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# TGFβ, Wnt/β-catenin and Hippo Pathway "Cross-talk": Myocardial Systems Biology Murmurings as the Pathways Converge

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author TGD designed and wrote the manuscript. Authors AH and DS provided expert critical commentary on the revisions. All authors read and approved the final manuscript.*

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## ABSTRACT

The heart failure (HF) diseaseasome is marked by substantial alteration in networked signaling pathways. Reductionist paradigm HF investigative approaches have focused on the regulation of a single pathway or single pathway components. In support of a more comprehensive systems biology investigative paradigm, rapidly evolving experimental evidence supports extensive regulatory cross-talk between the TGFβ-superfamily, Wnt/β-catenin and Hippo signaling systems in homeostasis, myocardial development and a variety of non-myocardial diseases. After reviewing the basic components and integrated regulation of each of these three pathways, we review landmark studies in diverse conditions and diseases highlighting the likely critical importance of regulatory cross-talk between these three pathways in the HF.

*Keywords: Heart failure; heart failure diseaseasome; TGFβ signaling pathway; Wnt/β-catenin signaling pathway; hippo signaling pathway; myocardial systems biology.*

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## 1. INTRODUCTION

Like all complex living tissue systems, the myocardium during homeostasis is an interactome marked by multiple networked signaling pathways [1-4]. During disease states like human heart failure (HF), the myocardial homeostatic interactome transitions to a "diseaseasome" marked by substantial alteration in networked signaling pathways and pathologic changes in myocardial structure and function [5-9]. While a reductionist paradigm to decipher the HF diseaseasome has yielded key insights into individual pathways and identified intersecting pathway hubs, this approach has not yielded new therapeutic strategies beyond combinatorial neurohormonal antagonism [10-12]. The discovery of new therapeutic strategies awaits maturation of a holistic systems biology paradigm approach to the myocardial interactome and diseaseasome [13-17]. Such an approach will provide a better chance to decipher the hierarchial, combinatorial epigenetic [18-23], proteomic [24,25], metabolomic [26-28], pathophenomic networks [29,30], time scales [31-35] and regulatory motifs [36-40] interacting in complex diseaseasomes like HF.

There are several challenges with executing a systems biology investigative approach, however. First, a comprehensive "parts list" of all the interacting epigenomic, proteomic, metabolomics and pathopheonomic components is lacking [13-17]. Second, systems biology bioinformatic analytic challenges remain formidable [41,42]. Pending the maturation of a systems biology perspective and investigative approach, a more delimited perspective such as a focus on the cross-talk between key signaling pathways can still inform meaningful investigative questions. In this review, we distill recent experimental evidence regarding cross-talk between the TGFβ, Wnt/β-catenin and Hippo signaling pathways to anticipate possible directions for future systems biology based HF diseaseasome research.

## 2. THE TGFβ SUPERFAMILY

TGFβ superfamily cytokines play critical, diverse roles in growth, adhesion, migration, apoptosis, differentiation and response to injury and fibrosis [43-56]. Since in the myocardium, TGFβ cytokines play key roles in hypertrophy, remodeling and fibrosis via the promotion of myofibroblast transdifferentiation and extra-cellular matrix synthesis [54,56-64], TGFβ signaling pathway components have emerged as attractive potential therapeutic targets in common myocardial diseases [65-67].

### 2.1 TGFβ Superfamily Ligands

The TGFβ cytokine superfamily includes over 30 secreted polypeptides in humans, including the prototypic TGFβ 1-3 isoforms, bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), activins, nodals and inhibitors as tabulated in Table 1 [44-48,68]. All members of the TGFβ superfamily share structural and functional characteristics [43-48]. Structurally, members possess a specific three-dimensional conformational fold and six precisely spaced C-terminus cysteine residues and an active form resulting from dimerization reinforced by a disulfide bridge [46]. Functionally, superfamily members have notable context-dependent effects on gene expression with the intracellular Smad signaling pathway discussed below serving as a central intra-cellular signal transduction "conduit" for gene expression responses [44]. Broadly, the contextual determinants conferring the wide-ranging effects of TGFβ signaling on gene expression include 1) the extra- and intracellular expression and activity of TGFβ canonical and non-canonical pathway signal transduction components, including concurrent cues regulating Smad function and triggering non-canonical pathways, 2) co-factors interacting with Smads to regulate transcription (e.g., FOXHI, OAZ) and 3) the epigenetic regulatory state of the cell dependent in turn on DNA methylation marks, histone modification, nucleosome positioning and chromatin conformation and miRNA and non-coding RNA expression [44,57].

TGFβ superfamily member ligands are translated in a wide variety of cell types, including fibroblasts and myofibroblasts [44-48,68]. Translated as precursors, ligands are cleaved precursors to form mature, dimeric ligands which largely function as paracrine factors on nearby cells [47]. Ligand access to receptors is controlled in a ligand-specific manner by a variety of extracellular binding factors and pathways [44]. In the extra-cellular matrix (ECM), for example, ECM proteoglycans and other matricellular proteins serve as "binding reservoirs" for a variety of growth factors, including FGFs, VEGFs, HGFs and TFGβ 1-3 isoforms [69]. These reservoired growth factors

may be both 1) released as soluble ligands following ECM degradation or 2) bound to appropriate signaling receptors with heparan sulfate as a co-factor [69]. In a well-mapped example of ECM protein-dependent growth factor signaling, secreted but inactive TGF-β1 associates with latency-associated peptide (LAP) to form a large latent complex (LLC) [70]. LLC then associates with a latent TGF-β-binding protein (LTBP) to form a larger complex. LTBP in turn binds to fibronectins and fibrillins, thereby incorporating "piggybacked" LLC inactive TGF-β into ECM scaffolding proteins. Several mechanisms may activate TGF-β, including 1) degradation of fibronectins or fibrillin, 2) LAP cleavage or conformational change, 3) TSP (another ECM protein) induction of metalloproteinase activity and 4) integrin binding to LAP sequences [69]. Incorporation of inactive TGF-β into ECM proteins thus permits TGF-β activation in response to ECM degradation, soluble factors and mechanical stimuli as transduced by integrins [70].

Factors external to the target cell which determine the overall effect of TGFβ cytokine signaling include 1) the level of ligand expression from the source (highly regulated in turn by contextual factors), 2) the ligand subtype (e.g., TGFβ1 and TGFβ2 differ in ligand affinity), 3) the expression of ligand-trapping proteins which fashion development ligand gradients and reservoirs in fully developed tissues, 4) the mediators of reservoir ligand release (e.g., mediators of latent TGFβ complex activation feed forward by mechanical signals transduced by cell surface integrins), 5) the level of expression and activation state of antagonistic ligands (e.g., inhibiins, Lefty, 6) the accessory receptor presentation of ligands to extra-cellular domain of cell-surface receptors (e.g., β-glycan presents TGFβ to receptors) and 7) the relative combinatorial affinities of the expressed membrane receptors themselves [44].

## 2.2 TGF-β Superfamily Receptors and Ligand-receptor Interaction

Two modes of ligand-receptor interaction exist within the TGFβsuperfamily (Figs. 1A and 1B) [46]. TGFβ and the activins have higher affinity for type II receptors and binding to a type II receptor permits assembly of a heterotrimeric complex of two type I and two type II Ser/Thr protein kinase TGFβ super-family receptors and the dimerized ligand [44]. In the process, one of the seven different types of type 1 "signal-propagating" receptors are phosphorylated by either ligand-occupancy or non-ligand dependent activation of one the five different types of type II "activator" receptors [44]. In contrast to TGFβ and the activins, the BMPs have higher affinity for the type I receptors, and the BMP-type I complex then recruits type II receptors to bind to complete the heterotrimeric complex and dimerized ligand [46].

## 2.3 Smads Signaling Cycle

The intracellular mediators of TGFβ signaling are members of the Smad protein family, categorized as 1) R-Smads if they function as phosphorylation-dependent intraceullar signal transducers for TGFβ heterotetrameric receptors, 2) Co-Smads if they function as cofactors in R-Smad signaling and 3) I-Smads if they function in an inhibitory manner [71]. There are eight Smads in humans, two TGFβ R-Smads (Smads 2, 3), three BMP R-Smads (Smads 1, 5, 8), one co-Smad (Smad 4) and two I-Smads (Smads 6, 7) as shown in Table 1. R-Smads and Co-Smad bind DNA with low affinity via a hairpin structure in their MHI domains, which also mediates interactions with other transcription factors [44,47]. Although the DNA-binding hairpins in Smads 1, 2, 3 and 5 are identical and recognize the DNA motif CAGAC (the Smad-binding element, SBE), the positioning of the SBE within Smads differs, and confers upon different Smads different preferences for different DNA-binding partners and different preferences for specific DNA sequences (e.g., Smad1 can bind to GC-rich sequences likely due to differential positioning of the SBE) [44].

Given the generally low affinity of Smad SBEs for DNA seqeuences, interaction with specific, higher-affinity DNA binding proteins is required for Smad complex recruitment to appropriate target gene promoters [47,48,72]. Thus in concert with other tissue and/or context dependent co-regulatory factors such as basic chromatin remodeling complexes and histone modifiers, Smad complexes mediate TGFβ-receptor mediated changes in gene expression and when associated with specific binding partners serve as hubs for multiple levels of regulatory inputs, conferring context-dependent modulation of TGFβ signaling [48,73]. Master transcription factors including FOXH1, EOMES, OCT4, MYOD1 and NANOG are among the many factors utilizing Smad complexes as

transcriptional co-factors [23,43,48,53,72]. The "hub" function of Smads is discussed in more detail below regarding the Wnt/β-catenin and Hippo pathways. Smads also interact with interact and recruit a wide variety of chromatin modifying enzymes to DNA as reviewed in detail elsewhere [72]. Smads require chromatin to assemble basal transcription machinery and activate transcription and important Smad-related chromatin remodelers include the histone acetylase p300 and the SWI/SNF component Brg1.

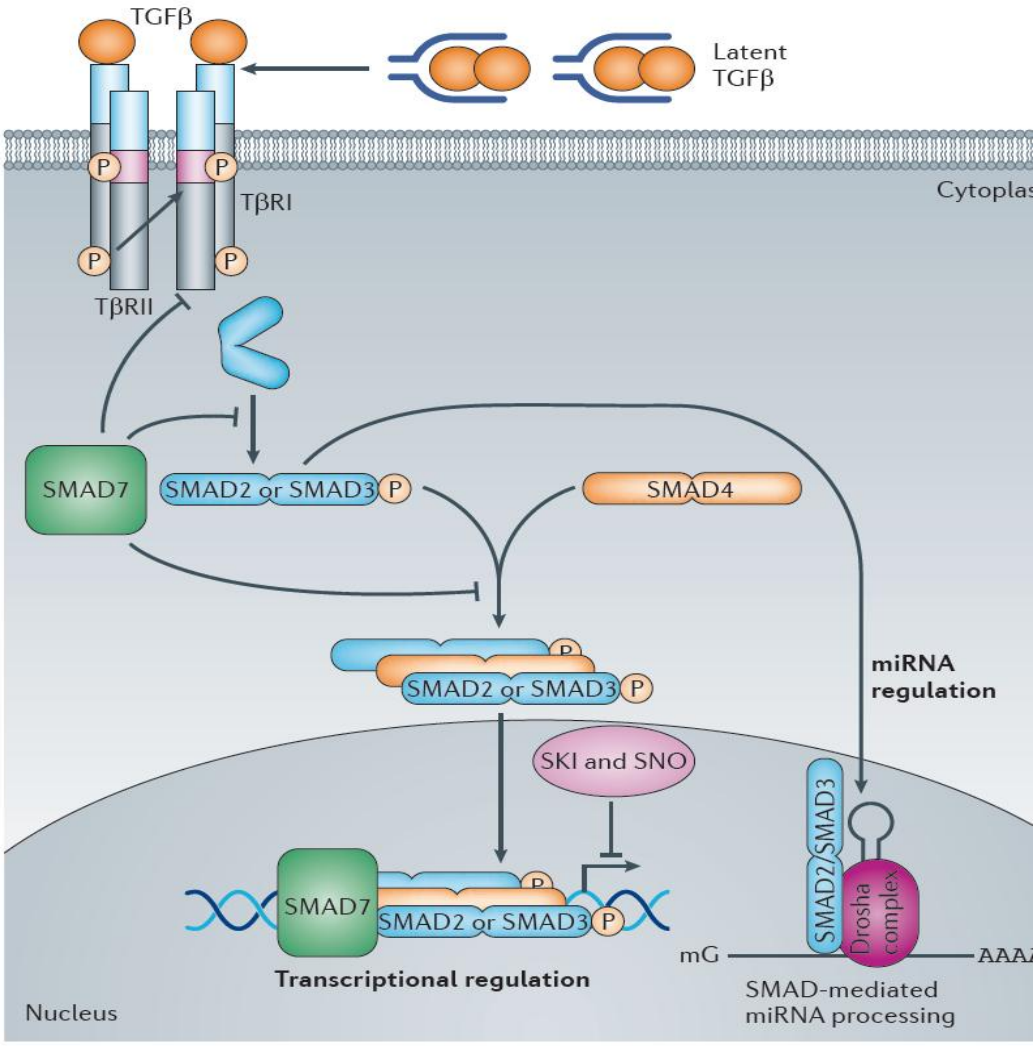


**Fig. 1A. The TGFβ canonical signaling pathway**

*Upon activation, TGFb dimers induce heteromeric complex formation between type II and type I receptors. Type II receptors transphosphorylate type I receptors, which propagage the signal by phosphorylating TGFb specific R-Smads 2/3. R-Smads complex with Co-Smad4 and translocate to the nucleus. The R-Smad-Co-Smad complex associates with genomic Smad-binding elements and transcription factors in a sequence-specific manner. The nuclear proteins SKI and SNO antagonize transcriptional regulation by Smads. The inhibitory I-Smad 7, induced by R-Smad-Co-Smad signaling, inhibits the TGFb pathway by multiple mechanisms, including mediating degradation of type I receptor, inhibiting R-Smad phosphorylation by type I kinase receptor kinase and inhibiting formation of the R-Smad-Co-Smad complex. R-Smads also modulate miRNA biogenesis. mG, 5' capping; AAAAA, 3' polyadenylation of mRNA*

