated and coordinated signalling network that allows cells to read a limited set of extrinsic cues, but mount the diverse responses that underpin successful development and homeostasis. Moreover, this design principle confounds the development of effective therapeutic interventions in complex diseases, such as cancer.

The TGF-β and WNT pathways

Transforming growth factor-beta (TGF-β) was initially discovered in a hunt for autocrine factors that might enhance transformation of normal cells [1,2]. Surprisingly, it soon became apparent that this "transforming" factor regulated myriad diverse and often contradictory cellular responses, most famously as both a potent wound-healing factor [3] and a putative tumour-suppressing antiproliferative factor [4]. The rapid expansion of the TGF-β family to include Activins, Nodals and bone morphogenetic proteins (BMPs) led to an explosion of studies showing key roles for these factors in virtually every facet of developmental biology and homeostasis [5-11]. In the 1990s, efforts to identify TGF-β superfamily receptors and intracellular mediators were anxiously pursued with the expectation that knowledge of the molecular components of the pathway would help illuminate how such diversity in biological responses was achieved. Identification of the TGF-β cell-surface receptors as a family of transmembrane serine/threonine (Ser/Thr) kinases, classified as type I or type II receptors, revealed that engagement of distinct combinations of type I/II receptor complexes, aided in some cases by ancillary proteins such as betaglyan or endoglin [12], provided for some diversity of responses. However, the genome contains surprisingly few very closely related receptors, challenging the notion that diversity of responses might be explained by a similarly diverse set of receptors. Even more streamlined is the Smad family of intracellular proteins [13]. Smads are direct receptor substrates that, upon phosphorylation, accumulate in the nucleus to regulate transcription through interactions with DNA-binding partners. While non-Smad pathways that were subsequently uncovered are important for aspects of cell behaviour such as polarity and motility [14], the Smad pathway is key for directing TGF-β transcriptional responses. Moreover, the limited set of Ser/Thr kinase receptors in fact funnel signals from multiple ligands to one of only two classes of receptor-regulated Smads, R-Smad2/3 for TGF-β-like ligands or R-Smad1, 5 and 8 for BMP-like ligands, confounding efforts to explain complexity through a diversity of signalling pathways.

The first member of the WNT (Wingless-type MMTV integration site) family of secreted factors was described 30 years ago [15], roughly at the same time as TGF-β [1].

One arm of WNT signalling, the so-called canonical pathway, signals through β-catenin, whose protein levels are controlled by a destruction complex comprising proteins that include adenomatous polyposis coli (APC), Axin, Dishevelled and glycogen synthase kinase 3 (GSK3) [16-18]. WNT stimulation induces stabilization of β-catenin that in turn, and like Smads, accumulates in the nucleus, where it promotes transcription in partnership with the DNA binding factors lymphoid enhancer binding factor/T-cell-specific transcription factor (Lef1/TCF). In fact, while the molecular components of morphogen signalling pathways including TGF-β, WNT, Notch, Hedgehog and the Hippo tissue size control pathway discussed below might bear little molecular resemblance, membrane and/or cytosolic regulation of a transcriptional modulator is a shared principle. Similarly, the concept that cellular outcomes are significantly impacted by interactions with other signalling cascades is another common theme. The specific molecular components that mediate inter-pathway communication are varied and a description of these encompasses a huge literature. Here, we will focus on some of the general features of pathway crosstalk using examples from the TGF-β and WNT pathways, and then extend our discussion to recent advances on how these pathways intersect with the Hippo tissue size and growth control pathway.

Pathways communicate with each other through a variety of mechanisms

Signalling pathway crosstalk allows for maximal plasticity and versatility in cellular responses. There are myriad ways in which crosstalk is molecularly manifested, with points of regulation occurring throughout the signalling cascade from the extracellular space right through to the nucleus. Here, a few illustrative examples of how signalling pathways are integrated will be discussed using TGF-β and WNT as examples, with the details more extensively reviewed elsewhere [19-21].

