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Smads – the Intracellular Hubs of Signalling in Regulation of Pluripotency and Differentiation of Stem Cells

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1. Introduction

Signalling by the members of one of the largest groups of peptide signalling molecules, the transforming growth factor beta (TGF β) superfamily, has been implicated in the regulation of aspects of essentially all events in the life and death of an animal cell. Smad proteins are versatile intracellular mediators of those signals, responsible for their direct transmission from the TGF β superfamily receptor complexes on the membrane into the nucleus, resulting in specific changes in gene expression. Explicitly, two different Smad groups, transmitting BMP (bone morphogenetic protein)/GDF (growth and differentiation factor) and TGF β /activin signals, respectively, have been shown to be involved in the maintenance of pluripotency in the mouse and human and all other characterised vertebrate embryonic stem cells.

Smad proteins are subject to extensive post-translational modifications, which are often a result of activation of other important cellular signalling pathways, rendering Smads important hubs of the major signalling pathways. Signalling by the members of the TGF β superfamily starts with their binding to the complexes of type I and II (and in case of TGF β s type III) receptors, resulting in phosphorylation of the type I receptor by type II, in its turn leading to the phosphorylation of pathway-specific regulatory Smads (R-Smads). R-Smads then enter the nucleus in complexes with the co-Smad, Smad4, and activate or repress transcription of target genes, often after binding other transcription factors. Much of the intracellular regulation is achieved via regulation of the levels of Smad proteins available for signal transduction, mostly by competition for receptor binding, degradation or changes in phosphorylation status. The latter regulation is often achieved by phosphorylation by the kinases activated by other important signalling pathways, notably by cytokines FGF/EGF (fibroblast and epidermal growth factors, respectively) and Wnt proteins. It was recently discovered that some types of Smad linker phosphorylation accompany Smad activation, and act to ensure the transient nature of activated Smad action, thus maintaining constant sensitivity of the cell to changes in the levels of the TGF β /BMP signal.

2. The Smad family

The name of the family is a combination of designations of the first identified members of this family of intracellular effectors of signalling, *sma* ("small", in nematode worm

Caenorhabditis elegans) and *mad* (“mothers against decapentaplegic”) from the fruitfly *Drosophila melanogaster*. As apparent from the fruit fly gene’s name, it was identified in the mutant screen for modifiers of the mutation in the *Drosophila* BMP2/4 homologue, *decapentaplegic*, thus revealing its involvement in this signalling pathway (Derynck et al 1998). There are 3 types of *mad*-related Smads: receptor-regulated Smads which, in response to the ligand, are directly phosphorylated by type I receptors and shuttle into the nucleus after associating with the second type of Smad, the co-Smad, and finally the anti-Smads, acting as negative regulators of the signalling pathway (Shi & Massague 2003). All members of the Smad family share certain structural similarities: they are proteins of around 500 amino acids in length, consist of two globular domains connected by a linker. Smads of all three classes possess a C-terminal “Mad homology 2” domain 2 (MH2) that mediates protein-protein interactions (see Figure 1), and an N-terminal DNA-binding Mad homology domain 1 which is present in R-Smads and the co-Smad. In mammals, Smad genes represent a highly paralogous group of 8 genes. Not unexpectedly, considering its role in binding to the activated receptors and oligomerisation of phosphorylated Smads, the MH2 domain bears certain structural similarity to the phosphopeptide-binding forkhead-associated (FHA) domain (Durocher et al 2000). Conservation within classes of Smads is so high that human R-Smad-expressing transgenes were found to elicit similar phenotypes to their endogenous fruit fly’s counterparts in the *Drosophila* embryo (Marquez et al 2001). Human Smad1 protein shares a remarkable 82% of its amino acid sequence with its *Drosophila* Mad orthologue (Attisano & Lee-Hoeflich 2001). To highlight the incredible evolutionary conservation of the Smad signalling pathway, it has to be noted that organisms as basic and evolutionarily removed from mammals as pseudocoelomates, e.g. nematode *C. elegans*, possess a complement of Smads of all three classes: R-Smad, co-Smad and anti-Smad (Newfeld et al 1999). Regulation of Smad function is achieved mostly at the posttranscriptional level, at the level of Smad proteins, their post-translational modifications, most significantly R-Smad activation by receptor-dependent phosphorylation. Thus, at the transcriptional level, R-Smads and the co-Smad appear to be expressed relatively uniformly, at moderate levels in most tissues (e.g. see GNF BioGPS data, biogps.gnf.org). Less is known about post-transcriptional regulation of the inhibitory Smad activity, levels of transcription of these Smad genes appears more variable, for instance with significantly higher levels of *SMAD7* transcripts in placenta and natural killer (NK) immune cells compared to majority of other tissues.

2.1 Domain structure of Smad proteins

In addition to the above mentioned Mad homology (MH) domains 1 and 2, involved in DNA and protein binding, respectively, increased attention is being drawn to the central proline-rich linker region, thought to be a major site of post-translational modifications modulating the function of the Smad proteins (Burch et al 2010, Wrighton et al 2009). While the linker is only moderately conserved across Smad classes, a high degree of conservation is observed within a given class and especially a subclass across the animal kingdom, all the way down to insects and even nematodes (Newfeld et al 1999). Another feature, until recently often considered to be a part of the MH2 domain, is the receptor phosphorylation domain containing the SSxSCOO⁻ consensus, present at the C-termini of R-Smads. This domain is specifically targeted for phosphorylation by the type I TGFβ/BMP receptor serine/threonine kinases (Shi & Massague 2003). A number of other Smad protein features and motifs, mostly involved in regulation of Smad localisation and degradation, will be discussed in detail in corresponding sections of this review (Figure 1).

