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Survey

Few Smad proteins and many Smad-interacting proteins yield multiple functions and action modes in TGFβ/BMP signaling *in vivo*

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Abbreviations: Alk, activin receptor-like kinase; CPSF, cleavage polyadenylation specificity complex; HD, Hirschsprung disease; MR, mental retardation; PAH, pulmonary arterial hypertension; PD, Parkinson disease; SIP, Smad-interacting protein; Smicl, Smad-interacting CPSF-like protein; Tdp, Tyrosyl DNA phosphodiesterase; TF, transcription factor; Ttrap, TNF receptor and Traf-associated protein.*Keywords:* CPSF; Sip1; Smad; Tdp2; Transforming growth factor β.

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ABSTRACT

Signaling by the many ligands of the TGFβ family strongly converges towards only five receptor-activated, intracellular Smad proteins, which fall into two classes i.e. Smad2/3 and Smad1/5/8, respectively. These Smads bind to a surprisingly high number of Smad-interacting proteins (SIPs), many of which are transcription factors (TFs) that co-operate in Smad-controlled target gene transcription in a cell type and context specific manner. A combination of functional analyses *in vivo* as well as in cell cultures and biochemical studies has revealed the enormous versatility of the Smad proteins. Smads and their SIPs regulate diverse molecular and cellular processes and are also directly relevant to development and disease. In this survey, we selected appropriate examples on the BMP-Smads, with emphasis on Smad1 and Smad5, and on a number of SIPs, i.e. the CPSF subunit Smicl, Ttrap (Tdp2) and Sip1 (Zeb2, Zfhx1b) from our own research carried out in three different vertebrate models.

Contents

1. Strong convergence of TGFβ family signaling towards Smad proteins

1.1. General principles of Smad signaling

Ligands of the transforming growth factor type β (TGFβ), encoded by 33 genes in human, signal via a complex of transmembrane receptors with serine–threonine kinase activity that are composed of type I (7 in total; in the field still often referred to as Alks, activin receptor-like kinases) and type II (5 in total) receptors, which activate Smad and non-Smad intracellular signal transduction pathways (Fig. 1) [1–3]. The non-Smad signaling cascades have in many cases not been demonstrated as strictly Smad-independent. The activation, and the specificity thereof, of the few (5 in total) receptor-activated Smads (R-Smads) is executed by the type I receptors in liganded receptor complexes. The R-Smads fall into two classes: Smad2/3 are known to signal TGFβ/Activin/Nodal activity and are activated by Alk4, Alk5 and Alk7 containing receptor complexes, and Smad1/5/8 activated by Alk2, Alk3 and Alk6 do this for bone morphogenetic proteins (BMPs)/growth differentiation factors (GDFs). In addition, two other Smads, referred to as the inhibitory Smads (Smad6/7, I-Smads), use different action mechanisms for – in their case – negative regulation of receptor/R-Smad signaling and are no substrates for the kinase activity of the liganded receptors [4,5].

For many of the available pure and bio-active ligands the binding receptors have been identified either in cells over-producing type I and/or type II receptor combinations or in cells with endogenous levels of receptors, combined in most cases with the downstream activation of one of the two R-Smad classes. This work has led to a complex binding pattern and variable affinities, with many of the ligands being able to bind to many receptors [2]. The BMPs are known to bind to 4 of the 7 type I receptors, and to 3 of the 5 type II receptors. Interestingly, in endothelial cells TGFβ when bound to an Alk1-TβRII complex, and the circulating ligand BMP9 when bound to an Alk1-BMPRII complex, both activate the BMP-Smads Smad1/5/8 [6–10]. Depending on the ligand–receptor combinations different modes of ligand–receptor contacts and of the assembly of receptor complexes have been proposed, mainly following a combination of studies involving structural as well as cell biology [11,12]. Also endocytosis of liganded receptor complexes through different routes, which are insufficiently characterized still for the many ligand-receptor combinations, is accepted to contribute to spatial-temporal regulation of the signaling. It likely also contributes to the specificity of the ultimate Smad-driven transcriptional response in the nucleus, and perhaps even the coupling of Smad with non-Smad signaling in the cytoplasm, and thus serves more than the mere degradation of internalized receptors [13,14].

Both classes of activated R-Smad accumulate in the nucleus as a complex with Smad4. In the case of transcriptional regulation of direct target genes, R-Smads mainly do this by low-affinity binding to Smad-binding elements (SBEs) in the proximal 5′ regulatory regions of the target genes in co-operation with a long list of DNA-binding SIP-TFs and their own co-factors. Several groups have investigated the target DNA sequence for R-Smads. An SBE (the 8 bp-long palindrome sequence 5′-GTCTAGAC-3′) was identified in a random screening as a consensus binding sequence for Smad3 and Smad4 [15]. Characterization of the *PAI-1* promoter, a known target gene for TGFβ, revealed 5′-AG$^{C}/_{A}$CAGACA-3′ as a direct

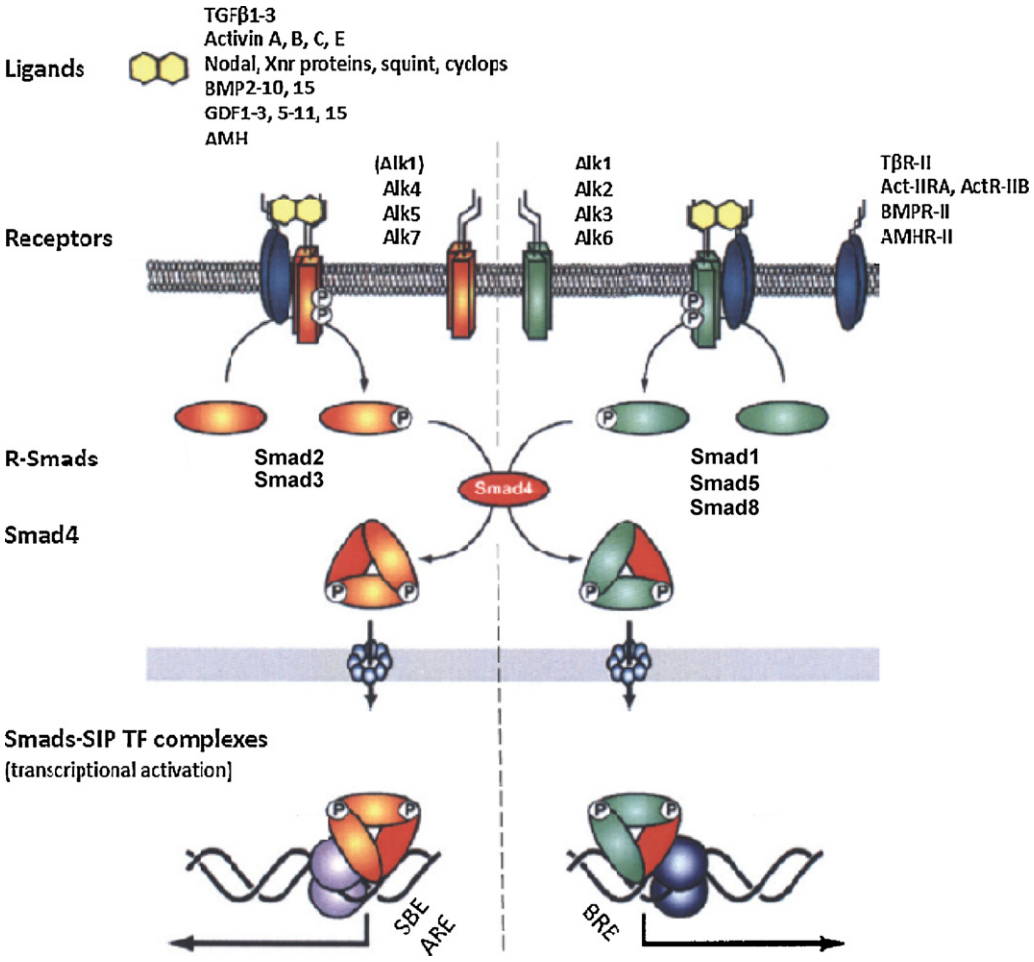

Fig. 1. General principles of Smad signaling. With the exception of the Lefty ligands, dimers of the mature ligands (only those for which receptor binding has been documented in the literature are shown here) bind at the cell surface to tetrameric receptor complexes composed of two type I (Alk) and two type II receptors. The activated serine-threonine kinase activity of the type I receptor activates one of two classes of latent R-Smad, which then bind to Smad4. This results in a net accumulation of active Smad complexes in the nucleus, where they participate in transcriptional regulation by teaming up with Smad-interacting DNA-binding transcription factors and co-factors of the latter (only transcriptional activation is shown here). Note that neither non-Smad signaling nor additional regulations (by ligand-binding proteins, co-receptors, trafficking, post-translational modification; for details, see text) are shown here (modified from CS Hill).