Perhaps the simplest form of signal integration occurs when activation of one signalling pathway regulates the transcription of the ligand or key components of the second pathway, thereby amplifying or attenuating signalling. For instance, in numerous developmental contexts, BMP and WNT ligands cross-regulate each other, thus controlling the extracellular milieu, or "niche", and hence cellular responses [22-24]. However, this type of "crosstalk at a distance" probably does not provide sufficiently dynamic mechanisms to allow for the complex contextual responses that are required by sophisticated biological systems. For this, more intimate pathway integration is required, and many studies have uncovered a wealth of direct physical interactions between pathway components. One way in which these physical links modulate responses is via synergistic convergence of pathways on a common target. Indeed, TGF-β-regulated Smads and the WNT mediator β-catenin can interact and converge on a common DNA binding partner, Lef/TCF to cooperatively induce expression of genes that control cell fate. For instance, simultaneous stimulation of Smads and β-catenin, whose pathways display overlapping activation in the Xenopus Spemann organizer, serves to localize and control expression of organizer-specific genes such as Siamois and XTwn during gastrulation [25,26], while in mammalian cells, examples of cooperative Smad/β-catenin targets include Msx2, Myc and CTGF [27-29]. This type of cooperativity is particularly important for ensuring that expression of master regulators that initiate cell fate decisions is tightly constrained to the appropriate time and place.

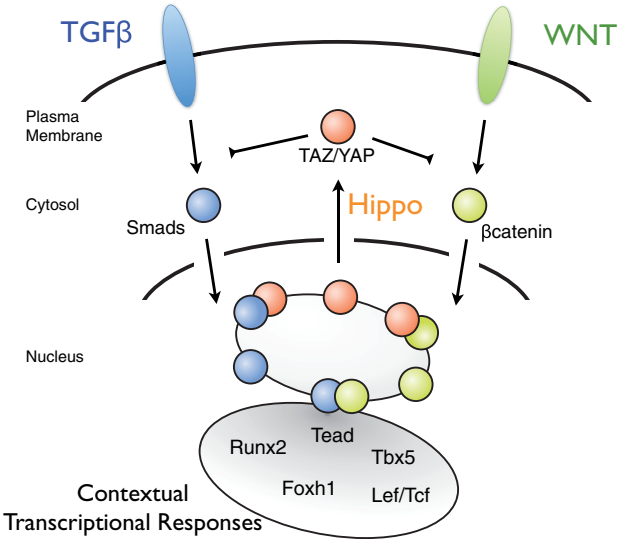
Sharing, however, can also be a means of inhibiting pathway responsiveness in the context of integrated signalling. For example, distinct arms of a signalling pathway may use the same component. In the case of WNT, Dishevelled proteins are important for both the canonical β-catenin and the non-canonical β-catenin-independent planar cell polarity (PCP) arms of the pathway [30-32], while in the TGF-β family, the common Smad Smad4 is required for both the BMP and TGF-β branches of the Smad cascade. Component sharing between distinct pathways is also well known, an example being the WNT negative regulator Axin, a core component of the β-catenin destruction complex, which has also been reported to associate with various Smads to modulate TGF-β signalling [33,34]. Competition for limiting levels of these common components has been proposed to mediate inhibitory crosstalk, such as the well-described ability of non-canonical WNT signalling to inhibit the canonical β-catenin pathway and the antagonistic interactions between TGF-βs and BMPs. However, it is important to note that identification of a point of crosstalk does not equate to a universal mechanism of cross-regulation. For instance, β-catenin is also a functional component of cell-cell adhesion complexes and is localized to the plasma membrane, but this function appears to be distinct from its role in WNT signalling [35]. Similarly, GSK3-mediated phosphorylation of β-catenin is required for promoting β-catenin degradation, and phosphorylation of GSK3 on a serine within its amino terminus by phosphatidylinositide-3 (PI3) kinase-activated pathways inhibits GSK3 activity [36]. This might lead to the assumption that PI3 kinase must always modulate WNT signalling. However, several studies indicate that there are distinct pools of GSK3 that compartmentalize its function and, thus, PI3 kinase-dependent inhibition is not universally manifested in all GSK3 activities [37-40].