2.2 Receptor-regulated, or R-Smads

In mammals, this group of Smads consists of 2 subclasses, or subfamilies: one primarily involved in the mediation of the BMP/GDF signalling (BMP Smads), and the others transmitting TGF β /activin/nodal signals (TGF β Smads). The first group is represented by 3 members – the closely related *Smad1* and *Smad5* genes and slightly more divergent *Smad8*. On the phylogenetic tree, BMP Smads cluster with the *D. melanogaster* Mad protein, the archetypal Smad involved in signalling by the product of the fly's orthologue of mammalian BMPs 2 and 4, *decapentaplegic* (Derynck et al 1998). Members of the other group, encompassing the TGF β /activin/nodal Smads, *Smad2* and *Smad3*, are more closely related to their *Drosophila* homologue, dSmad2, than to proteins from different classes of Smads (Newfeld et al 1999). It is interesting to note that even in insects, as it is known to be the case in the fruit fly and the bee, there exists a paralogue for the Smads of both BMP and TGF β subfamilies in higher organisms. Consistent with the concept of those two groups of Smads transmitting different signals, they are thought to serve as substrates for different sets of type I receptor kinases: BMP Smads are primarily phosphorylated by the activin receptor-like kinase (ALK) 3 and ALK6 (also known as BMP receptor type I A and B) and in some cases ALK2, while TGF β Smads 2 and 3 are activated by ALKs 4, 5 and 7 (Derynck & Zhang 2003). Interestingly, in some cell types ALK1 was shown as being able to transmit TGF β signals via BMP Smads 1 and 5, highlighting the complexity of the signalling by the members of the TGF β superfamily (Goumans et al 2002). Also at the N-terminal end of the MH2 domain is situated a “basic pocket”, which functions to enable binding of the R-Smads to GS-domain of the type I receptors which have been activated by phosphorylation (Wu et al 2000). In R-Smads this pocket is utilised to accommodate R-Smads' phosphorylated SSxP motif during the Smad oligomerisation, and it is believed that a similar motif exists in the co-Smad's MH2 domain (Massague et al 2005).

Upon phosphorylation by the receptor, the R-Smads are thought to normally form first homodimeric, and then heterotrimeric, complexes with incorporation of the co-Smad, Smad4, thus consisting of 2 R-Smad and 1 co-Smad proteins (ten Dijke & Hill 2004). It is believed that while the MH1 domain of Smads (specifically shown for 3 and 4) confers specific binding to the specific sequence called the Smad-binding element (SBE), recently defined as 5'-GTCT-3' (or its complement), the binding is relatively weak, and relies upon complexing with other transcription factors, thus achieving activation or repression of downstream gene promoters (Derynck et al 1998, Massague et al 2005, Shi et al 1998). It is believed that Smads 1 and 5 complexed with Smad4 bind an asymmetrical site composed of one SBE and one GC-rich consensus, 5'-GRCGNC-3' (Pyrowolakis et al 2004).

2.3 Common mediator, or co-Smad

Structurally, co-Smad protein is very similar to the R-Smads, as it consists of the N-terminal MH1 domain, central proline-rich linker and the C-terminal MH2 domain. Co-Smad's function is two-fold: to serve as an oligomerisation partner facilitating nuclear translocation of activated R-Smads, and to augment binding of R-Smad-containing complexes to target genes in the nucleus (Massague et al 2005). The important function of the recruitment of the transcription activating or repressing factors is performed by the so-called “Smad4 activation domain” (SAD), residing in the N-terminal portion of the MH2 domain.

This class of Smads is very well conserved evolutionarily, with clear orthologues of vertebrate Smad4 genes present in *Drosophila* (*medea*) and *C. elegans* (*sma-4*) (Newfeld et al 1999). Human Smad4 is a well-established tumour suppressor gene, often found mutated in

human pancreatic and intestinal cancers, and was originally called “deleted in pancreatic cancer 4”, DPC4 (Hahn et al 1996, Howe et al 1998). As one would expect, based on a proposed function as a co-factor for R-Smads, the *Smad4* gene is expressed ubiquitously and uniformly across all tissues in both mouse and human (e.g. GFN SymAtlas public access and authors’ own data).

2.4 Inhibitory Smads

Inhibitory Smads (I-Smads) are the more “distantly related” group of Smads, lacking the DNA-binding MH1 domain and displaying a higher degree of internal divergence. In contrast to the case with other Smads, *Drosophila*’s inhibitory Smad, *Dad*, appears to be more phylogenetically distant from vertebrate genes than one of the genes from *C. elegans*, 1L81 (Newfeld et al 1999). While the MH2 domain bears substantial homology to analogous regions of R-Smads and co-Smad, the N-terminal half of I-Smads is divergent enough not to be considered as a functional MH1. At the same time, curiously, a reliable alignment of N-terminal halves of all Smads could be performed, demonstrating few homologous motifs, including a stretch of basic amino acids, mostly lysine, interrupted by an insertion of a glutamate residue present in I-Smads and co-Smad and possibly compromising the ability of this region to function as a potential nuclear localisation signal. As the name of the family indicates, Smads of this group act as antagonists of the signalling by the TGF β superfamily members, and in mammals it consists of two family members: Smads 6 and 7. Currently, there are up to four different mechanisms by which inhibitory, or anti-Smads, exert their effects. Initially they were found to both compete with R-Smads for binding to activated type I receptors (Hayashi et al 1997) and promote proteasomal degradation of those receptors via recruitment of ubiquitin ligases, Smurf (Shi & Massague 2003). I-Smads were also found to be capable of mediating dephosphorylation of the type I TGF β superfamily receptors by recruitment of complexes of GADD34 with the catalytic subunit of the protein phosphatase 1 (Shi et al 2004). In addition, I-Smads were found to potentially have a role to play in the nucleus, where Smad6 has been shown to promote repression of the BMP target genes via interaction with co-repressor CtBP (Lin et al 2003).

3. Modulation of Smad signalling

Last decade saw the discovery of a significant number of mechanisms involved in the regulation of TGF β signalling at the Smad level, from the regulation of Smad binding to the activated receptors on the outer cell membrane to modification of the repertoire of transcriptional co-factors binding to Smad complexes in the nucleus. This does not come as a surprise, since the importance of the pathway in the regulation of the cell’s behaviour is difficult to overestimate. In this section, we will discuss some of the better understood mechanisms that cells employ to modulate Smad activity.