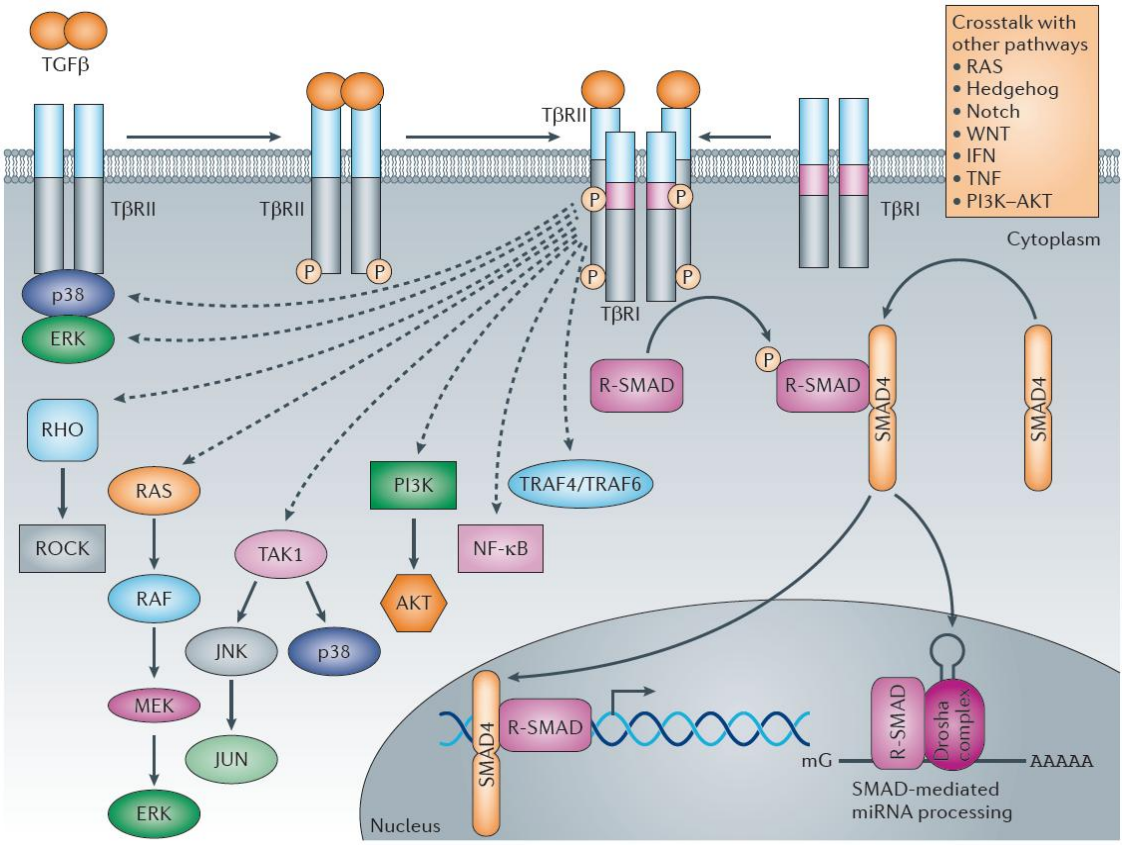


**Fig. 1B. The TGFb non-canonical signaling pathway (Akhurst et al. [65])**

*In the non-canonical pathways, the activated TGFb receptor complex signals through non-Smad factors, including TNF receptor associated factor 4 or 6 (TRAF4/6), TGFb-activated kinase 1 (TAK1), p38 mitogen-activated protein kinse (p38 MAPK), RHO, phosphoinositide 3-kinase (PI3K)-AKT, extracellular signal-related kinase (ERK), JUN N-terminal kinase (JNK) of nuclear factor κB (NF-κB). TGFb signaling is influenced by a variety of other pathways, including WNT, Hedgehog, NOTCH, interferon (INF), tumor necrosis factor (TNF) and RAS pathways. miRNA, microRNA; ROCK, RHO-associated protein kinase; R-Smad, receptor-specific Smad; TβR, TGFβ receptor; mG; 5' capping; AAAAA, 3' polyadenylation of mRNA*

For genes involved in cellular homeostasis, the net effect of TGFβ or BMP activated Smads is to increase or decrease RNA Polymerase II (Pol II) action and the transcription of targeted genes, typically within a five-fold range [68].

## 2.4 Canonical TGFβ Superfamily SMAD Signaling

In canonical Smad signaling, ligand-occupancy of the heterotetrameric TGFβ receptor activates Smads via Smad C-terminal receptor-mediated phosphorylation [52,71]. Following receptor-mediated phosphorylation, R- Smads appropriate for the ligand assemble into trimeric complexes with Co-Smad 4 (e.g., Smad2-Smad2-Smad4, Smad3-Smad3-Smad4 or Smad2-Smad3, Smad4 trimeric complexes for TGFβ/activin ligands, Smad 5-Smad-5-Smad4 trimeric complex for BMP ligands) [47]. The activated R-Smad-Co-Smad complex is tranlocated to the nucleus via nuclear import factors [74], where in concert with transcription factors, co-activators, co-repressors and epigenetic regulatory mechanisms the activated Smad complex targets specific promoters [47] and effects differential patterns of gene expression in a context-dependent manner [53,75].

## 2.5 TGFβSuperfamily Non-canonical (non-Smad) Signaling

TGFβ mainly signals via the canonical Smad pathway, but a variety of non-canonical (non-Smad) TGFβ signaling pathways, including pathways to activate small GTPases, MAP

kinases and PI3K exist, due in part to the dual functionality of TGFβR1 as both a serine/threonine and tyrosine kinase [44,47,76]. In cells undergoing epithelial to mesenchymal transition (EMT), ligand-occupied TGFβR2 directly phosphorylates the cell polarity factor PAR6, which recruits Smurf1 to target RhoA GTPase at tight junctions, resulting in tight junction dissolution [77]. TGFβs and BMPs may also activate Rho GTPases in a variety of cell types, thereby playing potential roles in actin cytoskeletal remodeling [78]. Non-Smad signaling can also activate a variety of members of the MAPK pathway, including ERK, JNK and p38, likely via type II and type I phosphorylation of tyrosine residues of ShcA or Src, resulting in the recruitment ofSH2-domain proteins and Erk and p38 activation [76,79]. TGFβ activates the p38/JNK pathway by inducing activation of TGFβ-associated kinase 1 (TAK1) via the ubiquitin ligase tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) [80,81]. TGFβ may also activate additional intracellular tyrosine kinases such as FAK, Src and Abl [47]. TGFβ activation of PI3K can be either Smad-mediated or non-Smad-mediated, although the mechanism of non-Smad dependent activation is not well characterized [47,76,79]. Activation of PI3K in turn leads to activation of Akt-mTOR pathway in the regulation of cell metabolism, growth, proliferation and survival [3,82,83]. In vitro, TGFβ and BMP non-Smad effects on MAPKs and PI3K can be immediate and transient or delayed and secondary, depending on the cell type and culture conditions [48]. It is unclear at present whether activation of MAPK and PI3K pathways is directly coupled to TGFβ receptors or due to network-wide context-dependent signaling crosstalk [44]. Finally, given that 1) TGFβ regulates the expression of integrins, 2) integrins spatially activate TGFβ1 and 3) integrins increase TGFβ, TGFβRI and TGFβRII transcription and hence overall TGFβ signaling, significant cross-talk between TGFβ-induced non-Smad signaling and integrins have been shown to play important roles in EMT and tumor progression [49,50,55,70,76,84].

## 2.6 Regulation of TGFβ Signaling

Smads undergo a variety of post-translational modifications which regulate the magnitude and duration of Smad signaling [47,85]. Although phosphorylation of R-Smads in the SXS motif by the type I receptor activates R-Smads, phosphorylation at other sites by a variety of kinases (e.g., Erk1/2, p38, JNK, TAK-1, GRK-2) results in inhibition of R-Smad signaling [71]. Co-activators p300 and CBP enhance Smad DNA binding by acetylating Smad lysine residues in the MH1 domain whereas poly (ADP)-ribosylation of the MHI domain inhibits DNA binding [44]. Smad proteins also undergo sumoylation, the functional significance of which is unknown [44]. CDK8/9 phosphorylation of Smads engaged in active transcription within the Smad H1 linker region both recruits co-transcriptional factors such as YAP but also primes the linker for subsequent GSK3 phosphorylation, which in turn provides binding sites for the E3 ubiquitin protein ligase Smurf1 and subsequent polyubiquitylation and proteasome-mediated degradation [86]. Several phosphatases, including PPMIA and small C-terminal phosphatases, dephosphorylate and inactivate Smads in either the cytoplasm or nucleus, providing for “fine-tuning” or inactivation of Smad signaling [85]. Regulatory proteosomal degradation of non-activated R-Smads also occurs via ubiquination (by HECT ubiquitin ligases Smurf1 and 2) and ubiquination of Smad4 occurs via E3 ligases JAB1/CSN5 [87].

Endogenous inhibitors of TGF-β signal transduction include I-Smad6, 7 and k-Ski. Signaling via the R-Smad-Co-Smad pathway induces transcription of I-Smads (Smads 6, 7), which invoke a negative feedback loop to inhibit TGFβ signaling under appropriate conditions by competitive binding to type I receptors and blocking R-Smad phosphorylation [88]. Smad 7 also recruits the ubiquitin ligases Smurf 1, 2 to the type I receptor, resulting in type I receptor ubiquitination, endocytosis and proteosomal degradation, a negative feedback loop blocking TGF-β signal transduction [89]. The deubiquitinases USP4, -11 and -15 can counter Smurf ubiquitylation, and USP15 also deubiquitylates R-Smads [90].

c-Ski is a proto-oncoprotein which regulates transcription in differentiating and proliferating cardiac cells [91]. C-Ski inhibits binds to phosphorylated Smad2 (p-Smad2) and “traps” the p-Smad2/c-Ski complex in the nucleus, thereby preventing p-Smad2 complex modulation of transcription [89]. Finally, the ubiquitin ligase Arkadia in turn regulates Smad7 level and Ski and SnoN, other inhibitors of R-Smad signaling [92,93].

## 3. THE WNT/β-CATENIN PATHWAY

The WNT/β-catenin signaling system, in contradistinction to the TGFβ superfamily signaling system, is marked by a variety of cytokine growth factor ligands (the secreted Wnt glycoproteins), a larger repertoire of receptors (Frizzled proteins) and co-receptors, canonical (β-catenin dependent) and non-canonical (non-β-catenin dependent) activation pathways and complex combinatorial networks of intracellular transduction regulatory components [94-96]. The Wnt pathway plays critical roles in myocardial differentiation and development, angiogenesis, stem cell renewal, homeostasis, hypertrophy and remodeling, heart failure and aging [97-99], and selected pathway components may provide novel opportunities for therapeutic targeting [100-103].

### 3.1 Wnt Ligands

Humans possess 19 Wnt protein genes which encode the evolutionarily conserved Wnt proteins critical to cell proliferation, migration, differentiation, apoptosis and polarity [97,104]. The secreted Wnt ≈ 40 kDa hydrophobic glycoproteins are characterized by a nearly invariant positioning of 22 cysteine residues which promote in disulfide bridges and confer specific globular secondary structures [94,104]. Some of the Wnts have isoforms with distinct 5'UTRs expressed from alternative promoters [104].