binding site for Smad3 and Smad4 [16]. These two sequences have the "CAGA" sequence in common, which is also found in many other acknowledged direct TGFβ-responsive genes. Smad1 has also been shown to bind weakly to this sequence. The binding of R-Smads to these sequences is relatively weak ($K_d = 1.14 \times 10^{-7}$ M for Smad3 and Smad4, and $K_d = 4.9 \times 10^{-7}$ M for Smad1), and hence multiple copies of SBEs are required for efficient transcriptional activation of SBE-based promoter-reporter plasmids [15]. This was one of the indications that interaction with other DNA-binding proteins would be necessary for stabilizing the interaction of R-Smads with DNA. In addition, BMP-Smads were subsequently found to bind preferentially GC-rich sequences, i.e. GCCGNC or GRCGNC, which are found in known BMP-regulated direct target genes like *Smad6, Id1* and *Msx2* [17–19]. A BMP-responsive element (BRE) was isolated from the *Id1* promoter and was shown in a reporter assay to be responsive to BMP, but not to TGFβ and activin [20].

One of the first examples of a co-activator for R-Smad proteins in the nucleus is P300/CBP, which contains histone acetyltransferase activity (HAT). Acetylation of histones diminishes the chromosome condensation, which releases the DNA from the tight chromatin structure, and renders the DNA accessible to TFs. P300/CBP directly binds to R-Smads via their MH2 domain and enhances transcriptional activation by TGFβ/BMP signaling [21]. Smad4 itself can act as a key co-activator of ligand-dependent transcription by stabilizing the interaction of the R-Smads with DNA and P300/CBP [22,23]. Of course, many of the meanwhile identified SIP–TFs function as subunits of larger complexes as well, including chromatin remodeling complexes, and also enhancer-based long-range control of target genes for Smad, SIP and Smad–SIP complexes accompanied by chromosome conformational changes remains to be thoroughly investigated in the field.

1.2. Regulation of TGFβ family signaling at different levels of the pathway

While the signal transduction towards Smad activation is fairly straightforward and convergent, the entire signaling system itself is tightly regulated at multiple levels of the pathway (for reviews, see [24–27]) other than by endocytosis and I-Smads already mentioned above (Section 1.1). For example, the bio-activity of nearly all ligands is dependent on their protease (mainly furin) based processing of the precursor polypeptide to the mature factor. Many of the ligands bind to extracellular matrix (ECM) proteins. ECM interaction with cells is controlled by TGFβ family signaling also, as the transcription of ECM-encoding genes, of genes encoding their proteases or receptors (integrin receptor chains) is also subject to ligands and regulated by their downstream Smads. In addition, a large, diverse, and still growing group of highly specific ligand-binding secreted proteins, some of which are

degraded by specific proteases, prevents the binding of ligands (mainly BMPs) to their receptor ectodomains.

Additional fine-tuning of signaling is achieved through incorporation into the receptor complex of a pseudo-receptor like the Alk2-like membrane protein Bambi and/or a growing list of non-signaling co-receptors. Ubiquitination coupled to proteasome-mediated degradation, as well as regulated nucleo-cytoplasmic shuttling and various post-translational modifications in Smad proteins (including phosphorylation and acetylation of Smads) further control the Smad pathway. Smads also link to molecular processes other than mere target gene transcription, for mainly through the work on SIPs novel activities of Smads have been identified in other processes. For example, Smads bind to proteins that are associated with the inner nuclear lamina [28,29], and they are candidate direct regulators of miRNA biogenesis [30]. They also influence the activity of cleavage-polyadenylation specificity factor (CPSF) complexes, which couple transcription induction to maturation of pre-mRNAs at the 3′-end, by binding to some of their subunits (like the SIPs CPSF-30 and Smicl) in ligand-activated cells (see Section 3.2).

2. Functional analysis of BMP-Smads using knockout mice

2.1. Novel lessons from BMP-Smad knockout mice

Seen the critical functions of BMP2/4 signaling in embryogenesis, the respective ubiquitous knockout mice for these BMPs (with several mutant and floxed alleles available, as well as studies performed in different genetic backgrounds), but also their receptors (Alk3, BmprII) and BMP-Smads (Smad1, Smad5) are early embryonic lethal. Homozygous null *Bmp2* mutants die at embryonic day (E) 7.5–9 with failure of the proamniotic canal to close, and display abnormal development of the heart in the exocoelomic cavity [31]. *Bmp4* is the most widely and extremely dynamically expressed *Bmp* gene throughout development. Ubiquitous inactivation of *Bmp4* resulted in two major extra-embryonic defects during gastrulation, i.e. a reduction in extra-embryonic mesoderm typified by a lack of or a very small allantois, and a complete lack of primordial germ cells (PGCs). Additionally, *Bmp4* mutants display reduced proliferation of the epiblast, resulting in a retarded growth and vestigial mesoderm differentiation [32,33].

Regarding the two key BMP receptors in early mouse embryos, *Alk3/BmprIa* knockout mutants die by E9.5, are smaller than normal, and form no mesoderm [34], while *BmprII* knockouts arrest at the egg cylinder stage, die before E9.5, with failure to form any organized structures and lacking mesoderm [35]. This necessitates the use of conditional strategies to study their function in later stages of development and in adult mice. In addition, many developing organs, like the mouse heart at mid-gestation, express multiple Bmp genes of the large Bmp subgroup. This shifted the developed set of knockout models towards single or combined conditional knockouts for the (still fewer) receptors, the few BMP-Smads or – in a number of cases – Smad4. The targeting of Smad4 of course also affects Smad2/3 signaling.

Smad8^{ex2,3} knockout mice are viable [36], so most embryology studies focus on single knockout mice for *Smad1* and *Smad5* and, more recently, *Smad1;Smad5* compound knockout mice, including double homozygous (“full”) knockouts, either as such or even in a Smad8 knockout background. The genetic inactivation of *Smad1* or *Smad5* in mice results in embryonic lethality around mid-gestation due to several embryonic and extraembryonic defects that include cardiovascular malformations. *Smad5*^{ex2} and *Smad5*^{ex6} knockout mouse embryos display identical phenotypes [37,38]. These mice die between E9.5 and E11.5 and develop defects already at E8.0 in the amnion, gut and heart. Later, these embryos have defects in heart looping and embryonic turning, defects of which are the first signs of left-right asymmetry defects in mice. After E9.0, the yolk sac of the mutant embryos contains red blood cells but fails to develop a robust vasculature. Within the embryo, the blood vessels are enlarged and surrounded by lower numbers of vascular smooth muscle cells. The endothelium-specific inactivation (using a Tie2-Cre approach) of *Smad5* results in normal and viable animals, which suggests that Smad1 functionally compensates for Smad5 absence in angiogenic endothelium [39].

Similar to *Smad5* knockout mouse embryos, *Smad1*^{ex1} or *Smad1*^{ex3} knockouts die from E10.5 onwards [40–42]. These mutant embryos pattern normally but exhibit pronounced defects in morphogenesis and proliferation of extra-embryonic tissues, leading to a dramatic reduction in the size of the allantois and the concomitant failure to form a proper umbilical cord and placenta, and they fail to establish a definitive embryonic blood circulation. In addition, they display a marked reduction in the number of PGCs and a defect in left-right asymmetry which upon further study, using a conditional Mesp1-Cre approach, reveals the repressive role of BMP-Smad signaling in Nodal auto-activation in the lateral plate mesoderm, suggested to occur by competition for Smad4, which has been proposed to become limiting [43]. The relative late onset of *Smad1* and *Smad5* mutant phenotypes in comparison with those observed for *Bmp2* and *Bmp4* conventional knockouts, suggests that Smad1 and Smad5 share interchangeable roles as transcriptional modulators of BMP target genes. This is supported by their very strong amino acid sequence conservation and shared biochemical activities in cell culture. This is also further demonstrated by the fact that, although *Smad1*$^{+/-}$ and *Smad5*$^{+/-}$ mice are each viable and fertile, the double m*Smad1*$^{+/-}$;*Smad5*$^{+/-}$ mutant embryos die around E10.5 and display defects in allantois morphogenesis, cardiac looping and PGC specification [36].