3.1 Regulation of receptor-Smad interactions

Transmission of the TGF β /BMP signal is dependent on direct association of the unphosphorylated, inactive R-Smad with the receptor complex. This process is essential for phosphorylation of the R-Smads, and is facilitated by the SARA (Smad anchor for receptor activation) protein (Shi 2001, Wu et al 2000). The introduction of a specific point mutation, into the MH2 domain of Smad 2 which is known to be critical for its efficient interaction with SARA, appeared to abolish its ability to transmit TGF β signalling (Wu et al 2000).

Some mutations in the SARA protein were shown to lead to a mislocalisation of Smad2 and consequently to compromised Smad2-mediated signalling, highlighting an essential role for this SARA-mediated R-Smad receptor targeting. The SARA protein also contains a FYVE phospholipid-binding domain, with a particularly high affinity towards phosphatidylinositol-3'-phosphate, a phospholipid highly enriched on endosomal membranes. This is consistent with the model in which the majority of Smad activation by the receptor complex might be taking place at the early endosome, where most of the SARA protein appears to be localised (Di Guglielmo et al 2003, Itoh & ten Dijke 2007). Another FYVE domain-containing protein, Hgs, has been implicated in promoting Smad phosphorylation in cooperation with SARA (Miura et al 2000). A whole array of factors, proposed to amplify the interactions of Smads with the receptor complexes, has been identified, including Disabled-2, Dok-1, Axin, ELF β -spectrin and cytoplasmic PML (Lin et al 2004, Massague et al 2005). Other proteins, such as TRAP1 (TGF β receptor-associated protein 1) and TRAP1-like protein (TLP), have been proposed as adaptors interacting with the inactive receptor complexes whilst also promoting the formation of heteromeric R-Smad-coSmad complexes by acting as Smad4 chaperones (Wurthner et al 2001). It has to be noted that there is still a lack of reliable, especially genetic, evidence of the requirement of these proteins for normal Smad signalling, in particular for the BMP pathway, as the role for SARA and other proteins has been demonstrated for the receptors and R-Smads normally associated with transmission of the TGF β signal.

A different mechanism of regulation of R-Smad activity by its sequestration from binding to the activated type I TGF β /BMP receptor was described in the interaction of Akt with Smad3 (Conery et al 2004, Remy et al 2004). Recently, a different model was put forward for a mechanism explaining this Akt-Smad interaction, proposing that an Akt kinase substrate, mTOR (mammalian target of rapamycin), acts to inhibit Smad3 phosphorylation by ALK5, the main type I receptor for TGF β molecules 1-3 (Song et al 2006).

3.2 Modulation of Smad function by phosphorylation

Phosphorylation of the C-terminus of R-Smads by the type I receptor is the central event in intracellular transmission of the TGF β and BMP signals, as it serves as a major trigger for the oligomerisation of Smads and ensuing regulation of gene expression by Smad complexes upon their translocation into the nucleus (Massague et al 2005). The specificity of the signal transmitted from the membrane is determined by the interaction of specific domains in both the type I receptor and the R-Smad. From its inactive form, with the unphosphorylated GS domain serving as a binding site for FKBP12 molecules, the type I receptor becomes an active serine/threonine kinase upon phosphorylation of the GS domain and ensuing release of FKBP12 (Shi & Massague 2003). The phosphorylated GS domain then acts as one of the R-Smad binding sites, while the specificity of receptor-R-Smad interaction is determined by an L45 loop on the receptor's intracellular kinase domain and the L3 near the C-terminus of the MH2 domain of the R-Smad.

In addition to the C-terminal phosphorylation by the receptors, a significant portion of the activated R-Smads in the cell is subject to secondary phosphorylation events, often thought to ensure a limited lifespan or attenuate the function of the active form (Itoh & ten Dijke 2007, Wrighton et al 2009). Most of those phosphorylation events take place in the linker region, while a few occur at the N-terminus of the R-Smad protein, in the MH1 domain. In the majority of cases, kinases of two families are implicated in these phosphorylation events: the mitogen-activated protein kinases (MAPK) and cyclin-dependent kinases (CDKs).

MAPKs that were specifically shown to phosphoryllate the R-Smad linker include p38 MAPK, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), Rho-associated protein kinase (ROCK) and MEKK-1 (currently known as MAP3K1). Among other known R-Smad linker kinases are protein kinase C (PKC), G protein-coupled receptor kinase GRK2, Ca²⁺/calmodulin-dependent protein kinase (CaMKII) and casein kinases CK1 γ 2 and CK1 ϵ (e.g. Wrighton et al 2009).

Interestingly, nearly all of the phosphorylated Smad residues are serines with few known threonine targets and not a single tyrosine, continuing the common theme for the whole pathway of utilisation of serine/threonine kinases for propagation and attenuation of the signal. It might be possible to surmise that this serves as means of separation of this pathway from interference from many other cellular signalling pathways utilising tyrosine kinases. At the same time, a wide variety of serine-threonine kinases have been shown to be capable of Smad phosphorylation, providing potentially physiologically-important inputs from other pathways. There also appear to be “layers” of phosphorylation, with some events dependent on the preceding modifications (Wrighton et al 2009). A good example of such an arrangement is phosphorylation by the enzymes of the MAPK family, which primes the Smad1 linker for further phosphorylation by the glycogen synthase kinase 3 β (GSK-3 β), which targets the Smad for ubiquitination and proteasomal degradation (Fuentealba et al 2007). C-terminally phosphorylated Smads are translocated to the nucleus, where the secondary phosphorylation by the MAPKs (Erk, p38 and JNK) occurs. This event is thought to promote the nuclear exclusion, and tertiary phosphorylation by the GSK-3 β kinase maximises the affinity of the “triple-phosphorylated” Smad for a Smurf or NEDD ubiquitin ligases, eventuating in the efficient proteasome-dependent degradation. Strong support for the notion that MAPK phosphorylation has a negative effect on Smad-mediated signalling comes from the phenotypic analysis of mice bearing mutations disabling phosphorylation at 6 of the most frequently utilised sites in the linker of Smad1 (Aubin et al 2004). Analysis of the localisation of MAPK phosphorylation-resistant Smad1 revealed its abnormal preferential concentration on the membrane, suggesting that MAPK phosphorylation might be also important for proper subcellular localisation.