Intracellular processing of Wnts prior to secretion may include posttranslational modification by glycosylation or acylation, the latter of which is essential for Wnt activity [104] and likely for Wnt secretion [94]. Secretion of ER and Golgi-processed Wnts via endosomes requires several associated cargo proteins, including Porcn, members of the p24 protein family and the sorting receptor WI [104]. Once at the cell surface, Wnts encounter a wide variety of interacting molecules, including heparins and suramin, for which Wnts have a high affinity [104]. In addition, extracellular glypicans and biglycans modulate subsequent Wnt-receptor interactions [105]. In mammals, Wnts likely mediate close-range signaling between cells in proximity to each other [94]. As discussed below, the non-Wnt ligands Norrin and the R-spondins 1-4 can activate the Wnt pathway [97].

### 3.2 Wnt/β-catenin Receptors, Co-receptors and Ligand Interactions

Wnt glycoproteins bind a heterodimeric receptor complex consisting of a Frizzled protein and an associated co-receptor (Fig. 2) [94,106]. The high-affinity 10 member Frizzled protein transmembrane receptor class comprises the principal Wnt receptors [97]. Frizzled receptors are G-protein coupled [97] and possess a large extracellular cysteine-rich domain for Wnt binding [95]. Different Wnt heterodimeric receptor complexes may include in addition to a Frizzled protein either a low-density lipoprotein receptor-related protein 6 (LRP6), receptor tyrosine kinase-like orphan receptor (ROR), protein tyrosine kinase 7 (PTK7), receptor tyrosine kinase (RYK), muscle skeletal receptor tyrosine kinase (MUSK) or proteoglycan family co-receptor [106].

Frizzled proteins use specific co-receptors to channel downstream signaling along particular intracellular pathway branches [95]. Binding of Frizzled receptors to the cytoplasmic scaffolding protein Dishevelled (DVL), which interacts with most Wnt co-receptors and transduces all major Wnt sub-pathways, recruits additional cytoplasmic transducers including the scaffolding protein Axin, CKI and GSK3 binding protein to promote β-catenin-dependent signaling [95]. The particular combination of Wnt receptor, co-receptor and Wnt ligand determines downstream signaling and confers a rich combinatorial, context-dependent complexity to Wnt pathway signaling [96,106].

A single Wnt glycoprotein can bind to multiple Frizzled proteins with variable affinities, promoting possible competitive inhibitory regulation [94]. In general, Wnt1, Wnt3A and Wnt8 employ β-catenin dependent signaling and Wnt5A and Wnt 11 employ β-catenin independent signaling [95]. In general, Frizzled proteins serve as receptors for both β-catenin dependent and β-catenin independent pathways, LRP5 and PRT6 serve as co-receptors for β-catenin dependent pathways and ROR1 and ROR2 serve as co-receptors for β-catenin independent pathways [95].

### 3.3 Canonical Wnt/β-catenin Signaling Pathway

The interaction of a secreted Wnt glycoprotein with a Frizzled receptor and a LRP5 or LRP6 co-

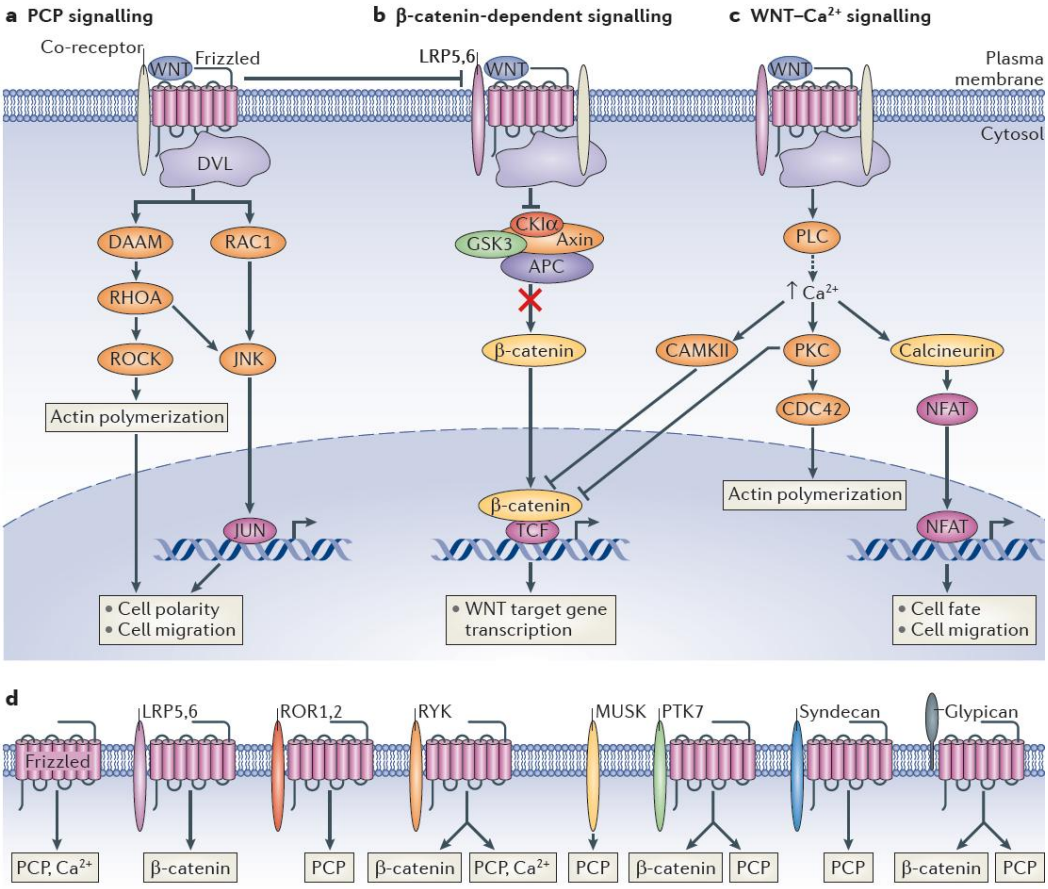


**Fig. 2. The Wnt/β–catenin canonical and non-canonical signaling systems (Niehrs [95])**

*(a)Planar cell polarity (PCP) signaling triggers activation of small GTPases RHOA and RAC1, which activate ROCK and JNK leading to actin polymerization and microtubule stabilization. b) In the absence of a WNT ligand, GSK3 phosphorylates β-catenin, which triggers its degradation. In the presence of a WNT ligand, the destruction complex containing GSK3, Axin, APC and CK1a is recruited to the occupied receptor and inactivated. β-catenin then accumulates in the nucleus where it activates the transcription of target genes in association with TCF and other factors. c) The WNT-$Ca^{2+}$ pathway activates $Ca^{2+}$-and calmodulin-dependent kinase II (CAMKII), protein kinase C and calcineurin. Calcineurin activates nuclear factor of activated T cells (NFAT), which then activates transcription of target genes. The PCP and Ca2+ pathways antagonize β-catenin signaling at multiple points. d) Major pathways used by Wnt receptors and co-receptors. DAAM, Dvl-associated activator of morphogenesis 1; DVL, Dishevelled; LRP, low –density lipoprotein receptor-related protein; MUSK, muscle skeletal receptor Tyr kinase; PLC, phospholipase C; PTK7, protein Tyr kinase 7; ROR, receptor Tyr kinase-like orphan receptor, RYK, receptor Tyr kinase.*

receptor leads to the inhibition of glycogen synthase kinase 3 (GSK3) and the inhibition of GSK3–mediated phosphorylation of a variety of substrates including the transcriptional co-activator β-catenin [95]. GSK3-mediated phosphorylation both inactivates β-catenin and targets β-catenin for proteosomal degradation in a "destruction complex" consisting of β-catenin, GSK3, adenomatosis polyposis coli (APC), the scaffold protein Axin and casein kinase 1α (CKIα) [107]. This destruction complex regulates the stability of cytoplasmic β-catenin and plays a key role in the regulation of the overall signaling output of the canonical Wnt pathway [94]. When Frizzled receptors are unoccupied, β-catenin is phosphorylated and sequestered in the destruction complex and thereby marked for proteosomal degradation [97,107]. Activation of the canonical Wnt/β-catenin pathway with Wnt ligand receptor binding recruits DVL and Axin to

the membrane, leading to disassembly of the destruction complex, decreased β-catenin phosphorylation and non-phosphorylated β-catenin cytoplasmic stabilization and accumulation [97,99,107].

Non-phosphorylated β-catenin gains entry into the nucleus where it accumulates and associates with a variety of transcription factors including T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family members to regulate transcription of target genes such as Wnt 1-inducible signaling-pathway protein I (WISP1), cell cycle-related proteins (c-myc, cyclinD) and the negative regulators DKK1 and Axin 2 in a context-dependent manner [94,95]. The current model of Wnt/β-catenin dependent signaling involves β-catenin "transcriptional switch activation" by TCF/LET-β-catenin binding and derepression of TCF/LET target gene repression [108]. TCF/LET target gene repression in the absence of β-catenin involves additional co-repressors, most commonly TLE family members but also Mtgr1, Coop and HIC5 [108]. The canonical pathway is prominently involved in cell differentiation and proliferation [95].

β-catenin is a constitutively expressed, multi-functional protein and found in several subcellular locations and exchangeable pools as illustrated in Fig. 3 [ 99,109]. Newly synthesized β-catenin may be sequestered in the destruction complex or immobilized by E-cadherin at adherens junctions where it interacts with α-catenin [109]. β-catenin in a phosphorylation dependent manner is also essential for E-cadherin adhesion and adherens junction attachment to the actin cytoskeleton [99]. β-catenin can be released from adherens junctions by protein kinases or E-cadherin down-regulation, and free excess β-catenin is immediately sequestered in destruction complexes in the absence of Wnt signaling [109]. β-catenin may also be "protected" in the cytoplasm by binding to APC and possibly Axin2 where it plays a role in cytoplasmic centrosomes [110,111].

## 3.4 Non-canonical β-catenin Independent Wnt Signaling Pathways

The non-canonical Wnt signaling pathways are β-catenin independent. To date, the best described include the planar cell polarity (PCP) and the Wnt-Ca2+ pathways, although a large number of additional pathways are likely to exist may likely exhibit significant overlapping components [112]. In PCP signaling, Frizzled receptors activate a cascade of phosphorylations via small GTPases Rac1, RhoA and JUN-N-terminal kinases that results in changes in cytoskeletal and cell polarity and activation of JNK-dependent transcription factors [95,112]. In the PCP pathway, DVL recruits PKC, atypical PKC (aPKC), DVL-associated activator of morphogenesis 1 (DAAM1) and partitioning defective 6 (PAR6), factors important in small Rho and Rac GTPase regulation, actin cytoskeleton tension and cell polarity [95,112]. The PCP and β-catenin-dependent pathways are regulated in part via ligand-receptor level antagonistic cross-talk, as inhibition of one pathway typically upregulates the other [95]. As an example of ligand-receptor antagonistic cross-talk, WNT5A, which preferentially activates the PCP signaling pathway, competes with WNT3A for binding to Frizzled 2, suppressing the WNT3A-Frizzled 2 β-catenin-dependent pathway [113]. In the Wnt-Ca2+ pathway, Wnt-Frizzled binding activates heterotrimeric G proteins, activating in series phospholipase C (PLC), diacylglycerol and inositol-1,4,5-triphosphate (Ins (1,4,5)) P3, Ca2+ release from intracellular stores, Ca2+- and calmodulin-dependent kinase II (CAMKII), calcineurin, protein kinase (PKC) and ultimate activation of the transcriptional regulator nuclear factor associated with T cells (NFAT) [114,115]. This pathway is invoked in cancer, inflammation and in neurodegenerative states [95,114].

## 3.5 Wnt/β-catenin Signaling Regulation

Wnt signaling is regulated at all pathway levels including in the 1) extracellular space by secreted extracellular antagonist and agonist soluble proteins [116], 2) cell membrane by Frizzled receptor modification and internalization and additional transmembrane proteins [106], 3) cytoplasm by β-catenin destruction complex activity [107] and 4) nucleus by complex combinatorial networks of transcriptional factors, DNA binding proteins, chromatin modifiers, co-activators and co-repressors [117,118].

### 3.5.1 Extracellular level regulation

Secreted proteins such as secreted Frizzled-related proteins (sFRPs 1-5) and Wnt inhibitory protein (WIF) bind and sequester secreted Wnt glycoproteins away from active receptor complexes [94].