The selected results demonstrate that Smad1 and Smad5 may function co-operatively to govern certain BMP-dependent processes, at least in development until midgestation. However, only the *Smad5* (and not the *Smad1*) deficient amnion develops a specific defect. Indeed, at early somite stages the *Smad5* mutant amnion thickens at the anterior side of the embryo, displays ectopic haematopoiesis and vasculogenesis, and develops *de novo* ectopic Oct4 and alkaline phosphatase positive cells resembling PGCs, a cell type normally present only at the posterior side of the embryo where BMP signaling occurs [37,44]. Recent investigation of this amniotic thickening indicates that in the absence of Smad5 the mechanisms that normally drive primitive streak formation, which are two positive feedback loops that are active only in the posterior part of the epiblast (Fig. 2), now become ectopically activated at the anterior side of the Smad5 mutant embryo (Pereira et al., unpublished results). Surprisingly, the underlying mechanism of the defect in the mutant mice is not the alteration of the expression levels of the antagonists of Nodal, which operate at the anterior side in wild-type embryos, for their mRNA levels remain unchanged in the *Smad5* mutant mice. Rather – as based on experiments in cultured cells exposed to a combination of Nodal and BMP – activated Smad5 can form unconventional complexes of activated Smad2–Smad5, which antagonize Nodal signaling by interfering with the previously identified active Nodal–Smad2/4–FoxH1 pathway [43].

This work, which started from the conventional *Smad5* knockout mouse embryo [37], represents a new and intracellular antagonistic mechanism that in this case prevents ectopic primitive streak formation in the mouse embryo. Thus, removal of *Smad5* results in ectopic (i.e. anterior) signaling of Nodal, which induces its two Nodal-supported positive feedback loops (the fast autoregulatory one, and the slower one which runs over Bmp and Wnt; see [45,46]). Ultimately, an ectopic primitive streak is formed in an extra-embryonic tissue. Unconventional Smad complexes (i.e. phospho-Smad1 with phospho-Smad2) have been

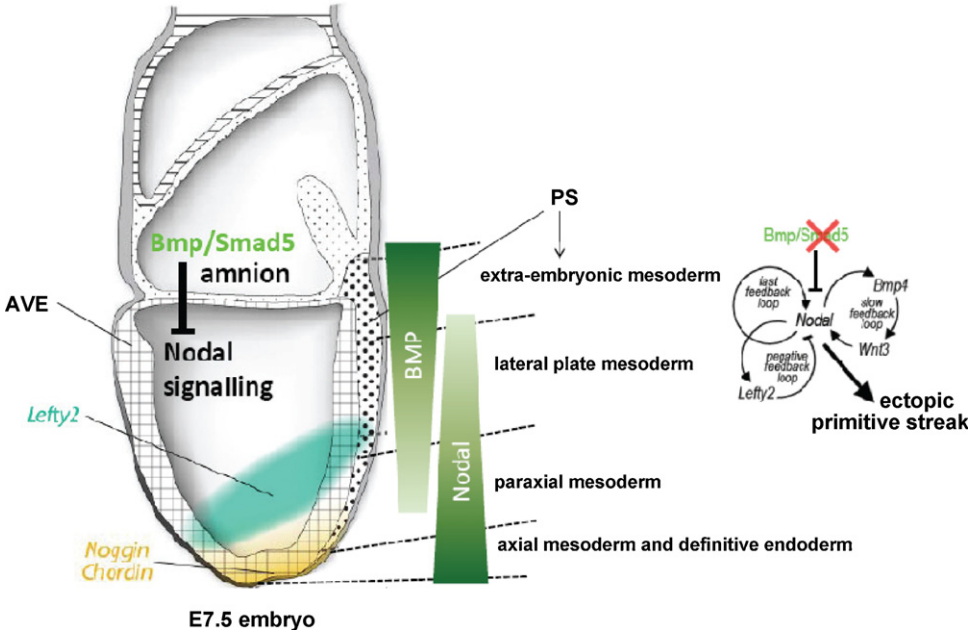

Fig. 2. Smad5 signaling as a mechanism to prevent ectopic streak formation. establish the antero-posterior axis by preventing ectopic primitive streak formation. Active Nodal and Bmp4 signaling in the embryo result in the induction of two positive feedback loops for *Nodal* expression at E6.5: a fast autoregulatory loop and a slow positive feedback loop [45,46]. These feedback loops are crucial for primitive streak induction in the posterior part of the epiblast. The slow feedback loop involves the expression and signaling of Bmp4 and Wnt3. The eventual allocation of the anterior visceral endoderm (AVE) to the prospective anterior side of the embryo is a well known event that is key to the establishment of the antero-posterior axis of the embryo. The AVE determines the anterior side by secreting antagonists of Nodal, Bmp and Wnt, thereby restricting the activity of these to the prospective posterior side for primitive streak (PS) induction. The putative patterns of graded Nodal and Bmp signaling in the streak at E7.5 are shown schematically, with the width of each triangle indicating the strength of the signaling activity. Lefty2, and Noggin and Chordin, are proteins that are locally produced in the embryo and help shaping the gradients of activity of Nodal and Bmp, respectively. At this stage, Nodal and Bmp are mainly involved in mesoderm/endoderm patterning. In the Smad5 knockout mouse an ectopic primitive streak is induced in the amnion, an extra-embryonic membrane that separates embryonic from extra-embryonic tissues. Based on experiments in transfected cultured 293 cells exposed to both BMP and Nodal we identified a new anti-Nodal role of phospho-Smad5. We propose that this occurs via the formation of mixed complexes between phospho-Smad5 and phospho-Smad2 thus preventing phospho-Smad2 from Nodal-activated Smad2–SIP (i.e. FoxH1; [43]) complexes. In Smad5 mutants, a lack of this anti-Nodal activity in amnion results in excess Nodal signaling and, as a result, ectopic primitive streak formation (Pereira et al., unpublished).

documented before in one study in cell culture. Indeed, TGFβ in epithelial cells can also bind to receptor complexes that contain Alk5 and Alk2 and/or Alk3, which leads to activation of the BMP–Smads. It was demonstrated that activated BMP–Smads can form a 'mixed' complex with activated TGFβ–Smads in epithelial cells [47]. Like for the proposed phospho-Smad5 with phospho-Smad2 complex, these remain to be demonstrated to occur *in vivo* at physiological levels.

Work with knockout mice has also significantly contributed to the notion that BMP signaling also plays important roles in soft tissues in embryogenesis and in adult mice, and deficiencies in BMP production or signal interpretation link directly to various human diseases. In addition to the impact of aberrant BMP signaling causing e.g. bone density diseases [29,48–51], and vascular diseases [8–10,52–56], exciting new BMP biology is for example emerging in cancer (e.g. gliomas in brain, but also ovarian (see Section 2.2 below), gastric and oesophagal cancer: for a review, see [57]), cardiac morphogenesis and possibly post-natal cardiac physiology and pathology [58–60], regulation of adult neurogenesis [61,62], and (negative) regulation by BMPs of repair of the central nervous system including cerebral ischemia and spinal cord [63,64], of (re)myelination [65–67], and of adult skeletal muscle repair [68–70]. S. Pangas and M. Matzuk (Houston) demonstrated that the removal of *Smad1;Smad5* from urogenital mesenchyme during embryogenesis (using an AmhrII-Cre based approach) yields adult mice. In the case of adult knockout females they develop granulosa cell derived, mostly unilateral ovarian tumors indicating that these BMP-Smads are candidate tumor suppressor genes [71,72]. A very interesting new aspect of BMP biology is based on the activities of the circulating BMP9 on endothelial cells of blood vessels (see Section 1.1) and the fact that pulmonary arterial hypertension (PAH) is caused by mutations in *BMPR-II*, while *SMAD8* mutations have been linked since then to PAH as well [55,73–75].