Traditionally, it was assumed that most Smad phosphorylation events taking place outside of the C-terminal SSxS domain, predominantly at the linker region, act to down-modulate Smad action (Itoh & ten Dijke 2007, Wrighton et al 2009). However, very recently it was demonstrated that some of those phosphorylation events could act to enhance nuclear functions of Smads, while ensuring their transient nature by priming Smads for rapid degradation (Alarcon et al 2009). In this case, the Smad linker is phosphorylated by one of the cyclin-dependent kinases (CDK8 or 9), and this phosphorylation event correlates with C-terminal Smad phosphorylation, and just like the C-terminal activation is induced by an agonist of the pathway, *i.e.* a member of the TGF β superfamily of signalling factors. This event, called the agonist-induced linker phosphorylation (ALP), occurs precisely at the serine and threonine residues, the phosphorylation of which is known to facilitate proteasomal degradation of Smads transmitting both TGF β and BMP signals (Gao et al 2009, Sapkota et al 2007). Experimental evidence supports the model in which double (C-terminally and ALP)-phosphorylated Smads are efficiently targeted by the ubiquitin ligases Smurf1 and NEDD4L for proteasomal degradation. Interestingly, while in the nucleus, activity of the double-phosphorylated Smads is augmented by ALP-dependent binding of the transcriptional co-activator Yap, and this interaction appears to play an important role in regulation of downstream targets, specifically demonstrated for the BMP-induced Smad1-

regulated genes of the *Id* family of repressors of gene activity (Alarcon et al 2009). Smad4 appears to exist in most cells in a constitutively-active state, and while most phosphorylation sites are unknown, phosphorylation by ERK at Thr277 have been shown to be required for efficient nuclear translocation of Smad4-containing complexes (Roelen et al 2003).

3.3 Regulation of Smad activity by phosphatases

The presence of phosphatases capable of specific dephosphorylation of the Smad linker region adds yet another level to the dynamic regulation of the levels of Smad signalling. Originally, the major focus in the interpretation of the phospho-regulation of R-Smad function was on their nuclear export and subsequent degradation. The presence of a large pool of Smads that are at least partially dephosphorylated prior to their export from the nucleus was acknowledged only relatively recently (Inman et al 2002), indicating the existence of phosphatases acting on phospho-Smads. It was also demonstrated that nuclear export-mediating proteins display a preference for the C-terminally dephosphorylated R-Smads (Xu et al 2002). One of the first Smad C-terminal phosphatases was identified in an RNAi screen in *Drosophila* for modifiers of the BMP-Smad mutant, *Mad*, and turned out to be the pyruvate dehydrogenase phosphatase (PDP), previously known for its function in mitochondria (Chen et al 2006). Interestingly, this phosphatase appears to be specific for BMP signal-transmitting Smads in mammals, and has no activity on TGF β Smads 2 and 3.

The phosphatase that acts on Smads relaying TGF β /Activin/nodal signals is protein phosphatase 1A (PPM1A, aka PP2C α), which was found to also function as a facilitator of the nuclear export of C-terminally dephosphorylated Smads 2 and 3 (Lin et al 2006). Depletion of PPM1A lead to an enhanced TGF β response, confirming the role for this phosphatase in the normal modulation of TGF β signalling which, in its turn, is known to regulate PPM1A stability (Bu et al 2008). Interestingly, stabilisation of PPM1A is performed by an important regulator of normal, and especially abnormal, cell growth PTEN (phosphatase and tensin homologue), known for its strong positive correlation with carcinogenesis and malignancy.

Another important group of Smad phosphatases is the so-called small C-terminal domain phosphatases (SCPs 1-3). While they have been originally identified for their ability to dephosphorylate the C-termini of BMP signal-transmitting R-Smads (primarily Smad1), SCPs were also able to dephosphorylate the linker regions of all R-Smads (Knockaert et al 2006, Sapkota et al 2006), thus being able to completely erase the phosphorylation marks from the Smad1 protein. Curiously, the evidence that SCPs can dephosphorylate the linker is much more consistent than that on its ability to erase activating C-terminal signatures, as in some systems there was no evidence of such activity of the SCPs (Wrighton et al 2006).

3.4 Targeting Smads for degradation

Control of Smad levels via proteosomal degradation is a well-established important mechanism for regulation of the Smad availability and consequently their signalling. It is likely that, together with controlled dephosphorylation, it could serve as one of the key mechanisms by which the cell maintains and/or limits its sensitivity to the changing level(s) of extracellular ligands. It is important to note that both C-terminally unphosphorylated and phosphorylated (activated) forms of Smads are targeted for degradation by the 26S proteasome (Itoh & ten Dijke 2007, Wrighton et al 2006). A number of E3-class ubiquitin

ligases were found to be capable of mediating Smad degradation: the HECT-domain ligases Smurfs (Smad-ubiquitin regulatory factors) 1 and 2 and related proteins, including NEDDs, Itch, WWP/Tiul1, as well as a few other ubiquitin-ligases such as CHIP, Skp1-Cul-F-box (SCF)/Roc1 complex and Arkadia, a U-box E3 ligase (reviewed in Itoh & ten Dijke 2007, Izzi & Attisano 2004).