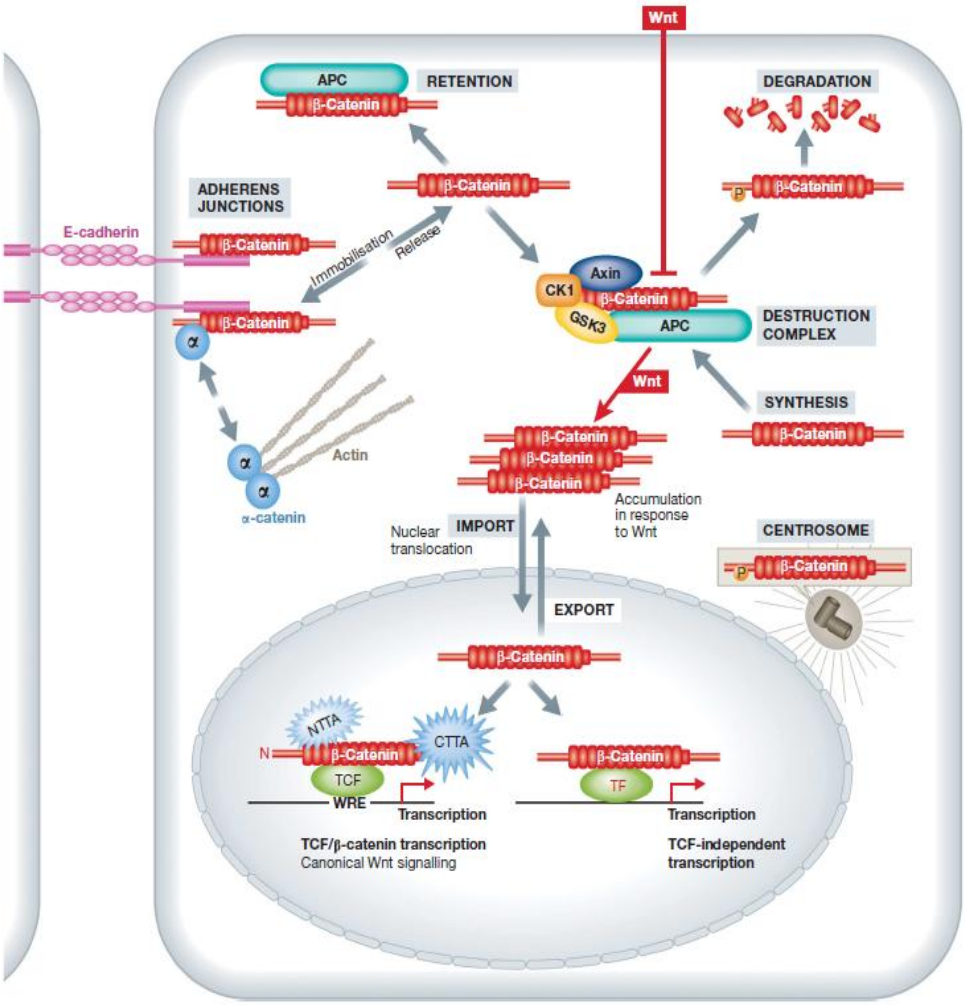


**Fig. 3. The many roles of β-catenin (Valenta et al. [109])**

*Newly synthesized β-catenin is immobilized by E-cadherin and interacts with α-catenin at adherens junctions (AJ), modulating the actin cytoskeleton. β-catenin can be released from AJ by protein kinases or downregulation of E-cadherin. Free β-catenin is immediately phosphorylated and marked for degradation in the GSK3, Axin, APC and CK1a destruction complex. A portion of free β-catenin may be sequestered by APC. Wnt signaling results in inactivation of the destruction complex, increased cytoplasmic β-catenin and β-catenin translocation into the nucleus to activate transcription. b-catenin may also play an important role in the centrosome. CTTA, C-terminal transcriptional activators; NTTA, N-terminal transcriptional activators*

Wnt regulation also occurs via 1) other extracellular secreted small protein antagonists including Cerberus, Dickopf-related protein 1, -2 (Dkk 1, -2), sclerostin and its homologue Wise, IGFBP-4 and 2) the transmembrane proteins Shisa, Waif1, APCDD1 and Tiki 1 [95,116]. The Dickkopf proteins 1,-2,-4 are potent secreted Wnt modulators that generally inhibit Wnt signaling, although Dkk2 may in certain contexts activate Wnt signaling [99,116,119]. Dkk1 disrupts Wnt-Frizzled-LRP6 formation, likely by binding to LRP6 and thereby disrupts the Wnt-induced Frizzled-LPR6 complex formation [116,119]. Dkk1 also binds with high affinity to Kremen1, -2, (Krm1, -2) single-pass transmembrane receptors [119]. Dkk1 binding to Krm1, -2 greatly potentiates Dkk1-mediated Wnt inhibition, likely by the formation of Dkk1-Krm2-LRP6 ternary complex which induces rapid endocytosis and LPR6 membrane clearance [119]. Sclerostin and Wise likely inhibit Wnt signaling by Dkk1-like mechanism [116]. IGFBP-4 binds directly to LPR6 and Frizzled 8 and blocks Wnt3a receptor binding [120]. As examples of transmembrane

Wnt inhibition, the transmembrane glycoprotein APCDD1 binds both Wnt and LRP thereby inhibiting Wnt signaling [94,97] and transmembrane metalloprotease Tiki1 cleaves the eight amino-terminal residues from Wnt, forming oxidized Wnt oligomers with reduced Frizzled receptor binding affinities [116].

Wnt signaling is also regulated by secreted small protein agonists including the R-spondins and Norrin [116]. The R-spondins 1-4 promote both β-catenin-dependent and β-catenin-independent Wnt signaling [95]. The mechanism of R-spondin promotion of β-catenin-dependent signaling is controversial, and proposed mechanisms include R-spondin binding to LRP6 or the DKK1 co-receptor Kremen serving as an R-spondin receptor [116]. In β-catenin-independent Wnt signaling, high-affinity syndecans function as receptors for R-spondins 2, 3 [95]. Following syndecan-R-spondin binding, syndecans trigger R-spondin endocytotic internalization, likely by clustering Wnt receptor complexes and DVL, and event necessary for R-spondin 3 to induce PCP signaling [121]. Frizzled 4 can be regulated by Norrin, which activates β-catenin-dependent signaling in an LRP5, LRP6 and WNT independent manner [116].

### 3.5.2 Receptor level regulation

At the receptor level, both Frizzled and Wnt co-receptors are regulated intracellularly by a variety of mechanisms including phosphorylation (for Frizzled, LRP6, ROR, MUSK), ubiquitylation, endocytotic internalization and membrane clearance (Frizzled, RYK), proteolytic release of the cytoplasmic domain (Frizzled, LRP6, RYK) or regulated extracellularly by cleavage of a lipid anchoring moiety [95]. R-spondins may also derepress Wnt signaling by protecting Frizzled receptors from endocytotic internalization via degradation routes [95].

### 3.5.3 Cytoplasmic level regulation

In the absence of Wnt signaling, the cytoplasmic destruction complex sequesters newly synthesized β-catenin in a "futile cycle" of phosphorylation-targeted proteasomal degradation [96,107]. Upon Wnt-ligand activation of Frizzled-LPR5/6 complex, 1) the destruction complex is recruited to the membrane in part via WTX/AMER1 protein binding to destruction complex APC and PIP2, 2) the destruction complex components are relocalized and disassembled, in part via DVL scaffolding, 3) β-catenin is stabilized in part via reduced GSK3 phosphorylation and 4) stabilized β-catenin then enters the nucleus [107]. The precise mechanisms of destruction complex sequestered β-catenin derepression are not clearly established, but evolving experimental evidence supports roles for 1) dissociation of the destruction complex components, 2) GSK3 phosphorylation at Ser9 which inhibits GSK3 and GSK3-mediated β-catenin inhibitory phosphorylation, 3) LRP6 direct binding and inactivation of GSK3, 4) Axin degradation upon Wnt signaling activation and 5) global inhibition of GSK3 activity via sequestration of GSK3 in multivesicular bodies [96,122]. The scaffolding protein Axin in the destruction complex acts a critical negative regulator of β-catenin signaling as it directly binds many of the complex components directly [96,122], and since Axin stability and cytoplasmic concentration vary significantly in diverse cell types and conditions, Axin degradation likely represents a major regulatory mechanism [123].

The kinetic responses of β-catenin have been studied and modeled mathematically [124,125]. Continuous stimulation of cells by Wnt3A resulted in an increase in total β-catenin within 15-30 minutes with achievement of a new six-fold increase in β-catenin concentration within 2 hours and an 80% decrease in GSK3-phosphorylated β-catenin in 15-30 minutes which returned to its initial concentration in 2 hours in multiple cell lines. The amount of GSK3-phosphorylated β-catenin exhibited a strong negative correlation with the rate of β-catenin accumulation. These findings support that Wnt signaling inhibits β-catenin phosphorylation and also increases β-catenin half-life from 16 to 104 minutes due to partial inhibition of the destruction complex. Thus, the degree, time course and intensity of Wnt-Frizzled signaling determines a new steady state β-catenin concentration. Kinetic analysis of the in vitro system concluded that core signal transduction mechanism is relatively simple, with only two regulated phosphorylation steps (GSK3 and CK1α), partial destruction complex inhibition given the constitutive rate of β-catenin synthesis

### 3.5.4 Nuclear level regulation

Since β-catenin is normally constitutively transcribed and translated, the inhibition of destruction complex β-catenin sequestration and degradation results in a rapid cytoplasmic rise of

β-catenin cytoplasmic concentration [96]. Although the mechanisms by which β-catenin then accumulates in the nucleus are poorly understood and appear unrelated to classic nuclear import factors such as RanGTPase and the importins, differential rates of β-cantenin nuclear import and export likely have important effects on the overall output of the Wnt pathway [96].

Nuclear β-catenin accumulation results in interaction of β-catenin with the TCF and LEF co-transcriptional factors and a host of other factors resulting in target gene effects [117,118,126]. The context-dependent regulatory interactions of β-catenin with co-transcriptional factors, chromatin modifiers including histone methyltransferases and histone deacetylases, ATPase-dependent nucleosome and chromatin remodelers, transcriptional machinery components such Mediator complex subunits and RNA Polymerase II, enhancesomes and other transcriptional factors is remarkably complex as summarized in Table 2 from recent reviews. Despite the complexity of Wnt/β-catenin signaling, a few overarching themes have emerged from evolving research.

First, the Wnt/β-catenin pathway transmits transcriptional activation signals by altering nuclear β-catenin fold changes. Interestingly, approximate two-fold change differences in β-catenin nuclear concentration rather than the absolute nuclear β-catenin concentration itself dictates Wnt signaling output [127]. The downstream transcriptional machinery "computation" of the β-catenin fold change signal rather than "read" of the β-catenin absolute concentration likely buffers stochastic, genetic and environmental variation and confers robustness on Wnt signaling [127]. Fold-change detection as utilized in Wnt signaling is a common network motif in transcriptional networks, whereby in an " incoherent feedfoward loop", an activator such as β-catenin (as described below) regulates both a gene and a repressor of the gene, thereby defining the amplification, duration and entire shape of the transcriptional response [128].

Second, the two-fold rise in nuclear β-catenin appears to "throw a transcriptional activation switch" and activate TCF-repressed gene targets [129]. TCF binds to DNA recognition sequences termed Wnt response elements (WREs) and in the absence of sufficient β-catenin, TCF represses the transcription ofWnt/ β-catenin target genes [126]. TCF repression involves the recruitment of additional repressive co-factors, particularly TLE1, a long-range chromatin repressor that interacts with histone deacetylases (HDACs) to compress chromatin and inhibit transcription [126]. β-catenin competes with TLE1 for TCF binding, and thus the relative nuclear and/or fold changes of β-catenin and TLE1 determine whether TLE1 functions as repressive or activating co-transcriptional factor.

Third, the structure of β-catenin plays a critical role in scaffolding of co-transcriptional factors, their interactions with other regulatory components and chromatin remodeling to facilitate transcription (Fig. 3) [118,126]. β-catenin is a member of the Armadillo (ARM) repeat protein superfamily and possesses a 12 imperfect ARM repeats (R1-R12) as its core, an R12-core helical domain (R12-C) and distinct N-terminal (NTD) and C-terminal (CTD) domains beyond the R1-R12 core [126]. The R1-R12 core binds TCF and LEF1, the two central β-catenin activating co-transcriptional factors, and the NTD and CTD domains recruit and provide binding sites for additional co-factors [109]. β-catenin R12-C binds to the modular Mediator complex, which bridges enhancers and promoter transcriptional start sites and serves as a regulatory scaffold for RNA Polymerase II and other transcriptional factors and machinery, via the Mediator complex subunit 12 (MED12). The β-catenin NTD and CTD domains promote recruitment of a wide variety of auxiliary transcriptional factors and regulators [126] as summarized in Table 2. Briefly, the β-catenin CTD interacts with crucial chromatin-dependent factors, including 1) HAT proteins CBP and p300, 2) SWI2/SNF2 family proteins Brg1 and ISW1, ATPases which shuffle or disassemble nucleosomes, 3) MLL proteins containing SET1 COMPASS complex histone methyltransferase domains promoting H3K4me3 and other transcription-associated histone marks resulting in swift and widespread chromatin changes [118]. The β-catenin NTD recruits and interacts with Bcl9 and Pygo, proteins critical to β-catenin readouts [126]. Pygo may function as a 1) "pioneer" factor at WRE genes by tethering TCF-bound β-catenin to target genes and 2) "reader" of chromatin histone marks via Pygo PHD finger domain interactions with methylated histone tails [126]. There is evolving evidence that the β-catenin CTD and NTD domains engage in

transcriptional regulatory "cross-talk" including cooperative interdependence, suggesting that β-catenin serves as a multi-dimensional scaffold for the cyclical recruitment and sequential exchange of chromatin-remodeling factors, transcriptional machinery and transcriptional regulatory factors at WREs as it orchestrates and fine-tunes Wnt/β-catenin pathway output. Finally, TCF as a member of the high mobility group box protein family induces strong DNA bending following DNA binding [108]. TCF-β-catenin binding may create a local structural chromatin environment that facilitates enhanceosome formation due to cooperatively between distant or strategically positioned WREs [126].