Conditional *Smad1;Smad5* double knockout mice (see also Table 1) were recently used in the Zwijsen team for studying the role of these BMP–Smads in endothelium in the embryo (using a Tie2-Cre approach). Similar to previous work by others studying the effect of combined *Smad2;Smad3* mutations in mesoderm formation in the mouse [76], a dose-dependent phenotype is seen in these *Smad1;Smad5* mutant mice in angiogenesis (Moya et al., unpublished results). Most importantly, subsequent analysis of the growing blood vessels in the endothelium specific "full" double knockouts, combined with RNAi-based knockdown of Smad1/5 in cultured HUVECs, indicated a role of BMP–Smad activation, in addition to the well established role of Dll4-Notch signaling, to discriminate tip cells from stalk cells selection in angiogenic blood vessels in the embryo [77] (Fig. 3). Leading tip cells in angiogenic sprouts exposed to gradients of VEGF are selected and instruct the adjacent cells to become stalk cells via Dll4-Notch mediated lateral inhibition, a principle known from patterning during neurogenesis in e.g. the neural plate of the early vertebrate embryo. However, the conditional knockout of *Smad1;Smad5* resulted in impaired Dll4-Notch signaling, shifting the tip-stalk cell balance towards increased number of tip cell-like cells at the expense of stalk cells. The results point at an important role of BMP-activated Smad1/5 proteins, via the BMP-induced *Id* genes-encoded proteins, in the Notch-regulated expression of stalk cell enriched transcripts. Most importantly, these recent findings strongly suggest that BMPs, by virtue of Smad1/5, co-orchestrate with Notch signaling in a direct manner tip versus stalk cell specification and hence provide vessel plasticity as well.

Table 1
Published Smad1/5/8 knockout mouse models.

		References
Ubiquitous homozygous knockout		
Smad1		[40,41,127]
Smad5		[37,38,128]
Smad8 (in gene databases present as Smad9)		[36,129,130]
Heterozygous knockout		
Smad5		[131]
Smad1;Smad5		[36]
Conditional (compound) knockouts for Smad1/Smad5/Smad8		
Cardiovascular development	Tie2-Cre, Sm22-Cre	[39; Moya et al., unpublished]
Digestive system	Villin-Cre	[132,133]
Eye development	Le-Cre	[134,135]
Haematopoietic system	Mx-Cre, Vav-Cre	[133,136]
Lung development	SPC-rtTA/TetO-Cre	[137]
Reproductive system	Amh2-Cre	[71,72,78]
Skeleton	Col2a1-Cre, Col1a1-Cre	[138,139]

Taken together, the two selected examples from the recent work in *Smad1/5* mutant mouse models indicate the need to (re)investigate in-depth the regulatory mechanisms by which BMP signaling interferes with signaling by other ligands of the TGFβ family and other signaling pathways, respectively, preferably *in vivo* in normal processes first. Indeed, we feel that disease contexts either in human or animal models are inevitably even more complex for such studies at this stage. In addition, this type of work also identifies new roles of BMP and BMP-Smad signaling *in vivo*, in these cases anterior development and sprouting angiogenesis, respectively. The question will be whether similar regulatory functions and underlying molecular mechanisms, which regulate cell specification and cell activities, operate in other sites of the embryo or tissues/organs of the adult animal. In addition, the BMP signaling system connects at the same time to human disease but also to normal repair processes initiated by (re)activation of resident progenitor/stem cells.

2.2. Phenocopying between conditional BMP receptor and BMP-Smad knockout mice

Genetic analysis in knockout mouse models that eliminate BMP receptors (or combinations thereof) and comparison with the phenotypes of knockout mouse models for BMP ligands or the BMP–Smads (in particular *Smad1;Smad5*) have largely confirmed the preceding biochemical studies addressing the specificity of ligand–receptor interaction and downstream Smad activation to operate *in vivo*. A key and first example in the BMP field was the

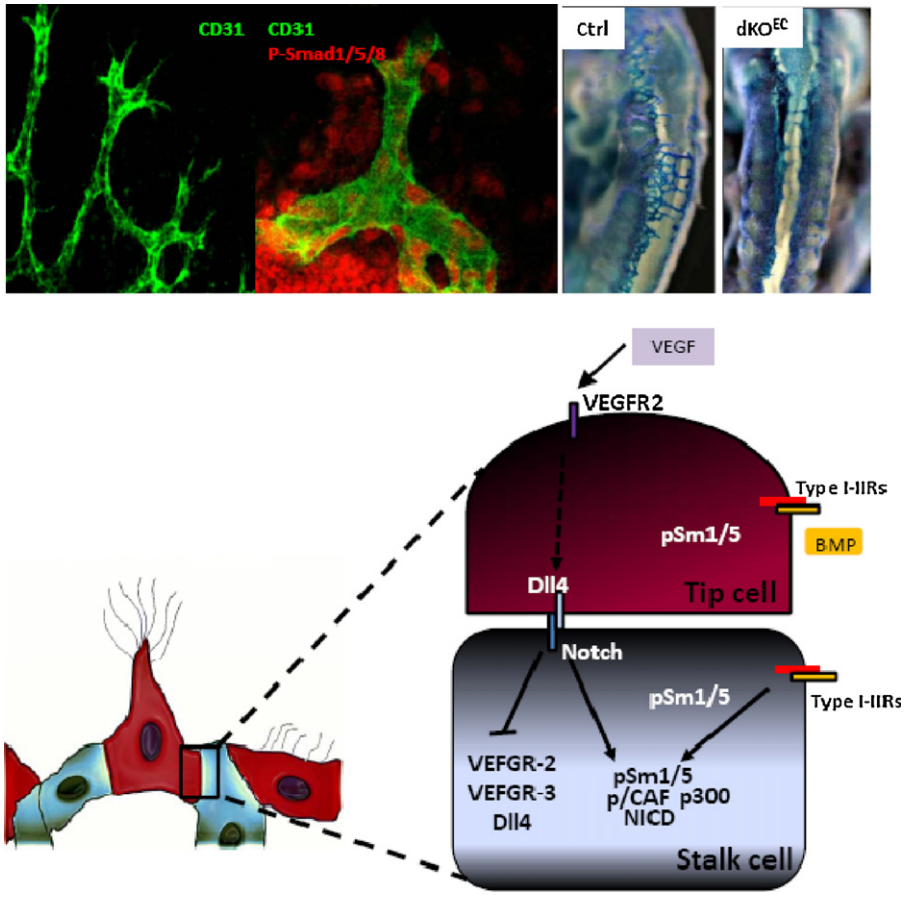

Fig. 3. Smad1and Smad5 mediated BMP signaling in embryonic vasculature. At midgestation the vasculature is expanded largely by sprouting angiogenesis, a process whereby new sprouts form from existing vessels. Selected endothelial 'tip' cells, or leader cells, guide each new sprout to areas with insufficient oxygen. The tip cells scan the environment with their multiple filopodia. The 'stalk' or following cells divide to provide new cells for tube extension and form the vascular lumen of the new vessel. The selection of tip and stalk cells is regulated by VEGF and Notch signaling levels [145]. Ubiquitous nuclear phospho-Smad1/5/8 localization demonstrates that tip and stalk cell undergo Smad1/5/8 mediated BMP signaling in endothelium in midgestation mouse embryos. Perturbed angiogenic sprouting in endothelium-specific R26R;Smad;Smad5 double knockout embryos (dKOEC) at E9.5. Fewer sprouts anastomose at the dorsal midline in mutants, and the capillaries form sinusoid-like vessels instead of a normal ramified network of capillaries. Based on experiments in HUVECs using Smad1/5-siRNA mediated knockdown, or overproduction of Id, NICD or Hes1 and the genetic mouse model, we propose an important role for Smad1/5 in the specification of stalk cell fate. Our results put forward that Smad1/5 mediated signaling and Notch signaling converge in stalk cells and affect target gene expression of both pathways. Our findings provide the first *in vivo* evidence for a regulatory loop between Smad1/5 and Notch signaling that orchestrates tip versus stalk cell specification.

ubiquitous inactivation of *Bmp4* on the one hand and *Bmpr-II* on the other hand in the mouse, both demonstrating that Bmp4–BmprII interaction in the mouse embryo is critical for mesoderm formation [32,35].

Our teams were involved in collaborative studies of this type as well. Striking examples of the phenocopying concept and the underlying functional compensation are the conditional combinatorial knockout mouse models for *Alk2;Alk3* (also named *Acvr-I* and *Bmpr-Ia*, respectively) and for the *Smad1;Smad5* double and *Smad1;Smad5;Smad8* triple knockouts. This work in mice was done primarily by R. Behringer and colleagues, using an AmhrII-Cre based approach ([78], see also Section 2.1). In mammals, the Sertoli cells of the fetal testes produce anti-Müllerian hormone (AMH, a member of the TGFβ family of ligands), which is known to signal through the BMP/Alk pathway involving a specific type II receptor, Amrh-II, in the Müllerian duct mesenchyme. This ultimately results in the induction of the regression of the Müllerian duct meso-epithelium, which on itself is needed in combination with the testosterone-induced (produced by the fetal Leydig cells) Wolffian duct differentiation in order to generate the male reproductive tract (for a review, see [79]). In humans mutations in either the ligand-encoding gene *AMH* of *AMHR-II* cause the majority of cases of persistent Müllerian duct syndrome [80], which was further confirmed by the respective single knockout male mice for these genes [81–83], in addition to the retention of the Müllerian duct in about half of *Alk3;AmhrII-Cre* mutant male mice [84].