One of the first observations linked the activated phosphorylation status of Smad2 with degradation, ensuring the turnover of the active form and serving as an example of the negative feedback ensuring transient nature of the activation of the pathway (Lin et al 2000, Lo & Massague 1999). Smurf2, which was identified as the protein performing this function, binds to both Smad2 and Smad3, but was found not to degrade the closely-related Smad3 itself (Bonni et al 2001). To add even more complexity to the subject, many of the Smad2-binding ubiquitin-ligases were found to stimulate degradation of this Smad and antagonise its activity (e.g. WWP1/Tiul1, NEDD4-2), some, e.g. Itch, were not shown to cause degradation and actually enhanced Smad2 signalling (Itch) (Bai et al 2004, Izzi & Attisano 2004). Other ubiquitin ligases were found to mediate the degradation of Smad3 but, curiously, most of them seem to either preferentially degrade the unphosphorylated form (Axin/GSK-3 β), or display no preference (CHIP, SCF/Roc1) (Guo et al 2008, Izzi & Attisano 2004).

Arkadia, a nuclear RING-domain E3 ubiquitin ligase, was found to be involved in the degradation of activated Smads 2 and 3 in mouse embryonic tissues (Mavrikakis et al 2007). Surprisingly, inactivation of Arkadia activity led to a nuclear accumulation of phospho-Smad2/3 accompanied by a decrease in the pathway's activity. Conversely, overexpression of Arkadia did not repress, and in some settings actually activated, the pathway (embryonic stem mouse cells), while leading to the decline in total abundance of the Smads (Mavrikakis et al 2007). Unlike Smurf2, which is thought to interact with both TGF β and BMP Smads, Smurf1 appears to specifically target Smads 1 and 5, while showing a strong preference for linker-phosphorylated forms, resulting in both exclusion from the nucleus and degradation (Sapkota et al 2007).

Much less is known about the regulation of the proteasome-dependent degradation of co-Smad Smad4, which is thought to exist in at least a partially-phosphorylated form. It was recently shown that phosphorylation by the JNK/p38 kinases, often enhanced for some oncogenic Smad4 mutants, has been shown to promote Smad4's degradation via the SCF complex-mediated polyubiquitination mechanism (Yang et al 2006). Other ubiquitin E3 ligases performing this function include Jab1 and CHIP (Itoh & ten Dijke 2007). Many other ligases can degrade Smad4 via Smad7 interaction, including Smurfs 1 and 2, NEDD4-2 and Tiul1 (Moren et al 2005). Another important RING-type ubiquitin ligase, Ectoderm (also known as TIF1 γ), was found to efficiently antagonise TGF β /BMP signalling by degrading Smad4 in settings ranging from developing embryos to transformed neoplastic cells (Dupont et al 2005).

In *Drosophila*, an unexpected player-translation initiation factor eIF4A-was found to directly interact with the fly's Smad1 and 4 homologues and enhance their degradation, acting synergistically but independently of the *Drosophila*'s Smurf homologue (Li & Li 2006).

3.5 Controlling the nucleo-cytoplasmic shuttling of Smads

One of the important properties of the intracellular distribution of Smads is the very dynamic nature of their shuttling between the nucleus and the cytoplasm. Even in a cell with active Smad signalling, when most Smads and their complexes are localised to the nucleus, a fraction of them appear to be dephosphorylated and exported from the nucleus (Xu et al 2002).

Despite the presence of what appears to be a nuclear localisation signal (NLS) in the MH1 domain, it has been shown that the nuclear import of co- and R-Smads does not involve the participation of the conventional nuclear transport factors, importins (Xu et al 2000, Xu et al 2002). It becomes possible due to the ability of Smads to directly interact with nucleoporins, the proteins forming the actual nuclear pore complex, as has been specifically shown for nucleoporins Nup153 and Nup214 (Xu et al 2000, Xu et al 2002). The so-called hydrophobic corridor, a contiguous set of hydrophobic areas of the MH2 domain, of R-Smads is responsible for direct interaction with the FG repeat region on nucleoporin (normally interacting with importins) (Xu et al 2002).

Some evidence suggests the existence of the Smad shuttling involving conventional nuclear transport receptors. For instance, Smad3 was found to undergo importin- β dependent translocation, but direct comparison of the contribution of importin-dependent and -independent processes revealed significant dominance of the latter (Xu et al 2003). Another important example of Smad “assisted” redistribution is CRM1-dependent export of Smad4 (Pierreux et al 2000). This process is known to depend on a leucine-rich nuclear export signal (NES), located in the N-terminal portion of the linker region (Watanabe et al 2000). Smad4 NES mutant variants are indeed retained in the nucleus permanently (Watanabe et al 2000). The nuclear import of Smad4 also differs from that of R-Smads, with its basic bipartite NLS binding to importin- α leading to nuclear localisation following the conventional pathway (Reguly & Wrana 2003).