The "off switch" for Wnt/β-catenin signaling is not yet identified [126]. Regulatory mechanisms may include 1) Pygo-dependent binding of β-catenin to methylated histones "frees" TCF to recruit TLE1 co-repressors with counteract β-catenin induced chromatin remodeling, particularly as nuclear β-catenin falls as upstream destruction complex β-catenin sequestration increases in response to diminished Wnt-Frizzled receptor activation [130] and 2) APC or Axin recruitment to WRE loci, which is associated with disappearance of β-catenin from WREs and appearance of TCF-TLE1 complexes [131,132] and 3) inhibition of WRE-associated kinase CK2 which in turn blocks β-catenin and TLE1 recruitment to WREs [133]. Many DNA-binding transcription factors such as Smad3, AP-1, Rxr and Kaiso bind β-catenin to activate or suppress β-catenin target genes, providing an additional regulatory level of control [96,108]. Vertebrates possess four TCF/LEF transcribed in a variety of isoforms to confer context-specific regulation of TCF/LEF-β-catenin and WRE binding and differential target gene regulation [108]. β-catenin dependent kinases such as HIPK2, NLK and TNIK may phosphorylate TFC/LEF binding partners in a Wnt- and β-catenin dependent manner, resulting typically in enhanced target gene activation but also in negative regulation (e.g., NLK phosphorylation of TCF4 and LEF1 triggers their dissociation from DNA) [134]. Finally, nuclear β-catenin also interacts with non-TCF/LEF co-transcription factors including Sox family members, FOXO proteins, HIF1α, type I and type II nuclear receptors, Prop1, Oct3/4, Pitx2, MyoD and Mitf in in various contexts including cardiac development and angiogenesis [99,108]. These non-TCF/LEF co-transcription factors compete for binding to the available β-catenin nuclear pool, affording yet another level of target gene specific regulatory specification and complexity to β-catenin signaling [108].

Although the Wnt signaling pathway is typically associated with gene activation, Wnt pathway activation may also result in gene repression in *Drosophila* [135] and vertebrates [117]. Repression also requires β-catenin binding to TCF, but binding to DNA motifs also allosterically regulates signal repression [118]. Allosteric conversion of the novel motifs to standard TCF-binding sites resulted in activation of Wnt gene expression and reversal of gene repression [117].

## 4. HIPPO PATHWAY SIGNALING

The molecular pathways that inhibit organ development and growth beyond appropriate physiologic check points were largely unknown until the discovery of the tumor-suppressor Hippo pathway in *Drosophila* and mammals [136-139]. Dysregulation of the highly conserved Hippo pathway results in cellular proliferation and often massive organ growth. Beyond the regulation of organ size, the Hippo pathway has been shown to play critical regulatory roles in apoptosis, proliferation, stem cell self-renewal and expansion and tissue regeneration. Interestingly, the Hippo pathway is regulated in part by diverse physical signals including cell geometry, cytoskeleton tension, matrixrigidity and other mechanical signals and functions as a transducer of information regarding cell confluence, cell polarity and the cytoskeletal and extra-cellular mechanical environment [140].

### 4.1 Hippo Pathway Components

The intracellular Hippo pathway which converges on the transcriptional co-activators Yap and Taz consists of a cascade of the two core kinases Mst1/2 and Lats1/2 and associated adaptor/activating proteins Sav1 and MOBK1A/B (MOB1A/B) as shown in Fig. 4 [136,138,139]. The role of WWC1/2/3 and associated proteins such as FRMD and Mer is uncertain at present. Mst1/2 are pro-apoptotic kinases activated by by caspase cleavage during apoptotic stress, binding to the activator/adaptor protein Sav1 or binding to Ras associated domain family (Rassf) proteins and inhibited by Akt [141]. Mst1/2 substrates include Lats1/2, ndr1/2, Mob1A/B, FOXO1/FOXO3 [141]. Sav1 scaffolds Mst1/2 via SARAH domains and activates Mst1/2 by an unknown mechanism [139]. Activated Mst1/2 phosphorylates and activates Lats1/2

[138,139,141]. Activated Mst1/2 also phosphorylates MOB1A/B, which complexes with Lats1/2 [138,139]. Activated Lats1/2 then phosphorylates, and thereby inhibits, the Yap/Taz transcription co-activators, the final effectors of the pathway [142]. By inhibiting Yap/Taz, activation of the Hippo pathway inhibits the Yap/Taz induced networks of gene expression which typically effect an increase in cell proliferation and an increase in organ size [143]. By regulating Yap/Taz localization and activation, the Hippo pathway "fine-tunes" cell proliferation, cell death and cell-fate decisions and integrates these signals to help specify organ size.

## 4.2 Hippo Pathway Activation

The triggers for Hippo pathway activation and signaling remain controversial. Proposed mechanisms include 1) cell-cell contacts/junction formation, 2) cell morphology, 3) mechanotransduction of signals from adjacent cells and matrices and 4) ligand-binding cell membrane G-protein coupled receptors found in *Drosophila* but not found to date in humans (Fig. 4) [144]. The mammalian genome contains homologs for the reported upstream regulators of the *Drosophila* Hippo pathway, but only the tumor suppressor NF2/Mer, mutations of which cause autosomal dominant neurofibromatosis 2, have been widely studied to date 138. Mammalian Hippo pathway extra-cellular components, including Hippo pathway receptors and associated ligands, remain unknown.

Compelling evidence supports roles for cell-cell confluence in Hippo pathway activation and Hippo pathway dependent Yap/Taz inhibition. A variety of Hippo pathway components are found in transmembrane complexes (e.g., Crumbs complex), cell-to-cell tight junctions and adherens junctions (Fig. 4) [139,142]. The presence of Hippo components in cell-to-cell adhesions may facilitate Hippo pathway activation based upon cell polarity and cell-to-cell and cell contact, confluence and/or density signals, vital mechanical and spatial inputs in the regulation of organ size [138]. Cell-cell interactions upon reaching confluence, for example, may trigger a cascade of signaling events resulting in Hippo pathway activation, phosphorylation and cytoplasmic sequestration of Yap via 14-3-3- binding [142]. In tissue culture, high cell density induces Yap phosphorylation and cytoplasmic sequestration [145] and disruption of cell-cell communications results in Yap nuclear accumulation [146].

In landmark studies in isolated cells, however, a variety of mechanical cues including increased extracellular matrix (ECM) stiffness, cell geometry characteristic of spreading cells, increased Rho activity and increased actin cytoskeletal tension resulted in Yap/Taz nuclear localization independent of canonical Hippo pathway activity [147,148]. The investigators concluded that cells "read" ECM stiffness, cell shape and cytoskeletal forces as Hippo-pathway independent Yap/Taz activity levels regulated in turn by a cytoskeletal pathway requiring the small Rho GTPAses. The non-Hippo pathway dependent increase in Yap/Taz activity resulting from increasing cell density, increased cytoskeletal tension and spreading cell geometry results in activation of Yap/Taz gene expression programs and an appropriate proliferative response [147-149]. The integration of 1) cell-confluence mediated Hippo pathway dependent Yap/Taz inhibition, 2) mechanotransduction-mediated Hippo-independent Yap/Taz activation, 3) small Rho GTPAse activity and actin cytoskeletal tension, 4) cell morphology and 5) cell attachments in determining aggregate Yap/Taz signaling will be an important focus of future research. Of note, failing myocardium is marked by myocyte hypertrophy, increased extracellular matrix stiffness, increased cytoskeletal and matrix tension, altered mechanotransduction, and altered cell-cell and cell-matrix attachments and anchoring. Although not investigated to date in detail, it would be consistent with the known mechanisms of Hippo pathway activation that in failing myocardium Hippo pathway activation occurs in response to these aggregate myocyte and matrix structural and functional alterations.

## 4.3 Hippo Pathway and Yap/Taz Inhibition

Contact inhibition is frequently antagonized by mitogenic growth factor signaling. The mitogenic growth factor EGF reduces Lats1/2 phosphorylation of Yap and stimulates Yap nuclear accumulation [150].

Inhibition of PI3K and PDK1, but not AKT, blocked EGF-mediated Yap nuclear accumulation, suggesting that EGF inhibits the Hippo pathway via PI3K and PDK1. PDK1 was shown to associate with the core Hippo pathway kinase complex through the scaffold protein Salv. In this manner, mitogenic signaling pathways inactivate Hippo-dependent Yap inhibition.

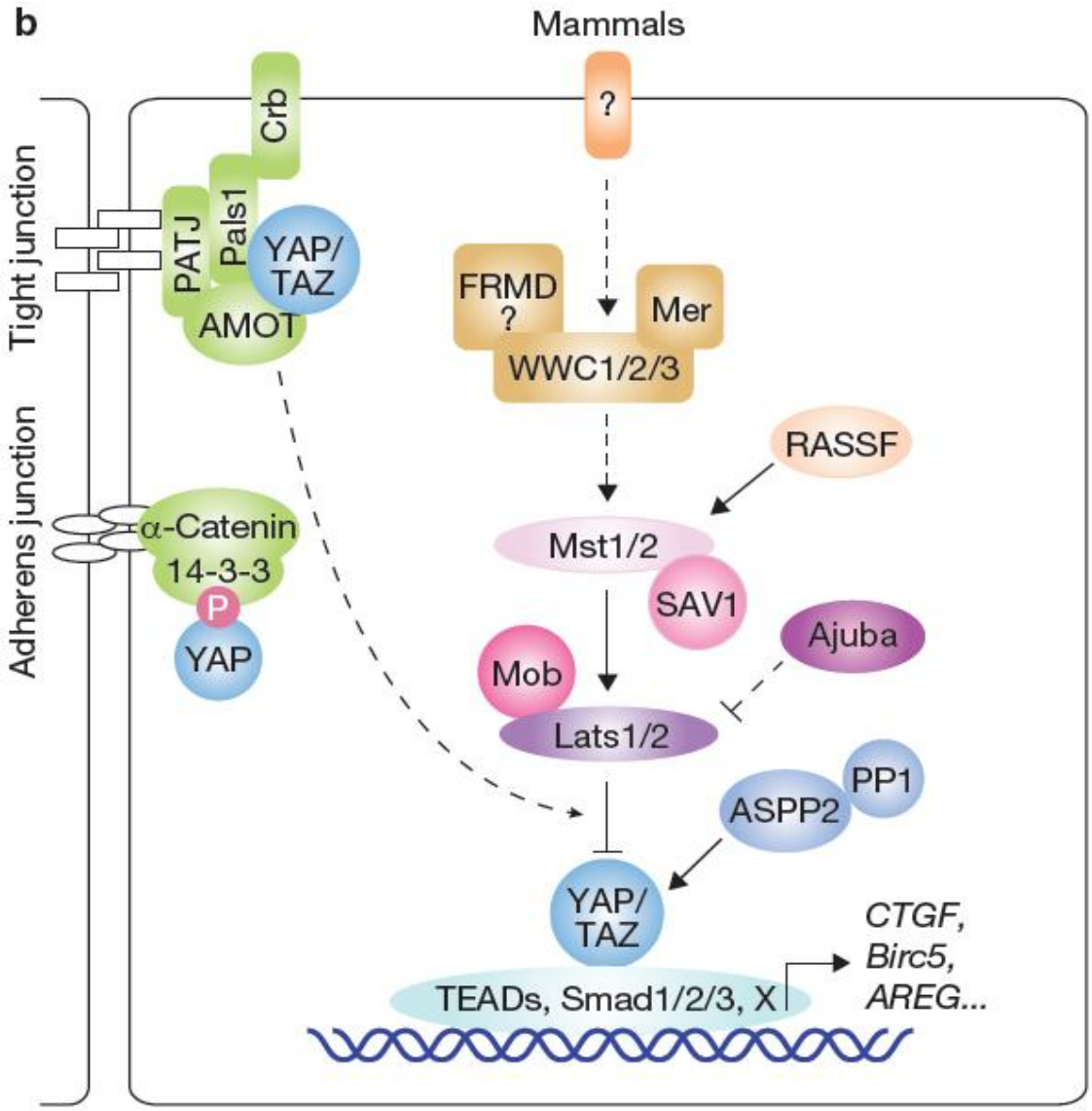


**Fig. 4. The hippo signaling system (Zhao et al. [139])**

*The mammalian Hippo signaling pathway. Crb, Crumbs complex which includes associated proteins PATJ and Pals1; AMOT, AMOT family proteins (tight-junction protein complex including PALS1, PATJ/MPDZ, Lin7);FRMD, FERM domain proteins homologue; Mer, Merlin homologue; WWC 1/2/3, homologues of Kibra; RASSF, Ras association domain family proteins; Mst1/2, homologues of Hpo Ste20 Ser/Thr kinase; SAV1, homologue of Salvador; Mob, homologue of Mats; Ajuba, homologue of dJuba; Lats 1/2, homologue of Wts NDR Ser/Thr kinase; PP1, phosphate; ASPP2, ; TEADs, homologue of Scalloped ; CTGF, Birc5, AREG, genes typically expressed via Yap/Taz activation. Homologue refers to homologue of Drosophila component. Direct protein-protein interactions in solid lines, possible protein-protein interactions dashed lines*

Inhibition of Yap and Taz, the final effectors of the Hippo pathway, may result from a variety of mechanisms, including 1) phosphorylation dependent cytoplasmic retention, 2) phorphorylation-independent cytoplasmic retention and 3) ubiquitylation and degradation [139]. Yap and Taz phosphorylation by Lats1/2 in the canonical pathway is strongly inhibitory, and promotes phosphorylated Yap and Taz binding and subsequent cytoplasmic sequestration and inactivation by binding to cytoplasmic 14-3-3 [139]. Yap and Taz binding to the cytoplasmic angiomotin (AMOT) family of proteins results in cytoplasmic sequestration, often at tight junctions [139]. Taz binding to Z0-2 localizes Taz to tight junctions. Finally, Yap phosphorylation promotes

recruitment of E3 ubiquitin ligase SCFb-TRCP and resultant Yap polyubiquitylation and proteosomal degradataion.