Degradation of key signalling components is another commonly reiterated theme in the context of pathway crosstalk. The E3 HECT-domain ubiquitin ligases, Smurfs, were originally characterized for their ability to mediate degradation of Smads and receptor complexes in the TGF-β pathway [41-43]. Interestingly, Smurf antagonism of Smad signalling also synergizes with mitogen-activated protein (MAP) kinase signalling which, together with WNT/GSK3, leads to hyper-phosphorylation of Smads, which then promotes Smurf association and Smad degradation [44,45]. Importantly, this link is critical for modulating Smad activity in certain biological contexts, such as epidermal and neural patterning in Xenopus and osteoblast differentiation in mammalian cells. Smurfs can also associate with Par6, a complex that functions both in TGF-β-induced epithelial to mesenchymal transitions and in the non-canonical WNT pathway to induce localized degradation of PCP pathway components [46,47], thus providing a key node that coordinates cell fate with planar polarity. In summary, these examples of multiple and diverse contacts between TGF-β and WNT pathways point to a molecular framework of pathway integration that is structured to allow for maximal plasticity and versatility in cellular responses.

Hippo, TGF-β and WNT crosstalk is context-dependent

In order to properly form tissues and organs, cells must not only integrate morphogenic signals provided by TGF-β, WNTs and other factors but also must incorporate information on the status of control pathways that govern overall cell and tissue growth. The Hippo pathway, initially identified in Drosophila, but well conserved in mammals, is one such pathway that acts as a major regulator of tissue growth and organ size [48-54]. Activation of the Hippo pathway, such as through cell-cell contact or upon polarization of epithelial cells, activates a cascade comprising the core kinases MST1/2 (encoded by the STK3 and STK4 genes) and LATS1/2 (Large tumor suppressor, homolog 1/2) that leads to phosphorylation of the related proteins TAZ (transcriptional co-activator with PDZ-binding motif) and YAP (Yes-associated protein), resulting in their cytoplasmic retention. In the absence of Hippo signalling, TAZ/YAP accumulate in the nucleus and in complex with various DNA binding factors, including TEADs (TEA domain family members) and Runx2 (runt related transcription factor 2), amongst others, that promote transcription of numerous target genes. Thus, unlike the TGF-β and WNT pathways that promote the nuclear activity of their respective mediators, Smads and β-catenin, activation of the Hippo pathway serves to turn off the nuclear functions of TAZ/YAP (see Figure 1). However, similar

Figure 1. Integration of Cell Signalling Pathways into Higher Order Networks Sculpts Transcriptional Landscapes



A simplified schematic of Hippo, TGF-β and WNT pathway interactions is shown. Each of the indicated pathways converge on transcriptional modulators that act in the nucleus to regulate transcription of target genes by interacting with DNA-binding partners, a selection of which are indicated (lowest cluster). Extensive physical interactions between Smads, β-catenin and TAZ/YAP (nuclear halo of components) describe a network of extensive crosstalk that provides for contextual transcriptional responses. In the presence of Hippo pathway activity, TAZ/YAP are sequestered in the cytosol, where they limit both TGF-β and WNT-β-catenin activity.

to the TGF-β and WNT pathways, a key transcriptional role for TAZ/YAP, primarily in cooperation with TEADs, in regulating stem and progenitor cell maintenance and differentiation has emerged [55-66].