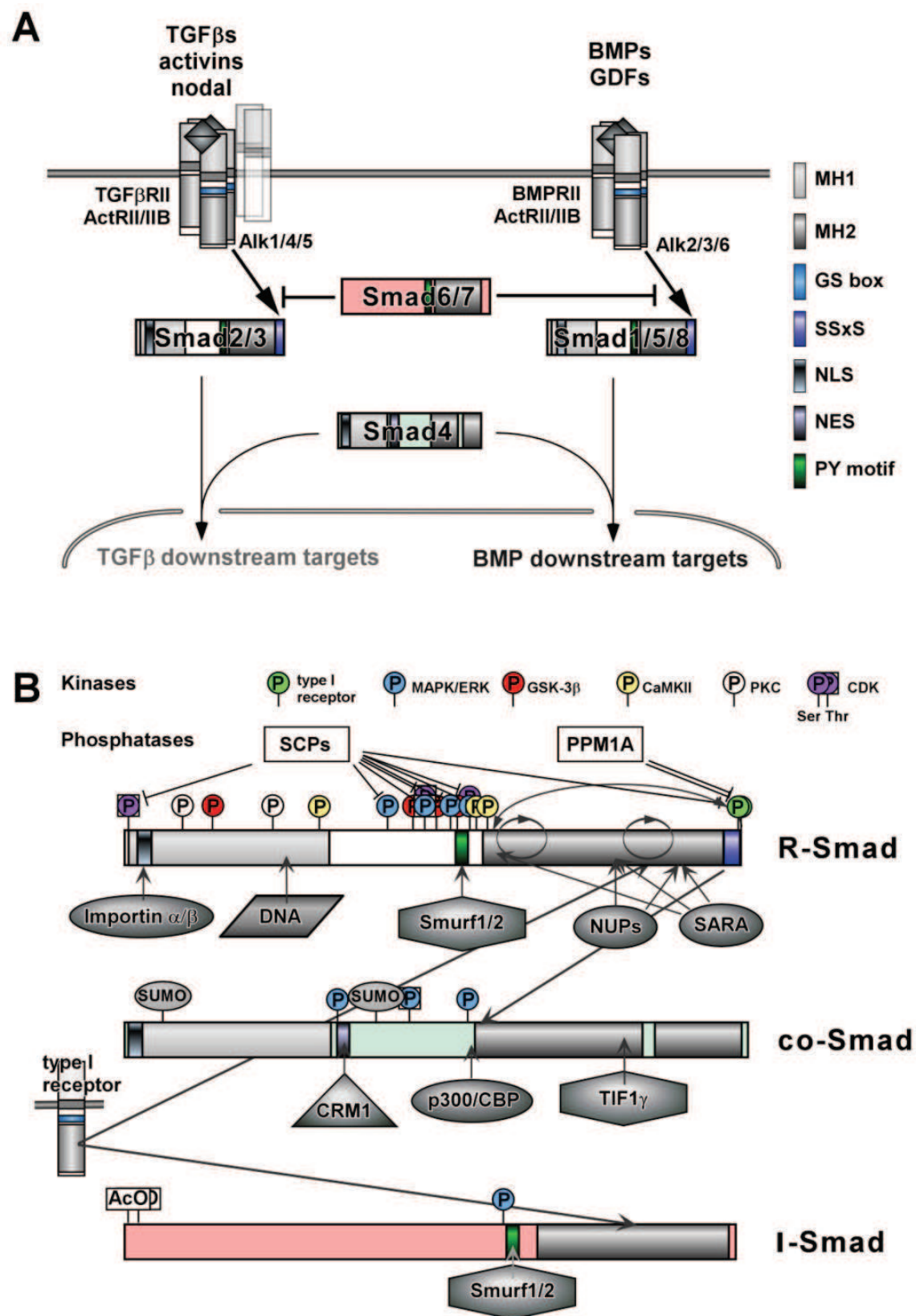
3.6 Sequestration of Smads from signalling

By definition, essentially any high-affinity interaction may be construed as a sequestration from signalling if it renders Smads unable to transmit signal by regulating the activity of the downstream target gene. Many of these interactions act to prevent shuttling of Smads into the nucleus, while others target them to particular locales in the cell, such as membranes and the nuclear envelope, or compete for binding, for instance between R-Smads and the co-Smad. Some of the better-characterised cases include the transcriptional repressor SnoN (Ski-related novel protein N), known to cause retention of Smads in the cytoplasm (Krakowski et al 2005). One of the integral proteins of the internal nuclear membrane, Man1 (also known as LEMD3), can sequester R-Smads to the inner nuclear membrane and down-modulate the levels of TGF β signalling (Lin et al 2005, Pan et al 2005).

Another important negative regulator of Smad activity, Ectodermin (TIF1 γ), was identified recently in the haematopoietic system, where it plays a pivotal role in controlling haematopoietic stem cell proliferation and differentiation. Using proteomics analysis, it was found to act as a competing binding partner with Smad4 for the phosphorylated form of Smad2 (He et al 2006). As we mentioned earlier, Ectodermin also appears to function as a ubiquitin ligase for Smad4 itself, thus antagonising the Smad4 function via two distinct mechanisms (Itoh & ten Dijke 2007).

3.7 Alternative splicing of Smads

In recent years, an understanding of the important role of alternative transcripts, their functions and expression patterns has received increased attention. As a result, a significant amount of data has been accumulated on alternative splice isoforms of the Smad genes (reviewed in Tao and Sampath, 2010). We will draw on a few examples that illustrate how even minimal changes in splicing patterns can lead to the generation of proteins with



modifications and interactions. Kinases targeting known colour-coded phosphorylation sites in Smads are shown on the top of the panel. Smad domain coding as in panel A. Hammerheaded lines show specificity of phosphatases towards particular phosphoserine/threonine residues. Selected major interacting proteins shown under the Smad of each type. Oval-shaped proteins positively, and triangle shaped-negatively regulate the Smad signalling. Two shown ubiquitin-ligases (Smurf and TIF1 γ /ectodermin) shown as hexagons. Arrows indicate interacting region of proteins, arrowed circle – regions involved in homotypic interactions. Abbreviations: NUPs-nucleoporins, SUMO-sumoylation and AcO-acetylation sites on Smad4 and inhibitory Smads, respectively. Modifications and interactions often differ for different Smads of the same type, and only selected examples shown. Drawings are not to exact scale. See text for more details.

significantly different properties. The first example is the retention of exon 3 in the majority of *Smad2* transcripts. As this is indeed the prevalent transcript form, it was designated *Smad2*, even though the corresponding protein appears to lack any DNA-binding activity due to an insertion of the polypeptide encoded by exon 3 near the β -hairpin in the MH1 domain, normally responsible for this interaction (Shi et al 1998). Interestingly, despite the fact that the *Smad2* transcript is normally 3 to 10 times more abundant than the one lacking exon 3 (*Smad2 Δ 3*), mice homozygous for the allele where exon 3 is deleted appear to be viable, unlike *Smad2*-deficient animals, suggesting that *Smad2* transcript-encoded protein does not play a unique vital role in development (Dunn 2002). This raises questions about the actual function of the longer Smad2 protein, as its *Drosophila* orthologue, *DSmad2*, also contains a similar “insertion” (Brummel et al 1999). It was reported that the long Smad2 can form an active transcription activation complex (with Smad4 and a co-factor FoxH1/FAST1) at the promoter of a downstream gene, *Mix2*, however it is difficult to imagine it making a significant contribution to DNA binding. It could serve as an “adaptor” enhancing the interaction of other transcription factors, or it might even have a role as a competitive inhibitor of Smad2 Δ 3/Smad3 signalling.