Work involving the characterization of the *Alk2;Alk3* conditional knockout male mice, as well as of triple-conditional BMP-*Smad1/5/8* knockouts, clearly provided evidence for functional redundancy of these two receptors and these three Smads in Müllerian duct regression in males [78]. Furthermore, the double-conditional receptor mutant females are fertile, suggesting that the differentiation of the Müllerian duct into the female reproductive tract is not dependent on these receptors. Hence, the conditional removal of the R-Smad encoding genes, using AmhrII-Cre, indicates in various allele combinations functional redundancy between these BMP-Smads, but also a key need for Smad5 deficiency for obtaining partial or complete Müllerian duct retention, the latter requesting indeed inactivation of all three Smad genes. However, other female-specific signals in Müllerian duct differentiation into the adult female reproductive system may be involved, for the persistent Müllerian duct in "full" Smad-deficient males was histologically different.

Systematic comparative studies of this type, addressing the individual, collective or redundant roles of the upstream receptor combinations and the comparison with the activated BMP–Smads in many other regions of the embryo as well as of the adult animal remain to be done. Another region for doing this is to our opinion the developing heart, from mid-gestation in the mouse embryo onwards as well as in the early post-natal embryo. This is based on previous studies assessing the role of Alk3 and Smad5 in cardiac myocytes in conditional knockout mouse models [39,85,86].

3. Smad-interacting proteins: a selection

3.1. Smad proteins as extremely versatile binders of non-Smad proteins

TGFβ/BMPs regulate a plethora of biological processes despite a seemingly simple intracellular Smad-mediated signal transduction cascade (see Section 1). However, a strict regulation of the cascade takes place both at the extracellular and intracellular level. For example, appropriate modulation of the intracellular cascade is not only achieved by regulation of the synthesis levels of its different components, but is also determined by the activity, sub-cellular (re)localization, post-translational modification, and stability versus degradation of these components.

One of the major reasons for the Smad pathway being involved in many molecular and cellular processes is that each of the three domains (MH1, linker and MH2 domain, respectively) of the R-Smads can bind to a surprisingly high number of different non-Smad proteins. We have identified and studied many SIPs over many years, three of which we discuss further below. These examples illustrate the power of the combination of studies in an animal model/embryo with biochemistry, and at the same time identify Smads as candidate players in new molecular processes (e.g. coupling of Smad-based transcription with maturation of specific mRNAs at their 3′-end; Section 3.2 on CPSF), may link Smads to new field interfaces (e.g. extrinsic signaling in a context of inflammation and accompanied by DNA repair; Section 3.3 on Ttrap/Tdp2) or have at least enabled us to identify new important SIP/DNA-binding TFs (like Sip1; see Section 3.4). Our studies in this field contribute to the clearly emerging picture that Smads are extremely versatile proteins that are regulated by SIPs and *vice versa*, bearing in mind that for most SIPs, certainly in the case of complex multi-domain SIP-TF, it has not been documented whether each of sometimes many activities of the SIP-TF (and hence also its target genes) are Smad-interaction dependent.

3.2. Smad–CPSF interaction in Xenopus embryogenesis

Smicl is a nuclear SIP that potentiates Smad2/3-mediated signaling in ligand-activated cells [87]. It has a domain with 5 CCCH-type zinc fingers that is similar to a domain in CPSF-30, the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF). Functional CPSF consists of at least four core subunits (160, 100, 73 and 30 kDa, respectively) and participates in cleavage and polyadenyation of *de novo* transcripts [88]. The Smad-binding domain located in the N-terminal segment of Smicl also displays homology with a corresponding domain in CPSF-30, while like CPSF-30 also Smicl can associate with other core CPSF subunits that were characterized previously. However, Smad proteins affect the binding of Smicl to these other CPSF subunits. Thus, this work with Smicl and CPSF-30 may point to the existence of a mechanism that couples Smad-dependent transcription with pre-mRNA processing. However, CPSF activity was previously never assessed in growth factor activated mammalian cells and also no specific endogenous genes have been identified as targets as the CPSF field invariably used transfected reporter constructs, primarily based on viral sequences, for documenting CPSF activity.

When considering searching for such Smad–Smicl–CPSF targets in the vertebrate embryo, we decided first to knock down Smicl in *Xenopus* embryos. Smicl is present maternally in the *Xenopus* embryo and – by using antisense morpholino based knockdown – is later specifically required for transcription of, and directly regulates, Nodal/β-catenin-induced *Chordin* in the Spemann Organizer, encoding an important secreted protein that protects the organizer from ventralization by BMPs [89] (Fig. 4). Surprisingly, other key genes of the organizer are not dependent on intact Smicl function in *Xenopus*, demonstrating that the Smad–Smicl cooperation is specific for a set of genes. In the case of *Chordin* we have also shown that this gene is activated by Nodal-related protein signaling in the *Xenopus* embryo in an indirect manner, and that this occurs in two steps. In the first step, Smad3 activates expression of the TF-encoding gene *Xlim1* directly. Then, a complex containing Smicl and this Xlim1 induces *Chordin*. Based on our results in transfected cells and in the *Xenopus* embryo, we proposed a model where Smicl is recruited to the promoter of specific genes by Smad proteins during transcription initiation in TGFβ/Nodal-stimulated cells. Subsequently, Smicl would then translocate to the CPSF complex and participate in mRNA 3′-end

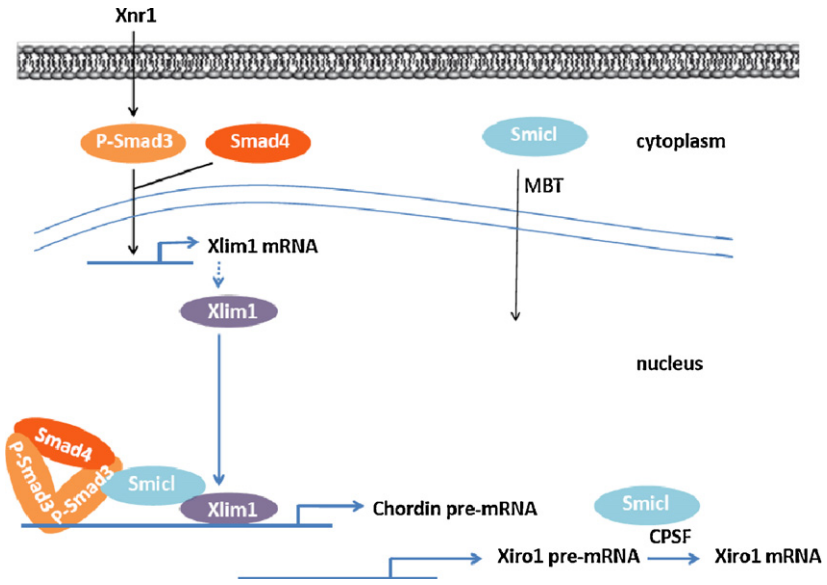

Fig. 4. Novel activities of Smad3 in the nucleus. Model for the participation during mesoderm induction in early amphibian embryogenesis of Xnr1,2,4(Nodal)-activated Smads in recruitment of the novel maternal CPSF subunit Smicl to the upstream region of specific Smad–SIP TF regulated zygotic genes from the mid-blastula transition (MBT) onwards. The case of the category I [90] target gene *Chordin* in the Spemann Organizer is shown here as its expression level is dependent on intact Smicl function in both *Xenopus laevis* and *Xenopus tropicalis* [87,89]. Many other candidate target genes, which fall into different categories (see [90]), for this type of Smad–CPSF co-operation have meanwhile been identified. *Chordin* expression has to be preceded by direct Smad3 (and not Smad2)-based activation of the TF gene Xlim1 [89]. Xlim1 also co-immunoprecipitates with Smicl [89], and the Smad3/Smicl and Smicl/CPSF interactions are mutually exclusive. Smicl is necessary for hyperphosphorylation of the C-terminal domain of Rbp1, the largest subunit of RNA Polymerase II. Smicl likely travels with RNA Polymerase II (not shown in the figure) to the 3′-end of the transcript, translocates to CPSF, and CPSF is then participating in coupled processing and polyadenylation at the 3′-end of category I transcripts like *Xiro1* [87,90]. There is evidence for polyadenylation factor CPSF-73 being the mRNA 3′-processing endonuclease of protein-coding transcripts [146]. Protein–protein and protein–RNA contacts of the core polyadenylation machinery assembled in a precleavage complex have been documented well [147], except for the new subunit Smicl.

processing coinciding with polyadenylation, which is exerted by other proteins. Doing so, we also identified a novel activity of Smads in the cell nucleus.