The upstream components and mechanisms whereby Hippo pathway activation is linked to cell density sensing are still under intensive investigation, but convergence of this pathway with those of TGF-β and WNT has been firmly established [50,51,53,67,68]. TAZ/YAP interact with the TGF-β-regulated Smads and, when Hippo is activated, the cytoplasmically localized TAZ/YAP prevents Smad nuclear accumulation and transcriptional activity [69]. In epithelial cells, this is critically dependent on the assembly of the Crumbs complex, which is a late event during establishment of apical-basal polarity and, thus, provides a sensor of cell density [70-76]. Consequently, in dense cells, Hippo activation and sequestration of TAZ/YAP and, in turn, Smads blunts transcriptional responses to TGF-β to prevent epithelial-to-mesenchymal transitions. Other components of polarity complexes, such as Scribble and cadherins [77-79], have also been linked to regulation of TAZ/YAP, indicating an intimate link

between cell-cell adhesion, polarity and Hippo pathway activity. Similar to observations in cultured cells, during early embryogenesis in mice, TAZ/YAP localization varies. In the blastocyst, TAZ/YAP is nuclear in trophectoderm precursors where it induces Cdx2 and the trophoblast fate, while in the inner cell mass it is cytosolic [55]. Importantly, this differential localization reflects and defines regions of active nuclear Smad complexes in inner cells that are exposed to the TGF-β-like ligand called Nodal [73]. Thus, interference with Hippo activity in the inner cell mass leads to concomitant nuclear accumulation of both TAZ/YAP and Smads. In the nucleus, a second important function of pathway crosstalk is manifested as TAZ/YAP cooperate with Smads to promote activation of specific target genes. Many of these cooperative targets control maintenance of stem cell pluripotency or induce differentiation. In human embryonic stem (ES) cells, cooperation of TAZ with Smad2/3 is required for TGF-β-like ligands to maintain pluripotency [69], while YAP, in partnership with Smad1, supports BMP maintenance of mouse ES cells and is required for BMP-induced osteoblastic differentiation of mesenchymal stem cells [56,80]. The composition of these Smad and TAZ/YAP-containing activation complexes, particularly with respect to the DNA-binding partners that are responsible for recruitment to specific target genes, is an important question requiring further investigation. However, it is known that TAZ recruits the mediator complex [69] and the TGF-β-regulated CTGF gene is induced by a YAP-TEAD4-Smad3-p300 promoter-bound complex [81]. Thus, by functioning to both control Smad nuclear accumulation and synergize transcriptionally in the nucleus, TAZ/YAP provides a mechanism to couple TGF-β Smad activity to Hippo pathway activity and cell density sensing.

Intimate connections between the Hippo and WNT/β-catenin pathways have also been delineated, in which Hippo activity antagonizes WNT signalling. Genetic manipulation of Hippo pathway components in both Drosophila and mice showed that this crosstalk is evident in diverse tissue and organ contexts. TAZ knockout mice have polycystic kidneys [82-84] that display increased levels of cytoplasmic β-catenin [85]. Heart-specific knockout of Salvador, [86], an MST1 binding protein, or ablation of the expression of MST1/2 in the intestine [87], result in nuclear accumulation of YAP and increased expression of WNT/β-catenin target genes. Moreover, during intestinal regeneration, transgenic overexpression of YAP restricts WNT signals, while loss of YAP leads to WNT hypersensitivity [88]. In Drosophila, a link between Wingless, a WNT family ligand, and Yorkie, the ortholog of TAZ/YAP, in patterning of imaginal discs has also been established [67,85,89]

Several molecular mechanisms have been proposed to explain how loss of Hippo pathway activity might promote WNT signalling. We described a mechanism in which TAZ binds and inhibits phosphorylation of the cytoplasmically localized WNT/β-catenin pathway component Dishevelled to dampen WNT signalling [85]. In the intestinal regeneration model, YAP binding to Dishevelled was shown to act to restrict Dishevelled nuclear translocation during regenerative growth [88]. Thus, Hippo pathway activation by cell-cell contact, for example, enforces cytoplasmic localization of TAZ/YAP, thereby attenuating WNT signalling through a cytoplasmic mechanism.