### 4.4 YAP and TAZ Transcriptional Regulation

In the absence of Hippo pathway inhibition, Yap/Taz are sufficient to induce gene expression programs resulting in proliferation and organ growth [138]. The TEAD 1-4 family of transcription factors are critical partners in Yap/Taz gene expression programs, as RNAi knockdown of TEADs or disruption of the Yap-TEAD interaction abrogates YAP-induced proliferation [139,151]. Direct target genes of Yap/Taz/TEAD include CTGF and Cyr61 in mammals [139]. Other PPXY motif containing transcription factors including Smad 1, RUNX, ErbB4 and p73 for YAP and RUNX, PPARγ, Pax3, TBX5 and TTF-1 for Taz interact with the WW domains of Yap/Taz [139]. Yap/Taz also bind Smad2/3, an interaction regulating the nucleocytoplasmic shuttling and nuclear accumulation of Smad2/3 [152]. As discussed below, Yap/Taz also interacts with β-catenin to induce expression of the canonical Wnt target genes SOX2 and SNAI2 in the mouse heart [153]. As transcriptional co-activators upon which the cross-talk from multiple pathways converge, Yap/Taz induce the expression of BIRC5, AREG and FGF1 and many other genes in a tissue-specific contextual manner [138].

### 4.5 Hippo in Development, Cancer and Fibrosis

Double knock-out of Yap/Taz resulted in embryonic lethality [154]. Specific deletion of Yap in the embryonic mouse heart resulted in cardiomyocyte hypoproliferation, cardiac hypocontractility, myocardial hypoplasia and lethality at embryonic stage 10.5 [155]. Constitutive over-expression of Yap in the embryonic mouse heart resulted in an increased proliferation and number of cardiomyocytes and increased myocardial mass and size [155]. Yap is both necessary and sufficient to regulate cardiomyocyte proliferation *in vitro* and *in vivo* 143. Yap over-expression is tumorigenic and Yap genomic amplification is often observed in human cancers [136,139,156,157]. Less is known about the role of the Hippo pathway in fibrosis compared to the TGFβ and WNT/β-catenin pathways. Rassf1A is an endogenous activator of Mst1 in the heart, promoting cardiomyocyte apoptosis and surprisingly inhibiting fibroblast proliferation and cardiac hypertrophy via cell-autonomous and autocrine/paracrine mechanisms [158]. In cardiac fibroblasts, Rassf1A/Mst1 negatively regulates TNF-α, a mediator of hypertrophy and fibrosis [158], and suppression of the Rassf1A/Mst1 pathway inhibited cardiomyocyte apoptosis but via TNF−α paracrine effects, promoted cardiac hypertrophy and fibrosis [158]. The investigators concluded that suppressing the Rassf1A/Mst1 pathway in cardiac fibroblasts could be detrimental [158].

## 5. TOWARDS MYOCARDIAL SYSTEMS BIOLOGY: EXAMPLES OF TGF-β SUPERFAMILY, WNT/β-CATENIN AND HIPPO SIGNALING PATHWAY CROSS-TALK

Experimental evidence of signal integration and cross-talk between the TGF-β, WNT/β-catenin and Hippo pathways is evolving rapidly as illustrated in Fig. 5 [144,159]. In the section that follows, we review selected landmark studies to date in diverse model systems, the myocardium, fibrosis and cancer.

### 5.1 Cross-talk in Diverse Model Systems

*Smad1-YAP interaction is facilitated by Mediator phosphorylation of the Smad1 linker region.* As receptor activated Smads form trimeric complexes, they can be phorphorylated in the linker region by CDK8 and CDK9, kinase components of the large transcriptional Mediator complex [86]. Upon phosphorylation in the linker region by by CDK8/9, Smad1 recruits Yap to the Smad1 phosphorylated Smad1 linker site [86]. The Smad1-Yap complex then binds to DNA in concert with additional transcriptional factors, co-activators and co-repressors to induce context-dependent transcriptional programs. Following transcription, the Smad1phosphorylated linker region is then recognized by ubiquitin ligases, leading to proteasome-mediated degradation and turnover of activated Smad1s [86].

Thus, Mediator via CDK8/9 phosphorylation of Smad1 linker region has a dual role, both 1) facilitating Smad1-Yap co-activated transcription, an integral feature of TGF-β and BMP canonical pathways and 2) promoting activated Smad1 turnover [86].

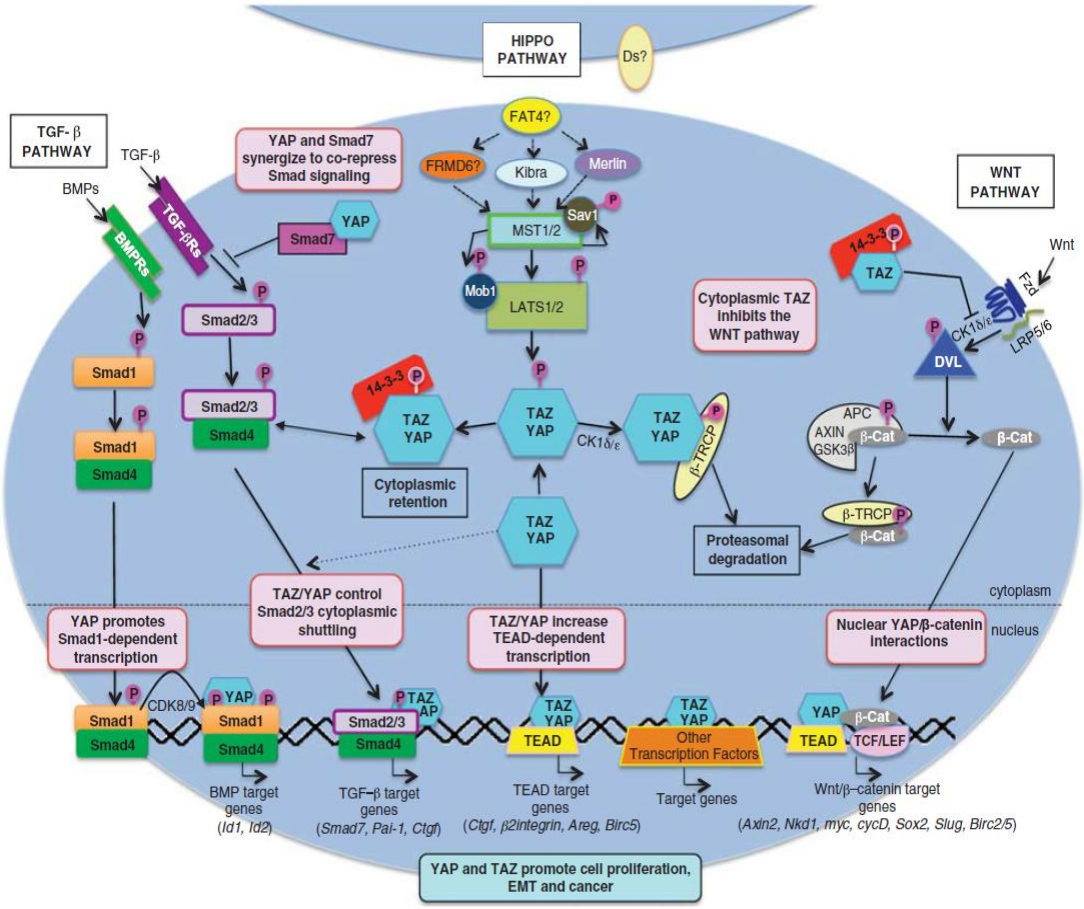


**Fig. 5. Cross-talk between the TGΦβ, WNT/β-catenin and Hippo pathways (Mauviel et al. [178])**

*This figure illustrates the TGF-β, Hippo and WNT/β-catenin pathway crosstalk with a focus on the role of the Hippo pathway. Central to the Hippo pathway are the transcriptional regulators TAZ and YAP. Nuclear TAZ and YAP associate with a number of DNA-binding transcription factors to mediate their transcriptional activity, including the TEAD and SMAD families. In a relatively unclear manner, upstream regulators of the Hippo pathway activate the MST 1/2 kinases, which phosphorylate and activate the LATS 1/2 kinases. LATS 1/2 then phosphorylates TAZ and YAP, leading to their binding 14-3-3 proteins and subsequent TAZ and YAP cytoplasmic sequestration. In the cytoplasm, TAZ and YAP perform an array of functions, including binding to SMAD 7 and TGF-β-activated SMAD complexes interfering with TGF-β signaling. Cytoplasmic TAZ also binds DVL proteins to inhibit WNT/β -catenin signaling*

*The Hippo pathway via TAZ regulates Wnt/β-catenin signaling.* Taz cytoplasmic localization is mediated by LATS-dependent phosphorylation, which drives phosphorylated Taz binding to the cytoplasmic retention factor 14-3-3 [160]. In mice, cytoplasmic Taz inhibited the cytoplasmic CK1δ/ε –mediated phosphorylation of DVL, thereby inhibiting Wnt/β-catenin signaling [161]. Decreased Taz levels or inhibition of Hippo signaling enhanced Wnt3A-stimulated DVL phosphorylation, nuclear β–catenin and Wnt target gene expression. Taz null transgenic mice developed polycystic kidneys with increased cytoplasmic and nuclear β-catenin. These findings taken together reveal a cytoplasmic function of Taz in Wnt/β-catenin signaling regulation [161]. The investigators concluded that the Hippo pathway functions to restrain and likely "fine-tune" Wnt/β-catenin signaling in a context dependent manner [161].

*The Hippo pathway via Taz regulates TGF-β signaling.* Like Smads, Taz undergoes nucleocytoplasmic shuttling, and in the nucleus Taz is recruited to sites of active transcription as discussed. The same group of investigators showed that in response to TFG-β stimulation, Taz binds to heteromeric Smad2/3-4 complexes

via the carboxyl-terminal coiled-coil Taz domain [152]. TGF-β induced Taz binding to the Smad2/3-4 complex binding was crucial for the nuclear accumulation of Smad complexes and the coupling of the Smad complexes to DNA binding proteins and transcriptional machinery such as the Mediator complex. By binding to the ARC105 subunit of Mediator, Taz appeared to function by scaffolding functionally distinct transcriptional regulatory complexes to target genes in a context-dependent manner. Knock-down of Taz by siRNA (siTaz, 90% reduction in Taz protein levels) markedly reduced 1) activation of the TGF-β responsive Smad reporters p3TP-lux and pARE-lux and the expression of TGF-β induced genes (e.g., Smad7, PAI-1, Id2) in a variety of human cell types and 2) reduced nuclear accumulation of Smads. Taz was shuttled in and out of the nucleus, and modulation of Taz localization also modulated Smad localization. The investigators concluded that Taz 1) associates with heteromeric Smad complexes in a TGF-β dependent manner, 2) plays an essential role in Smad complex nucleo-cytoplasmic shuttling and Smad nuclear accumulation, 3) couples Smad complexes to transcriptional machinery including Mediator and 4) the absence of Taz abrogates TGF-β signaling [152].