In a follow-up study, the team of J. Smith (Cambridge) searched for additional targets of Smicl in *Xenopus*, using microarray analysis on RNA derived from control embryos at the early gastrula stage and from embryos injected with Smicl antisense morpholinos [90]. They found that Smicl is essential for the onset of expression of many genes (about 70 in total), like *Xiro1*, at the mid-blastula transition (MBT, when zygotic gene expression in the amphibian embryo starts) and that are regulated by 3′-end processing of their mRNA in *Xenopus* embryogenesis. In addition, at MBT, Smicl was found to interact with the tail of Rpb1, the largest subunit of DNA-dependent RNA Polymerase II, like CPSF-30 does, and is required for phosphorylation of Rbp1's C-terminal domain between MBT and mid-gastrulation.

3.3. Negative regulation of Nodal–Smad signaling in zebrafish by the SIP Ttrap/Tdp2, a novel DNA repair enzyme

We identified Ttrap in a screen for interactors of the short intracytoplasmic domain of CD40, a member of the TNFR family. Ttrap also binds to Traf proteins, the effectors of TNFR/CD40 signaling, and its overproduction in mammalian cells was found to negatively regulate NFκB activation [91]. Subsequent work in our lab revealed that Ttrap binds also to receptors of the TGFβ family and to Smad proteins, and that Ttrap is phosphorylated by the Alk4 receptor for Activin/Nodal. This prompted us to take studies on this protein forward to a combination of functional studies and target gene analysis in ttrap morphant zebrafish embryos with biochemical analysis in cell culture and fish embryos [92]. The zebrafish work has shown that Ttrap negatively regulates smad3 in Nodal–Alk4–Smad3 signaling and is needed for normal gastrulation movement (through affected *snai* and downstream *e-cadherin* gene transcription) and left–right asymmetry establishment in fish embryogenesis (Fig. 5).

All the protein interactions listed above take place through the 125 aa-long N-terminal segment of Ttrap [91, 92; Ibrahimi, Vermeire et al., unpublished results], while the remaining C-terminal part of Ttrap ranks it as a new member of the family of divalent Mg^{2+}/Mn^{2+}-dependent phosphodiesterases, including subgroups of nucleases, inositol-phosphatases and sphingomyelinases, with the well-studied DNA repair protein APE1 (also named APEX1) being its closest relative [93,94]. Other teams have meanwhile reported the interaction of Ttrap with Ets/Fli TFs, its weak binding to Sumo-1 but strong binding to Sumo-2 and -3 and to Ubc9 and ubiquitin, HIV-1 integrase, and wild-type and missense Parkinson disease (PD) mutants of DJ1/PARK7 [95–98]. Xu et al. [99] have also shown that Ttrap interacts and colocalizes with three well-studies nuclear body proteins: promyelocytic leukaemia (PML) protein, Sp100 and Daxx in PML. Furthermore, Ttrap/Tdp2 was recently identified as the major and possibly unique 5′-Tyrosyl DNA phosphodiesterase (5′-Tdp) activity in vertebrate cells that is critical for resistance to topoisomerase2-induced DNA damage [100,101]. Mutations in *TDP1*, encoding a 3′-Tdp, cause spinocerebellar ataxia with axonal neuropathy (SCAN1), a progressive neurodegeneration in humans [102,103].

The early developmental defects of tdp2 knockdown fish and the modulation of nodal signaling by ttrap, and in general its function in TGFβ signaling prompted us to target *Tdp2* in mouse embryonic stem cells and make conditional knockout mice. Surprisingly, seen the defects in early embryos of morphant fish [92], homozygous *Tdp2* knockout mice are viable (Vermeire, Ibrahimi et al., unpublished). We also did send a large cohort of 14 weeks-old *Tdp2* knockout mice, and littermate control mice, to the German Mouse Clinic (GMC) (Vermeire, Gailus-Durner et al., unpublished; see also www.mouseclinic.de) for analysis in their dual pipeline for mouse phenotyping, which runs from week 16 till

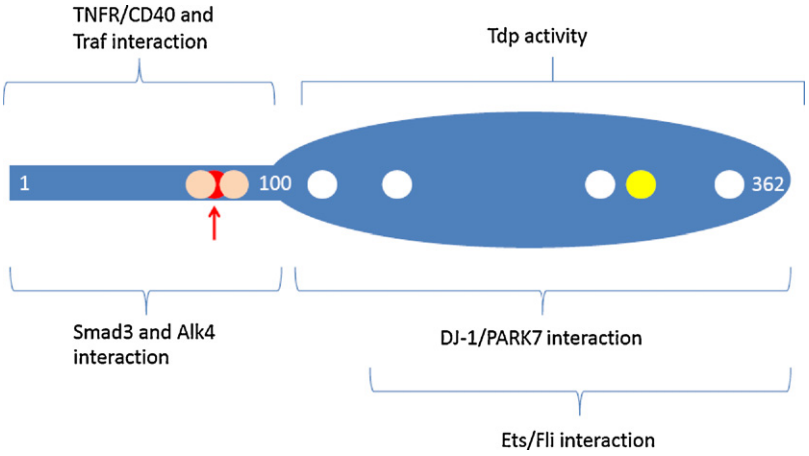

Fig. 5. Schematic representation of interaction domains and motifs in (human) TTRAP/TDP2 protein. Residues that are crucial for the interaction between (human) TTRAP and TRAF6 or CD40 have been mapped to the first 100 amino acids (AA) [91]. In contrast, the interaction between TTRAP and DJ-1/PARK7 maps to the large C-terminal domain (tested as AA 104–362; [98]). The interaction domain for Ets-type TFs has been mapped to AA 136–362 [95]. The motif for non-covalent binding to SUMO protein is mapped to AA 280–284 (yellow bullet) [96], and a potential cleavage motif for early caspases is present between AA 90–94 (red bullet and arrow) (Ibrahimi, Vermeire et al., unpublished results). The putative endonuclease domain of TTRAP starts at AA 118 and stretches until the end of the protein (with key segments AA 118–120, 150–152, 261–264 and 349–351 indicated as white bullet). Crucial functions that relate to the identified function of TTRAP in Nodal–Alk4–Smad3 signaling are the two Alk4-phosphorylation sites at residues 88 and 92, flanking the potential cleavage site for caspases [92].

26. The preliminary data from the GMC show that the *Tdp2* knockout mice do not display overt phenotypes. These mice are currently being monitored for development of pathology at older age, with special attention for development of tumors and of neurodegeneration in combination with neuro-inflammation.

3.4. Sip1, a multi-domain transcription factor with many functions, and with two faces, in the mouse

Our lab was the first to identify Sip1 (Smad-interacting protein-1) by virtue of its binding to the C-terminal MH2 domain of Smad1 in a yeast 2-hybrid screen [104]. Subsequent work has shown that Sip1 binds to Smads2/3 and to Smads1/5/8 in ligand-stimulated cells only, but many of Sip1's functions may be Smad-independent as well and hence underpin multiple mechanisms of action. Sip1 thus binds to both classes of R-Smads, and preliminary analysis of the initially 51 amino acid-long Smad-binding domain (SBD) including using SBD aptamers inserted in a thioredoxin scaffold, indicate that these both Smad classes need the same minimal domain for interaction (Conidi et al., unpublished data). Sip1 is a DNA-binding TF related to the previously isolated δEF1/Zfhx1a/Zeb1 protein. They both repress target gene transcription through binding with two zinc fingers within each of their two zinc finger clusters to a separated repeat of mainly CACCT(G) or sometimes CACANNT(G) in gene regulatory regions [105] (see Fig. 6). Full-length Sip1 and δEF1 bind to the co-repressor CtBP [106] and the chromatin-remodeling corepressor complex NuRD [107], and can become an activator by binding to P300/PCAF [108]. Sip1 levels are under control of miRNAs, including in epithelial–mesenchymal transition, which is relevant to invasive properties of epithelial-derived tumor cells [109–112].