Another interesting example is the splicing isoform of Smad4 lacking the nuclear export signal, NES, encoded by exon 3, or the adjacent sequences in exon 4, shown to have a similar effect on nuclear retention (Pierreux et al 2000). A number of splicing isoforms, including those lacking the NES, can be detected at a comparable, albeit somewhat lower, to the normal form levels in various tissues. The protein forms derived from transcripts lacking either exons 3 or 4 appeared to enhance the response to TGF β signals (Pierreux et al 2000). In the absence of the TGF β /BMP signal Smad4 is present in and shuttling between the nucleus and the cytoplasm, and nuclear Smad4 is thought to act to ensure low basal level of expression of the Smad target genes by forming complexes at their promoters with the SnoN transcriptional repressor (Stroschein et al 1999). Thus, expression of a population of the NES-deficient, nuclear-bound Smad4 might serve a dual purpose-to sensitize cells to the TGF β /BMP signal by minimizing the basal signal level and amplifying the response upon activation of the R-Smads.

Recent advent of the alternative transcript-detecting microarrays will greatly aid in shedding some light on regulation of the alternative promoter and splicing pattern usage in Smad signalling, with a particular interest in the signalling in pluripotent and undergoing directed or undirected differentiation stem cells.

4. Smads in the differentiation and maintenance of pluripotency

Both the maintenance of pluripotency by embryonic stem cells and the differentiation decisions they make are known to be greatly dependent on signalling by the members of the TGFβ superfamily. This appears to be true for both *in vitro* and *in vivo* settings. To date, all embryonic stem cells are known to be dependent on signalling by either molecules of the BMP/GDF family, (as is the case with mouse ES cells) or the TGFβ/activin/Nodal family (in case of human ES cells) to maintain their pluripotent state. Many of these signals are converted into cellular responses (such as changes in gene expression or cytoskeletal reorganisation) via the canonical, or Smad-mediated, signalling pathways. In this section, we will focus on the specific evidence linking roles of the Smad proteins to the maintenance of pluripotency and the differentiation of particular germ layers and cell types, drawn from both *in vivo* (to a large extent on the mouse model) and *in vitro* studies.

4.1 Role of Smads in early embryo patterning and development of germ layers

The advent of mouse molecular genetics, and in particular homologous recombination-based gene knockouts (KOs), enabled researchers to unequivocally address questions about

Phenotype	Smad knockout							
	1	2	3	4	5	6	7	8
Lethality at (dpc)	10.5	8.5	-	7	10	-	-	-
Epiblast proliferation				X				
Gastrulation				X				
Left-right axis defects	X				X			
Mesoderm formation				X				
Cardiac development					X	X		
Angiogenesis					X			
Vasculogenesis					X	X		
Hematopoietic system			X				X	
CNS development				X				
Definitive endoderm		X						
Primordial germ cells	X				X			
Extraembryonic tissues	X	X						
Tumours in hets (adult)			X	X				

Table 1. A brief summary of single knockout phenotypes for mouse *Smad* genes. See text for more details.

the essential roles of genes in mammalian development. The limitations of conventional KOs, associated with the early lethality of complete gene KO, or the functional redundancy of the target gene, can be overcome by the use of recombinase-dependent conditional knockouts (cKOs) and/or genetic intercrossing approaches. However, to the date, our insight into the requirements of Smads in mammalian development is limited to the phenotypes of single mutants, some of their combinations and selected tissue-specific cKOs. Simultaneous cKO in various tissues of all R-Smads involved in the transmission of the TGF β /activin/nodal (Smads 2/3) or BMP/GDF (1/5/8) signals will provide us with important insight into the interplay of signalling by these branches of the TGF β superfamily, addressing one of the most interesting questions in the field.

Manipulation of Smad4 function in mice has been extremely fruitful in allowing us to assess the function of the canonical, Smad-mediated TGF β superfamily signalling as a whole, as this Smad is thought to function as a co-Smad in all R-Smad-mediated events. Full knockout of Smad4 is early embryonic lethal (Sirard et al 1998, Yang et al 1998), with defects in epiblast proliferation and gastrulation. Interestingly, a conditional knockout of Smad4 in epiblast resulted in a much milder phenotype, and demonstrated that BMP signal-mediating Smad signalling is dispensable for some aspects of gastrulation (Chu et al 2004). Consistent with the role in carcinogenesis, mice heterozygous for the Smad4-null allele exhibited a high incidence of intestinal tumours (Yang et al 1998).

Smad1-deficient mice die at ~10.5 dpc due to implantation defects, and chimera experiments show that this Smad's function is essential in extraembryonic tissues and allantois (Tremblay et al 2001). Apart from a prominent defect in germ cell formation, the embryo proper appears to develop normally, suggesting a functional rescue by another Smad, e.g. Smad5, as BMP signalling is known to play a prominent role in early embryo patterning (Tremblay et al 2001).

Smad2 was found to be indispensable for the development of the endodermal lineages, with a clear inability of Smad2-deficient cells to contribute to definitive and visceral endoderm (Tremblay et al 2000). Interestingly, those experiments also confirmed a functional redundancy of Smad2 with other (almost certainly Smad3, expressed in the embryo proper and known as another TGF β /nodal Smad) transmitters of Nodal signalling in the embryo. At the same time, in the visceral endoderm the phenotype became apparent due to a known lack of potentially compensatory Smad3 expression in that tissue (Tremblay et al 2000). Another study suggested a role for Smad2 in the extraembryonic tissues, important for gastrulation, as well as in the regulation of embryo rotation and the later development of anterior structures (Heyer et al 1999).

Analysis of Smad3-deficient mice revealed that its function is dispensable during embryonic development, but is important for various aspects of normal immune responses, such as T-cell and splenocyte functions. These mice also show an improved wound healing response, defects in articular cartilage (Ashcroft et al 1999, Yang et al 1999b) and had a higher propensity to develop metastatic colorectal cancers (Zhu et al 1998).