*Taz mediates Wnt signaling*. In a separate study in HEK293 cells, Taz was also shown to serve as a downstream component of the Wnt/β-catenin signaling cascade independent of its role in Hippo signaling [162]. In the absence of Wnt activity, the components of the β-catenin destruction complex (APC, Axin and GSK3) were required to maintain Taz at low levels and Taz degradation was dependent in turn on phosphorylated β-catenin that bridged Taz to its ubiquitin ligase β-TrCP [162]. Wnt3A signaling induced an increase in Taz (but not Yap) levels, Taz protein stabilization and Taz-induced Wnt transcriptional responses. The investigators concluded that 1) Wnt activation resulted in parallel activation of β-catenin and Taz, both of which were required for full Wnt transcriptional response, 2) a substantial portion of the Wnt transcriptional response was in fact mediated by Taz and 3) that Taz stability determined at least in part by β-catenin represented a "cross-roads" between Wnt and Hippo signaling [162]. Interestingly, loss of β-catenin was not accompanied by events known to regulate Taz through the Hippo pathway, including downregulation of cadherin adherens junctions, epithelial-to-mesenchymal transition, altered apico-basal polarity or changes in LATS activity.

*Crumbs Integrates Cell Density with Hippo and TGFβ pathway signaling.* Crumbs is a large transmembrane protein complex that localizes to the apical region of cells adjacent to adherens junctions and functions as a polarity regulating module in poorly understood interactions with two other polarity modules, the atypical protein kinase C complex and the Scribble complex 140. In high cell density conditions, the Crumbs complex interacts with Yap/Taz promoting Yap/Taz phosphorylation, the cytoplasmic retention of Taz-Smad2/3 complexes and the suppression of TGFβ signaling. In low cell density conditions or following disruption of the Crumbs complex, there is less Yap/Tax phosphorylation which drives the nuclear accumulation of both Yap/Taz and Smad2/3 and enhanced TGFβ signaling which predisposes mouse embryonic epithelial cells to undergo TGFβ-mediated epithelial-to-mesenchymal transitions [146]. As above, the same investigators demonstrated that high cell density, resulting Hippo pathway-dependent prominent cytoplasmic localization of Yap/Yaz P results in Taz binding to Smad complexes to control Smad nuclear accumulation [152]. In this manner, Crumbs-dependent Hippo signaling couples cell density to regulation of TGFβ-Smad nuclear accumulation [146]. The investigators concluded that the Hippo pathway plays a key role in controlling TGFβ-Smad signaling [146].

## 5.2 Cross-talk in the Heart

*Yap serves as a nexus for IGF, Wnt and Hippo mediated myocyte proliferation.* Yap overexpression increases transcription of IGF-1, IGF-1 receptor, IGF binding proteins 2 and 3, β-catenin and genes involved in mitosis and cytokinesis such as Cyclin A2 and B, Cdc2 and Ect2 [155]. By increasing PI3K abundance and Akt phosphorylation Yap overexpression inactivates glycogen synthase kinase 3b thereby derepressing its downstream substrate β-catenin [155]. Yap is thus a nodal integrator for stimulatory coupling of the IGF, Wnt and Hippo signaling pathways in the heart and fosters activation of networked developmental programs for myocyte proliferation and regulation of heart size [155].

*Hippo pathway inhibits Wnt/β-catenin signaling in regulation of heart size. Salv* conditional

knockout (*Salv* CKO) mice exhibited reduced phosphorylated Yap without change in total Yap *in utero* denoting reduced Hippo activity, and expired postnatally with obvious cardiomegaly and evidence of increased myocyte proliferation [153]. Microarray analysis revealed up-regulation of canonical Wnt genes in Salv CKO embryos [153]. The Wnt-Hippo "signature" included cardiac repair and remodeling gene SOX2, tumorigenesis and EMT-promoting genes SNAI2/SLUG, the anti-apoptosis genes BIRC5/SURVIVIN in addition to cdc20, l-Myc, Birc5 [153]. Salv CKO had four-fold increase in nuclear β-catenin staining, indicated derepression of canonical Wnt signaling upon Salv deletion. Crossing the Salv CKO mice with β-catenin conditional null allele mice to yield Hippo-deficient, β-catenin deficient mouse embryos led to the suppression of Hippo-deficient myocardial overgrowth phenotype in such mice, indicating that Wnt/β-catenin signaling was required for Hippo-deficient cardiomyocyte proliferation and cardiac hypertrophy. These findings taken together suggest that Hippo pathway signaling inhibits Wnt/β-catenin signaling to regulate heart size [153].

## 5.3 Cross-talk in Fibrosis and EMT

*Activation of canonical Wnt signaling is required for TGFβ-mediated fibrosis.* The TGFβ signaling pathway is a critical mediator of myofibroblast induced myocardial fibrosis [62,63] and human tissue samples from diverse fibrotic disease states show enhanced expression of TGFβ[58]. The Wnt/β-catenin canonical signaling pathway is also upregulated in human fibrotic diseases with increased expression of Wnt-1 and Wnt10b, increased nuclear levels of β-catenin , increased transcriptional activity at TCF/LEF WREs and sharply decreased levels of the Wnt inhibitor Dkk1 (60% decline in mRNA, 80% decline in protein) 163. In an example of activation "cross-talk" induced in cultured human dermal fibroblasts, TGFβ signaling stimulated Wnt/β-catenin canonical pathway signaling by decreasing expression of the secreted Wnt antagonist Dkk1 via Smad3/4 canonical pathway p38 dependent mechanism [163]. As further evidence of regulatory TGFβ-Wnt cross-talk mediated by Dkk1 expression in the pathogenesis of fibrosis, Dkk1 transgenic overexpression in mice reduced fibrosis due to constitutive TGFβ type 1 receptor signaling [58].

*β-catenin and TGFβ pathways mediate EMT in a CREB-dependent manner in pulmonary alveolar epithelial cells.* Epithelial-mesenchymal transition (EMT) may serve as the source of α-smooth muscle actin expressing (αSMA) myofiboblasts in fibrotic lung disorders [164]. In cultured pulmonary alveolar epithelial cells (AECs), TGFβ-1 administration resulted in 1) TCF/LEF target gene activation and nuclear β-catenin accumulation, 2) evidence for direct interaction between Smad3 and β-catenin by co-immunoprecipitation and 3) evidence for a Smad3/β-catenin/CREB complex spatially and temporally associated with αSMA expression by chromatin immunopreciptation [164]. Knockdown of Smad3 or knockdown of β-catenin abrogated the effects of TGFβ-1 on α-smooth muscle actin expression in AECs [164]. Taken together, these findings led the investigators to conclude that 1) TGFβ-induced convergence of β-catenin dependent and canonical Smad3 signaling and 2) molecular interactions between Smad3 and the β-catenin/CREB complex are both critical to TGFβ-induced EMT in AECs [164].

*Dkk1 inhibits pericyte to myofibroblast differentiation and TGFβ activated MAPK/JNK cascades in injured kidneys.* Pericytes are the major progenitors of fibrosis-engendered myofibroblasts in the kidney following injury 165. In the injured kidney, the Wnt/β-catenin signaling system is markedly upregulated including the secreted Wnt inhibitor Dkk1 [165]. Dkk1 inhibits pericyte activation and differentiation in to myofibroblasts, resident myofibroblasts and PDGF-, TGFβ-, and CTGF-activated MAPK and JNK signaling pathways via an Wnt/LPR6 interaction mechanism independent of downstream β-catenin signaling [165].

## 5.4 Cross-talk in Cancer

*TGFβ, Wnt/β-catenin and Hippo pathways exhibit cross-talk in colonic epithelial stem cell renewal and cancer.* In mouse intestine, regeneration of crypts of Leiberkühn stem cells following injury was dependent upon the non-canonical Wnt5a ligand mediated by activation of TGFβ signaling [166]. The effects of Wnt5a required kinase activity of the TGFβ type I receptor and was associated with enhanced Smad3 phosphorylation and nuclear localization [166]. TGFβ type 1 receptor kinase inhibitor suppressed all Wnt5a-dependent effects on stem cell regeneration [166]. In a different model,

transgenic overexpression of Yap resulted in unexpected growth suppression and loss of crypts during intestinal regeneration via reducing Wnt target gene expression [167]. The inhibition of Wnt signaling stemmed from Yap inhibition of DVL activity and nuclear translocation and was independent of the Axin-APC-GSK3β complex [167]. Yap silencing was shown in a subset of highly aggressive, undifferentiated human colorectal cancers, leading the investigators to hypothesize that unopposed Wnt signaling led to excessive proliferative signals [167]. In another study, Wnt/β-catenin signaling was linked to increased Yap expression via activating β-catenin/TCF4 complexes binding to YAP enhancers [168]. Decreasing Wnt/β-catenin signaling via shRNAs was shown to decrease both Yap mRNA and protein levels [168].

*β-catenin-driven human colon cancers require Yap1 for survival and tumorigenesis*. Oncogenic Wnt/β-catenin signaling regulates many processes essential for the initiation and progression of malignant transformation of colonic epithelial cells. In 85 human colon cancer cell lines, genome-scale loss-of-function screens demonstrated that β-catenin active cell lines were dependent on a Yap1 transcriptional complex comprised of Yap1, the transcription factor TBX5 and β-catenin [169]. Yap1 phosphorylation by the tyrosine kinase Yes1 was 1) essential for the transforming properties of β-catenin cancers and 2) led to localization of the phosphorylated complex to the anti-apoptotic gene BCL2L1 and BIRC5 promoters where complex-mediated transcriptional activation of the expression of these genes promoted cancer survival [169].

## 6. CROSS-TALK IN ADULT HEART FAILURE

The complex biology of postnatal myocardial adaptive and maladaptive remodeling and transition to failure has multiple genetic, mechanical, paracrine and autocrine mechanistic determinants yet converges on a common phenotype. The postnatal failing heart morphologic phenotype is marked by geometric remodeling, typically ventricular dilation due to increasing intracavitary stress and strain and the adoption of a more spherical ventricular configuration. The postnatal failing heart histologic phenotype is marked by myocyte loss due to necrosis and/or apoptosis, hypertrophy of surviving myocytes and interstitial fibrosis. Since postnatal myocytes possess limited proliferative capacity, the postnatal heart adapts during remodeling primarily by myocyte hypertrophy rather than by myocyte hyperplasia [170]. In postnatal heart failure, myocyte hypertrophy and interstitial fibrosis are driven by cross-talk between multiple signaling systems, including the TGFβ and Wnt/β-catenin systems. To date, convergent cross-talk between the TGFβ, Wnt/β–catenin and Hippo systems has not been systematically studied in postnatal heart failure and much less is known about the role of the Hippo system in postnatal heart failure.

The central role of the Hippo system in regulating developmental cell proliferation and organ size, however, has garnered increasing attention given the limited regenerative capacity of the postnatal heart following injury and during remodeling (Fig. 6). In aggregate, the experimental evidence to date in the postnatal heart supports that 1) the canonical Hippo/Yap pathway is crucial for regulation of myocyte but does not likely promote significant myocyte proliferation in the postnatal heart and that 2) the Hippo pathway kinases MST and LATS play important roles in regulating postnatal myocyte hypertrophic growth [170]. As discussed, Yap regulates myocyte proliferation and cardiac growth by three known mechanisms: 1) physical interaction with β-catenin and stimulation of β-catenin/TCF transcriptional activity, 2) stimulation of the IGF axis and via 3) transcriptional regulation of a number of cell cycle regulators [170]. Yap also promotes cardiac regeneration by activation of the IGF axis and Wnt signaling and Yap deletion results in "default" fibrosis [171]. In animal models, Yap activation post-myocardial infarction results in less myocyte loss and improved contractility [171,172]. Yap mRNA is upregulated in both human hypertrophic cardiomyopathy and in mouse models of ventricular hypertrophy in response to transoaortic constriction [173]. Conversely, Hippo activation inhibits Wnt-signaling and restrains myocyte proliferation and heart size [174] and impedes adult heart regeneration [175]. Proapoptotic Mst1 signaling in cardiac fibroblasts protects against pressure overload hypertrophy in mice [176] and LATS2 is a negative regulator of myocyte size [177,178].

Evolving research is focused on ways to "harness" Hippo/Yap in postnatal heart disease to promote myocyte proliferative growth and myocardial recovery without stimulating adverse myocyte hypertrophy [170].