Mutations in one of the two alleles of *ZFHX1B* (mapped on chr2q22, spanning over 120 kb and divided into 10 exons, encoding a 1214 amino-acid long protein (1215 in mouse) named also SIP1 and ZEB2) cause Mowat–Wilson syndrome (MWS; www.mowatwilson.org) in humans [113–118]. Previously often called Hirschsprung Disease (HD)–mental retardation (MR) syndrome (MIM 235730), MWS has many clinical features in common with Goldberg–Shprintzen megacolon syndrome (MIM 609460) but the two disorders are genetically distinct. One of the most specific clinical signs in MWS is a distinctive deviant facial appearance and uplifted earlobes that, along with severe MR, prompts the clinician to investigate for the genetic defect. The precise incidence of MWS, previously suggested to be 1/4500 live births, is unknown but thought to be under recognized [119]. This single-gene disorder is characterized by various malformations, which not all appear in all patients. The malformations/malfunctions are clearly in the central nervous system (CNS) [MR, delayed motor development, absence of corpus callosum, microcephaly, occurrence of seizures and epilepsy] and combine with developmental defects in the neural crest cell (NCC) lineage [cranio-facial abnormalities, HD] and a wide and heterogeneous spectrum of other congenital anomalies. The latter include genital anomalies (particularly hypospadias in males), eye defects, and in few patients heart defects (e.g. tetralogy of fallot, septal defects, patent ductus arteriosis, pulmonary arterial sling), and cleft palate and sensorineural deafness. Analysis of about 220 MWS patients has shown that full genomic deletion of the *ZFHX1B* locus occurs in roughly 20% of known cases, 3/4th of which are detectable by FISH, but 1/4th being missed by this technique. The remaining near-80% of *ZFHX1B* known mutations create frameshift mutations that result in C-terminally truncated and likely unstable, undetectable mutant protein, and haplo-insufficiency has been postulated to be the major cause of the wide variety of symptoms of this disease. Only few missense mutations that affect the function of a domain of the multi-domain SIP1/ZFHX1B/ZEB2 protein are known, but a new one that affects NuRD binding is being studied in our lab.

Previous studies in our *Sip1* conventional knockout mice showed that these die early in postimplantation embryogenesis, i.e. at E9, and display severe neural plate and neural crest and somitogenesis defects [120–122]. Therefore, several conditional knockout mouse models were established (see Table 2). We summarize first a number of important general conclusions from these studies. First, some of the phenotypes found in the respective knockout mice correlate with defects found in Mowat–Wilson patients, but other ones reveal new roles of Sip1 in certain cell types/tissues, which have not been analyzed yet in patients. For example, our published data obtained in conditional *Sip1* knockout mice (using Wnt1-Cre, which is active in premigratory and migratory NCC) suggests that the HD and facial malformation have their origin in defects in NCC [121]. However, the same study has also revealed an important function of Sip1 in the

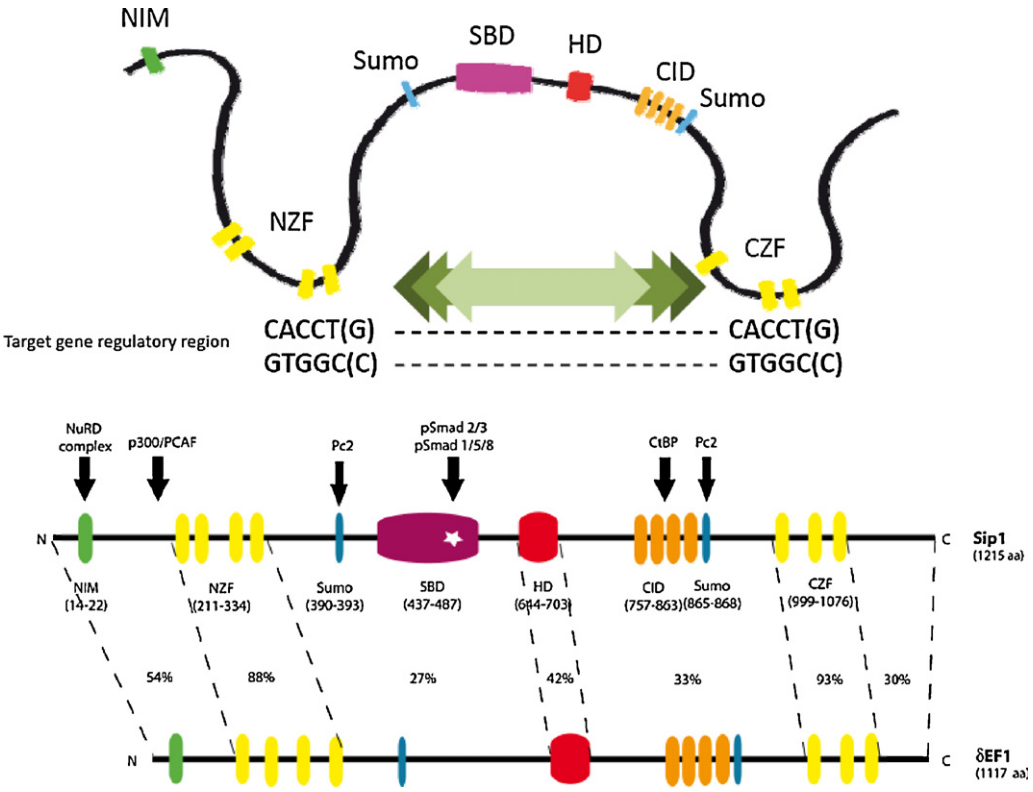

Fig. 6. Schematic representation of the Zfhx1 family member and zinc finger SIP–TF Sip1, also named Zeb2 and Zfhx1b. In the top panel the functional domains in the 1215 aa-long mouse Sip1 are indicated, while the lower panel presents more details, as well as a comparison with the very weak Smad-binder δEF1/Zeb1/Zfhx1a, and amino acid identity for the domains (in %). These are, respectively, the essential zinc fingers in the zinc finger clusters (NZF, CZF) located in the N-terminal and C-terminal segment of Sip1 and that each bind to E-box-like sequences on DNA ([105]; for details, see text). The spacing between the half-sites can vary [105]. The four binding sites (indicated as CID; PLXL($^{S}/_{T}$)) for the co-repressors CtBP [106] have been proposed to be less efficient in CtBP-binding when Sip1 is sumoylated [148]. The two demonstrated covalently modified sumoylation sites (indicated as Sumo) encompassing K^{391} and K^{866}, respectively, regulate transcriptional activity of Sip1 in a promoter-dependent context; the Polycomb group protein Pc2 can act as a small ubiquitin-like modifier E3 ligase for Sip1 [106]. Collective mutation of all binding sites of the CID however did not result in a less efficient repression of transfected E-cadherin promoter-reporters [149,150], while this was also the case for each individual sumoylation site out of a total of nine candidate sites (van Grunsven, Vanlandewijck et al., unpublished results). Sip1 binds to Smad2/3 and Smad1/5/8 via a Smad-binding domain (SBD) of 51 amino acids [104]. Recent studies on this SBD, using SBD-based aptamers in a thioredoxin scaffold, indicate that 14 amino acids (marked with a star) of the SBD are minimally required for binding to both classes of Smad (Conidi et al., unpublished results). Two-hybrid screening in yeast, using SBD-encompassing fragments of Sip1 as bait, has also led to the isolation of Smad2 as partner for Sip1 (van Grunsven et al., unpublished results). Full-length Sip1 and its related Zfhx1 family member δEF1 (also named Zeb1, Zfhx1a) [108], like fragments of δEF1 [151] also bind to P300/PCAF, suggesting that these DNA-binding repressors can also act as activators of transcription. Smad complexes are also P300-binding proteins. The homeodomain-like domain (HD), which likely does not bind to DNA, is also indicated. Sip1 also binds via a NuRD-interaction motif (NIM) to the corepressor complex NuRD [107].

adrenosympathic anlage and in the epicardial cells during cardiac development. In addition, Sip1 seems crucial for the formation of the transient boundary cap cells, which contains precursor cells for sensory neurons destined for the dorsal root ganglia, but also satellite glial cells and myelinated cells accompanying the motor axons (Cazzola, Van de Putte et al., unpublished results). The sensory neuron phenotype during embryogenesis seen in the Wnt1-Cre;Sip1−/− model may also offer an explanation for the pain phenotype seen in dorsal root ganglion neurons (in the nociceptive neurons) of Sip1+/− mice [123]. Another example is that selective removal of Sip1 from GABAergic interneurons in the ventral forebrain, at least with some of the used Cre strains, yield mice that three weeks after birth undergo myoclonic seizures and die immediately after (van den Berghe et al., unpublished results).