In certain developmental contexts, such as trophectoderm cells in the blastocyst, or in cancer cells where contact-dependent growth inhibition is bypassed, the absence of Hippo activity results in cells that display high levels of nuclear TAZ/YAP. TAZ/YAP can also interact with β-catenin, and exactly as delineated for TGF-β/Smads, in the nucleus it can cooperate with β-catenin to promote the transcriptional activation of a panel of target genes [86,87,90]. Thus, upon loss of Hippo pathway activity, this nuclear activity reinforces WNT-induced gene responses. However, the recurring concept of context-dependence arises yet again, as this transcriptional enhancement cannot be generalized to all gene targets, but rather is only relevant to a subset of genes. For instance, a recent report has shown that in β-catenin-driven cancers, β-catenin and YAP cooperate with TBX5 to transcriptionally activate only a subset of genes, including several anti-apoptotic targets that are essential for cancer cell transformation and survival [90]. Moreover, additional complexity in cellular responsiveness can arise from many other points of communication between WNT and Hippo pathways. The WNT/β-catenin pathway induces expression of CD44, which binds to Neurofibromatosis-2 (NF2; also known as Merlin) that, in turn, promotes Hippo activity to limit cell growth [91,92]. WNT/β-catenin signalling also induces YAP expression in colorectal cancer cells [93]. Also, a recent study reported that WNT signalling directly modulates TAZ, but not YAP, stability via β-catenin [94]. In contrast, in the study of β-catenin-driven cancers referred to above [90], only YAP (but not TAZ) was identified as cooperating with WNT to contribute to transformation. Thus, signalling outcomes may also depend on the relative levels of these two highly related proteins. Shared components such as GSK3 and CK1δ/ε that can phosphorylate β-catenin, Dishevelled, TAZ/YAP, as well as βTrCP, the E3 ligase that promotes β-catenin and TAZ/YAP degradation, also provide for additional potential mechanisms of signal integration [84,95].

Thus, while it is clear that the Hippo pathway targets TAZ and YAP converge with TGF-β and WNT signaling pathways, the impact of Hippo activity on cellular responses to these important morphogens is very much dependent on context. Regardless of its complexity, it is clear that pathway crosstalk is key for the diverse cellular responses observed during development and homeostasis. Furthermore, as exemplified above for the TGF-β, WNT and Hippo pathways, even the nature, extent and response to this crosstalk can vary in a context-dependent manner.

An integrated signalling network rather than individual signalling pathways

The experimental *modus operandi* for investigating cell signalling still tends to focus on a single pathway. This often leads to disparate observations on biological functions that are highly dependent on the cell type and model system under investigation; for example, TGF-β signalling regulating both ES cell maintenance and cell fate determination. Therefore, it is increasingly clear that understanding biological diversity requires understanding signalling pathways not only as isolated entities but also as integrated higher order networks that form the molecular framework required for contextual responses. The establishment of these contextual signalling environments is what is ultimately critical for allowing cells to robustly mount appropriate biological outcomes in space and time. Moreover, the inherent dynamics of these systems also provide an adaptive reservoir for diseases, such as cancer, to circumvent targeted therapeutics and ultimately thwart our attempts to affect lasting cures. Taking a network-wide view will undoubtedly provide insights that more adequately describe how a limited toolbox of extrinsic cues modulates cellular outcomes and will enhance our understanding of the complex physiological and pathophysiological processes that act to disturb these networks.

Abbreviations

BMP, bone morphogenetic protein; GSK3, Glycogen Synthase Kinase 3; LATS1/2, Large tumor suppressor, homolog 1/2; Lef1/TCF, lymphoid enhancer binding factor/T-cell-specific transcription factor; MAP, mitogen-activated protein; PCP, planar cell polarity; PI3, phosphatidylinositide-3; TAZ, transcriptional co-activator with PDZ-binding motif; TEADs, TEA domain family members; TGF-β, Transforming Growth Factor beta; WNT, (Wingless-type MMTV integration site); YAP, Yes-associated protein.

Disclosures

The authors declare that they have no disclosures.

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