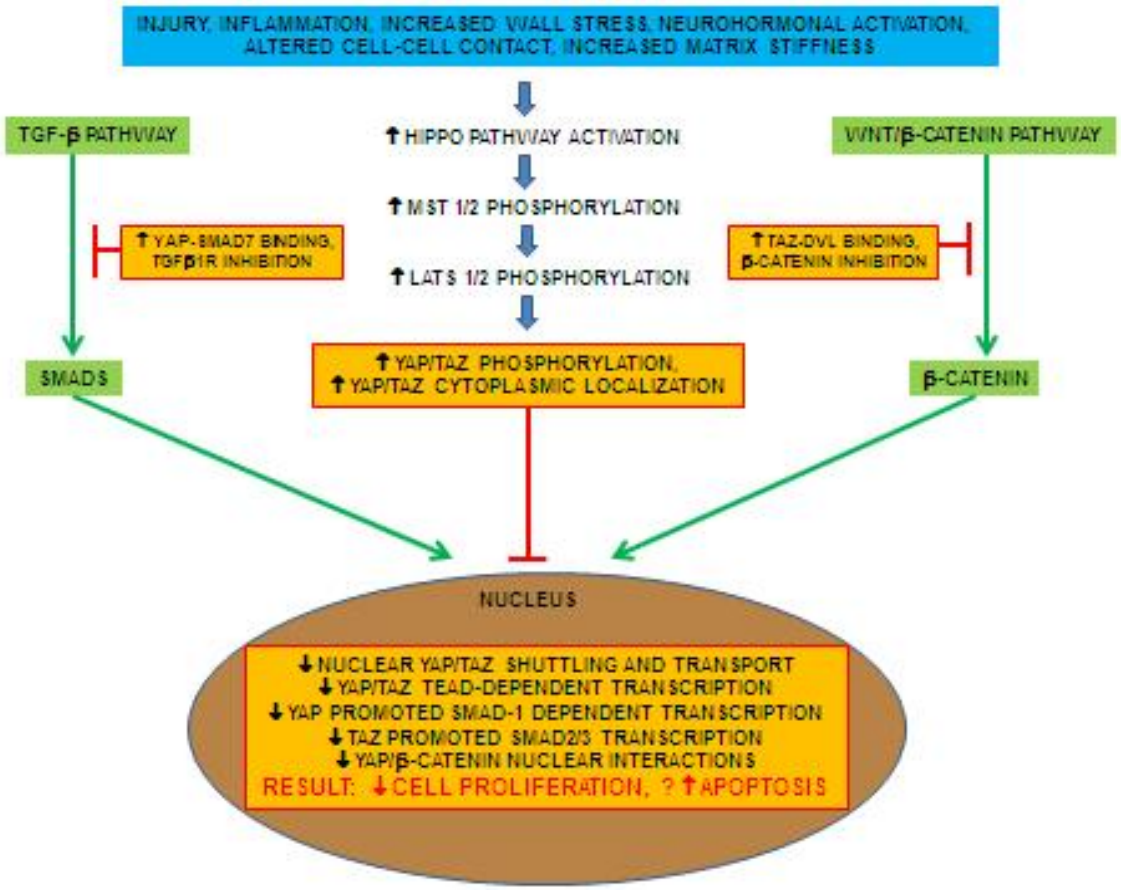


**Fig. 6. Hippo pathway mediated cross-talk in chronic heart failure**

*A variety of factors (top blue box) likely result in Hippo pathway activation in chronic heart failure. Increased YAP/TAZ phosphorylation results in increased cytoplasmic localization of YAP/TAZ and inhibition of some limbs of the TGF-β and WNT/β-catenin signaling pathways (red lines). The reduction of nucleus-localized YAP/TAZ results in a decrease in YAP/TAZ promoted or dependent TEAD, SMAD and β-catenin transcriptional programs and responses.The aggregate net effect may be a reduced proliferative and increased apoptotic response in the face of ongoing injury, inflammation, wall stress, neurohormonal activation, altered cell-cell contract and increased matrix stiffness characteristic of chronic heart failure*

In failing postnatal myocardium, multiple contextual signals and cues likely exist for Hippo pathway activation, including increased cytoskeletal tension, increased matrix rigidity, altered myocyte geometry and altered cell-cell confluence and contact points. In the relatively "hostile" microenvironment of postnatal failing myocardium, Hippo activation and resultant Yap inhibition may adversely modulate important Yap-dependent myocyte proliferative and survival pathways.

## 7. CONCLUSION

While the interactions of the TGFβ superfamily, Wnt/β-catenin and Hippo signaling pathways have not been widely studied in the myocardium to date, the studies reviewed above support likely critical roles for such interactions in both the myocardial homeostatic interactome and the HF diseaseasome [13-17]. Future systems biology approaches will be required to decipher more comprehensively the TGFβ, Wnt/β-catenin and Hippo signaling pathways and identify key HF diseaseasome module hubs, edges and/or nodes as potential therapeutic targets. Until such systems biology approaches mature, HF research intently pitched to the cross-talk between key signaling pathways such as the TGFβ, Wnt/β-catenin and Hippo pathways is likely to deepen the mechanistic understanding of the HF diseaseasome.

**Table 1. TGFβ superfamily ligand, receptor and smad associated components**

| Component | BMP | GDF | Activin | TGFβ | AMH | Inhibitors |
|---|---|---|---|---|---|---|
| **Ligand** | BMP2, 4<br>BMP5, 6, 7<br>BMP8A, 8B<br>BMP 9, 10 | GDF5, 6, 7<br>GDF9b<br>GDF 10, 11<br>GDF 15 (MIC 1)<br>----------<br>GFD 1, 3<br>GFD 8 (MYO) | Inhibin βA<br>Inhibiin βB<br>Nodal | TGFβ1<br>TGFβ2<br>TGFβ3 | AMH (MIS) | BMP3<br>Inhibin α<br>Inhibin βC<br>Inhibin βE<br>LEFTY A<br>LEFTY B |
| **Receptor II** | BMPRII<br>ActRIIA/IIB | BMPRII<br>ActRIIA/IIB | ActRIIA<br>ActRIIB | TβRII | AMHRII | N/A |
| **Receptor I** | BMPRIA (ALK3)<br>BMPRIB (ALK6)<br>ALK 2<br>ALK 1 | BMPRIA (ALK3)<br>BMPRIB (ALK6)<br>ALK 2<br>----------<br>ActRIb (ALK4)<br>ALK7<br>TβRI (ALK5) | ActRIB (ALK4) | TβRI (ALK5)<br>----------<br>ALK1<br>ALK2<br>BMPRIA (ALK3) | BMPRIA (ALK3)<br>BMPRiB (ALK6)<br>ALK2 | |
| **Receptor III** | RGMa, b, c (+) | Cripto 3 (+) | Cripto 3 (-)<br>Cripto 1 (+) | TβRII (+)<br>Endoglin (+)<br>Cripto 3 (-) | ? | TβRII (-)<br>Cripto 3 (-) |
| **R-SMAD** | SMAD 1, 5, 8 | SMAD 1, 5, 8<br>---------- | SMAD 2,3 | SMAD 2, 3<br>---------- | SMAD 1, 5, 8 | N/A |
| **Co-SMAD** | SMAD 4 | SMAD 4 | SMAD4 | SMAD 4 | SMAD4 | N/A |
| **I-SMAD** | SMAD6, 7 | SMAD 6, 7 | SMAD 7 | SMAD7 | SMAD6, 7 | N/A |

*BMP = bone morphogenic protein; GDF = growth and differentiation factors; TGFb = transforming growth factor beta; AMH = Anti-Mullerian Hormone; Receptor II = type II receptor; receptor 1 = type I receptor; receptor III = co-receptors; MYO = myostatin; MIC1 = macrophage inhibitory cytokine 1; Cripto 1 = TDGF1; Cripto 3 = TDGF3; in Receptor III row, (+) = "positive" effects and (-) + "negative" effects, respectively, on signaling by each co-receptor. Dashed lines separate groups of ligands or receptors based on the division into BMP and TGFβ/activin-like pathways. Ligands, type I receptors and R-SMADS are color-coded: blue, BMP-like pathways; red, TGFβ/activin-like pathways, Table is reprinted with permission from reference [45]*

**Table 2. TCF transcriptional repression of Wnt targets in absence of nuclear β-catenin [118]**

| **Balance between TCF repression and activation is not the same in all Wnt targets** |
|---|
| -WRE cis-regulatory elements contain information besides TCF-binding sites that control expression<br>-TCF repression may be the dominant determination of expression levels in some WREs, and β-catenin may relieve TCF-mediated repression to permit non-b-catenin factors to activate expression<br>-Likely both TCF mediated activation and repression are critical to regulating the full repertoire of β-catenin targets |
| **Factors contributing to TCF repression** |
| *Factors binding to TCF*<br>-Gro/TLEs act as TCF co-repressors and promote HDAC interactions and chromatin-silencing histone deacetylation<br>-Gro/TLEs and β-catenin compete for TCF binding, promoting transcriptional activation and repression, respectively<br>-Proteins analagous to Gro/TLEs: Coop, MTG family members (which bind HDACs), Hic-5<br>-Proteins preventing TCF to bind DNA: Osterix<br>-Proteins binding to TCF and b-catenin simultaneously: NCoR, SMRT<br>-Proteins binding to b-catenin alone and prevent TCF-b-catenin complex formation: Reptin, TIS7<br><br>*WRE-bound factors acting in parallel to TCFs* |

-Factors maintaining low target gene expression in absence of b-catenin: Kaiso (inhibited in turn by p120-catenin)
-Factors which recruit TCF4 and b-catenin away from target gene chromatin: CtBP and H1C1 proteins
-Factors that repress TCF targets genes: various HDACs (a recurring theme in TCF repression)

*TCF-β-catenin buffers*
-Competitors with b-catenin for TCF binding: Gro/TLE, Mtgr-1, Coop
-TCF acetylators: CBP (a HAT enzyme)
-β-catenin C-terminal binding proteins which inhibit β-catenin TCF binding: Chibby, ICAT
-β-catenin binding proteins which limit β-catenin access to TCF and/or promote β-catenin degradation: Sox9

**Factors contributing to TCF-b-catenin transcriptional activation**

*Factors facilitating β-catenin binding to TCF*
-Proteins promoting β-catenin binding to TCF N-terminal: TBL1, TBLR1
-Proteins determining β-catenin N-terminal domain transactivation: 1) Bcl9/Bcl9-2 proteins interact with Pygo proteins to form a Bcl9-Pygo complex which is recruited to TCF-β-catenin complexes on WRE chromatin in "chain of adaptors" activation model as NHD of Pygo in turn interacts with several factors and complexes such as Med12 and Med13 Mediator complex subunits bridging transcription factors and RNA polymerase II; 2) TAF4, a TFIID subunit, interacts with Pygo and is required for activation of Wnt target; 3) PHD Pygo domain can bind mono-, di-, trimethylated H3K4 and binding is allosterically enhanced by HD1 Bcl9 domain; 4) Pygo and Bcl9 may promote high nuclear levels of β-catenin
-Proteins determining β-catenin C-terminal domain transactivation: 1) CBP and p300 (both HATs) bind β-catenin C-terminal and also catalyze histone H3/H4 acetylation associated with transcriptional activation in a WRE-restricted manner; 2) other co-activator proteins interacting with b-catenin and p300/CBP to activate target genes include GRIP1, CARM1, CoCoA; 3) MLL2 histone methyltransferase ; 4) Brm and Brg-1 (ATPase subunits of SWI/SNF chromatin remodeling complex) promote β-catenin target effects; 5) TERT associates with Brg-1 and is required for maximal b-catenin target activation; 6) ISWI (chromatin-remodeling ATPase).

*Interactions between β-catenin transactivation domains*
-Mechanisms not well understood; the same co-regulators may participate in either activation or repression in a context-dependent manner; same factors can promote different transcriptional outputs due likely to chromatin state of target gene and/or presence and effects of additional WRE chromatin co-regulatory factors

*Role of chromatin modifications in activating β-catenin targets*
-HATs, HDACs and histone methyltransferases may also modify nonhistone proteins: 1) CBP and PCAF acetylate b-catenin which increases b-catenin stability and affinity for TCFs, 2) FHL2 binds to b-catenin and p300 potentiating b-catenin acetylation and b-catenin induced transcriptional activation, 3) b-catenin activation-dependent increase in H3 and H4 acetylation described at many WREs, especially H3K4me3 (perhaps due to MLL2), H4K30me, H3R17 methylation by CARM1, H3K79me2/me3 methylation by DOT1L (H3K79me3 may be the most crucial DOT1L-mediated chromatin mark promoting b-catenin transcription)

**Variations of the TCF transcriptional switch**

*Wnt/b-catenin asymmetry signaling*
Wnt signaling stimulated TAK1 and NLK activity in mammalian cells, which results in 2) down-regulation of nuclear POP-1 (NLK mediated POP-1 phosphorylation promotes pPOP-1 nuclear export) and promotes the stabilization and nuclear accumulation of SYS-1, both of which are required for Wnt target gene activation in the asymmetry developmental pathway and 2) NLK-mediated phosphorylation of TCFs, which leads to impaired TCF DNA binding ability and/or TCF ubiquitylation and degradation

*Wnt-dependent "TCF" exchange*
Mammals have four TCF genes (TCF1, 3, 4 and LEF1) with multiple isoforms with variable regulatory roles (e.g., TCF 1 and TCF 4 is typically activating but may also be repressive, TCF3 isoforms are repressive) such that different TCF isoforms occupying different WREs and/or exchanged at the same WREs enrich the Wnt target gene regulatory repertoire

*Reverse transcriptional switch for targets repressed by Wnt signaling*
Although TCF-b-catenin typically results in transcriptional activation of target genes, transcriptional repression may also result and some target genes may be regulated by a "reverse switch", whereby TCF

activates expression in the absence of Wnt/β-catenin signaling but TCF-β-catenin represses expression in the presence of Wnt upstream signaling.

## CONSENT

Not applicable.

## ETHICAL CONSIDERATIONS

Not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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