Second, in many cases the established conditional mouse models display phenotypes the molecular mechanisms of which reveal also new modes of action of Sip1. For example, a number of genes that help to explain the phenotype(s) are downregulated in the Sip1 knockout cells, while many more other genes are upregulated in the absence of Sip1, pointing at Sip1 as being an activator and for the majority of its target genes a repressor of target gene transcription [124]. Many of these genes are candidate direct target genes for Sip1 and/or point also at other cellular processes where Sip1 could play a role. For example, RNA-seq analysis of sorted Sip1-deficient embryonic forebrain cells, and comparison with sequencing data from control forebrains, suggest regulation of different classes of genes involved not only in neurogenesis but also the regulation of gene sets encoding GPCRs

Table 2
Published $Sip1^{ex7}$ knockout mouse models.

		References
Ubiquitous homozygous knockout		
Neural plate/neural crest		[120,140]
Somitogenesis		[122,140]
Heterozygous knockout		
Hirschssprung disease (x *Sox10*)		[141]
Early development (x δEF1)		[142]
Pain		[123]
Conditional knockouts		
Eye lens development	Pax6-Cre	[143]
Craniofacial development	Wnt1-Cre	[121]
Sensory development	Wnt1-Cre	[121]
Hippocampal anlage	Emx1-Cre	[144]
Embryonic brain cortex	Nestin-Cre, NEX-Cre, Emx1-Cre	[124]
Embryonic haematopoiesis	Tie2-Cre, Vav-iCre	[126]

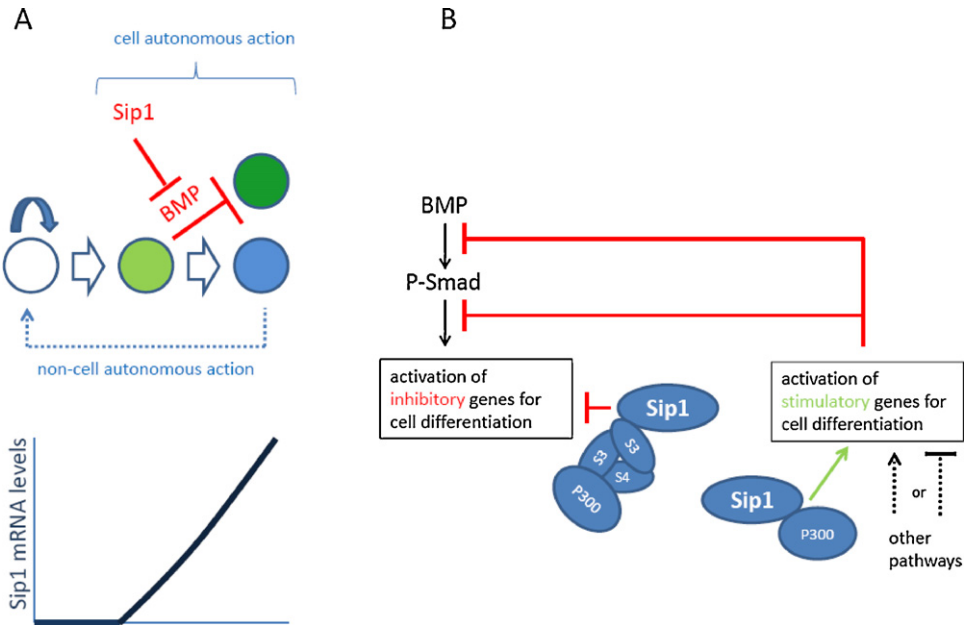

Fig. 7. Schematic representation of functions and action modes of Sip1. The present and emerging data indicate that Sip1 is – for example – not involved in pluripotency of e.g. mouse embryonic stem cells. Rather, in multi-potent progenitor cells (white cells in panel A) Sip1 mRNA levels often accumulate upon commitment (light green cells, e.g. neural commitment) and differentiation towards one cell type (dark green cells, e.g. neurons) (Stryjewska et al., unpublished results; [125]). In the case of cell-autonomous functions as, perhaps not all of its TF functions are accomplished in co-operation with bound R-Smads (BMP-Smads in the given example in panel A). The present biochemical data in transfected cells suggest that in the case of Sip1-Smad interaction Sip1 neutralizes P300-Smad4-RSmad complex based gene activation by turning the P300 complex with the Smads into a repression complex for the same target genes. In the case of BMP signaling, this would mean that Sip1 is capable of neutralizing a set of genes normally induced by BMP receptor signaling and that also inhibit differentiation. Hence, Sip1 is a negative regulator of BMP activity, which is for example in line with the neural-inducing activity of Sip1 and the anti-neural activity of BMPs [see e.g. [106]]. It is not clear yet whether the SBD and the NIM (see Fig. 6) in Sip1 co-operate in such action. As a TF, Sip1 has non-cell autonomous functions as well ([124]; for details, see text). In addition, and within the same cells where it acts as a transcriptional repressor in conjunction with Smads, Sip1 can however activate, likely in co-operation with either P300 or PCAF directly bound to it, but not R-Smads, other candidate target genes although these seem to be less in number than the repressed targets (panel B). This means that, when following the same logic, such Sip1-activated targets would encompass genes that encode proteins that negatively feedback to BMP signaling and/or stimulate cell differentiation, perhaps in combination with other (non-TGFβ family) signaling pathways (indicated as dashed lines) that are stimulatory or inhibitory. Similar diverse action modes may also underlie cellular phenotypes in migration/mobilization observed in conditional knockout embryos in neural crest and embryonic haematopoietic cells [120,121,126]. S3, phospho-Smad3; S4, Smad4.

and ion channels, vesicular trafficking proteins, and proteins involved in synaptogenesis and synaptic plasticity.

Third, the picture is emerging that Sip1 negatively regulates BMP–Smad signaling in a number of multipotent progenitor cell types where BMPs exert an anti-differentiation effect (Fig. 7A), e.g. anti-neural effects of BMPs in *Xenopus* embryos [108] and mouse embryonic stem cells (Stryjewska, Verstappen et al., unpublished results), while Sip1 is necessary for neuroectodermal differentiation of human ES cells [125], but also for embryonic haematopoiesis [126] and myelination (Weng et al., unpublished results). This means that evidence is accumulating that Sip1 is an intracellular negative regulatory mechanism of BMP–Smad signaling in the nucleus of ligand-activated cells by virtue of binding to the R-Smads, and where the candidate target genes for the Smad–Sip1 repressive interaction are genes that are otherwise BMP-induced and encode negative regulators of cell commitment/differentiation (Fig. 7B). Following the same logic, it would in the same cells also be very well possible that Sip1 as a transcriptional activator then directly activates a set of genes that promotes the differentiation process.

Fourth, it cannot be excluded that Sip1 has in addition to its cell-autonomous role also a non-cell autonomous function and hence in the knockout models its removal from a specific subset of cells has also consequences for other cells in the same region or niche when Sip1 is not expressed in these latter cells. This is clearly the case in the embryonic cortex in the forebrain, where Sip1 in neurons of the upper layers regulates the level of transcripts for the secreted proteins neurotrophin-3 and fibroblast growth factor-9, which regulate the timing of neurogenesis and gliogenesis, respectively, of the progenitor cells [124].

4. Conclusions and needs

The examples we selected for further discussion in this survey paper reveal new activities, and the complexity of their underlying molecular mechanisms, but also the next needs in the TGFβ/BMP field. Indeed, in addition to – for example – answering the question which *in vivo* activities and sets of target genes of SIP–TFs are truly Smad-dependent, and *vice versa*, another need is not only to map but also quantitate how the different components of the TGFβ system connect via autoregulation, synexpression and feedback control. Perhaps this is not experimentally approachable at a system scale *in vivo*, i.e. in an embryo context, but likely a progenitor/stem cell culture system wherein BMP induced or inhibited differentiation would be better suited for studies at the system level. A second need is clearly still to understand Smad-SIP cross-talk with other pathways. Both in the case of cultured stem/progenitor cells and *in vivo* in the mouse, we have experienced that this is particularly needed in the case of cross-talk with Wnt, Notch or inflammation pathways. Finally, a third need is the obligatory expansion from studies of signaling in one cell type towards multiple cell types in a given niche within either the embryo or embryonic and adult organs, including in normal processes of repair and in diseases such as e.g. PAH or PD.

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