Interestingly, ablation of the Smad5 protein in mice lead to a defect in germ cell development similar to that observed in Smad1-null mice, suggesting that either the maximal level of BMP signalling specifying those cells requires both Smad 1 and 5 functions or, despite the high similarity of the protein structure these Smads perform not fully overlapping (and thus redundant) functions (Chang et al 1999, Chang & Matzuk 2001). This hypothesis was tested by removing one copy of each of *Smad1* and *Smad5* genes, with the resulting double heterozygous animals displaying primordial germ cell, cardiovascular and

allantoic development defects (Arnold et al 2006). Other abnormalities found in Smad5-deficient mice include angiogenesis and vasculogenesis defects, left-right patterning abnormalities and multiple other extraembryonic and embryonic defects (Chang et al 1999, Chang & Matzuk 2001, Yang et al 1999a).

Unlike other Smad knockouts, Smad8 deficiency in mice does not lead to any obvious phenotype, nor does it enhance the phenotype of either Smad 1 or 5 heterozygous mice (Arnold et al 2006). Unlike Smads1/5, Smad8's expression appears to be very restricted, with only some expression in the visceral yolk sac during early development and very specific expression domains observed later on (Arnold et al 2006). It has to be noted that it is quite possible that in different vertebrates this highly conserved gene has an important and unique role to play, since the expression domains and levels are quite likely to vary between different taxa. Smad8 is somewhat divergent from the highly homologous Smads 1 and 5, and may perform other functions. For instance, epigenetic silencing of Smad8 was uncovered in 30% of breast and colon cancer samples (Cheng et al 2004), pointing to a potential role for Smad8 in modulating cell growth rate.

Importantly, inhibitory Smads 6 and 7 differ from other Smads in that their regulation often takes place at the transcriptional level. Consistent with this notion, observed mutant phenotypes are normally expected to be confined to those tissues with high levels of Smad 6 and 7 expression. In the case of Smad6, defects in mutant mice appear to be restricted to the cardiac mesenchyme and vasculature, and include cardiac valve and septation defects, hypertension and aortic ossifications (Galvin et al 2000). Similarly, mice with a hypomorphic mutation of Smad7 appear to display only B-cell specific abnormal up-regulation of TGF β signalling (Li et al 2006). Some interesting evidence about the importance of Smad7 comes from human sclerodermal tissues and fibroblasts, which have been found to be deficient in Smad7 function (Dong et al 2002). The restoration of Smad7 levels by adenoviral expression appears to be sufficient in this system to down-regulate the TGF β signalling to normal levels (Dong et al 2002).

4.2 Smad signalling in pluripotency

Until recently, it was presumed that much, if not all, of the TGF β superfamily signalling known to be required for the maintenance of pluripotency in all known embryonic stem cells (ESCs) is transmitted via the canonical Smad pathway. However, it is only recently that specific evidence supporting this notion has started to appear.

Significant progress has been made in understanding the molecular basis of the differential requirement for the TGF β superfamily members for the maintenance of pluripotency in the *in vitro* cultures of the two main ESC models, mouse and human ESCs. The two differ greatly in that while BMP/GDF activity is necessary in addition to leukemia inhibitory factor (LIF) for the maintenance of the pluripotent state of mouse ESCs (Ying et al 2003), it induces trophoectodermal differentiation of human ESCs (Ying et al 2003, Ying et al 2008). Conversely, signalling by members of another branch of the TGF β superfamily, TGF β /activin, is essential for the maintenance of the pluripotent state of human ESCs in combination with bFGF (FGF2) (Rao & Zandstra 2005). Our own studies show that other members of the TGF β superfamily, capable of inducing sufficient activation of Smads2/3 (namely GDF11 and GDF8/myostatin), are capable of substituting for the TGF β /activin activity originating from the feeder fibroblast cells (Hannan et al 2009). This is not totally unexpected, considering the notion that while mouse ESCs, as well as ESCs from most other

organisms, represent an equivalent of cells of the inner cell mass of the blastocyst, the human ESCs more closely resemble epiblast cells of a more advanced stage embryo (Nichols & Smith 2009).

The homeobox-containing transcription factor Nanog is a well-established key player, or even a master-regulator factor, in the maintenance, and as a marker, of pluripotency in human, mouse and most other ESCs (Chambers et al 2003). Loss of Nanog function leads to the unavoidable differentiation of ESCs into extraembryonic tissues, in both human and mouse models (Hyslop et al 2005, Mitsui et al 2003). Recently, a specific mechanism for the maintenance of pluripotency was put forward (and tested in mouse ESCs) implicating one of the BMP signal-transmitting Smads, Smad1, and Nanog (Suzuki et al 2006). In this model, Nanog, induced by LIF/Stat3 signalling, prevents persistence of the BMP mesodermal differentiation-inducing signal by directly binding to Smad1 and modulating its activity in transcriptional complexes. Interestingly, more recent transcriptomics studies showed a high coincidence of the Nanog and Smad1/5-binding sites in promoters of many genes involved in the regulation of pluripotency and early differentiation events in the mouse highlighting the possibility of a more complex interplay between the two (Chen et al 2008).

In human ESCs, NANOG plays an equally crucial a role in the maintenance of pluripotency and, just like in the mouse, its enforced expression is sufficient to maintain the pluripotent state of cells even in the absence of extrinsic factors, such as TGF β /activin and FGF2 (Chambers et al 2003). The NANOG locus was found to be a direct target of Smad-mediated TGF β /activin signalling in human ESCs, with SMADs 2 and 3 shown to directly interact with the promoter (Xu et al 2008). At the same time, BMP signalling induced repression of NANOG expression coincidental with the binding of Smad1/5 to its promoter.

Another important recent finding related to Smad2/3 signalling is the elucidation of the mechanism negating the well-known mesendoderm-inducing effect of TGF β /activin while simultaneously preserving its pluripotency-maintaining action. It was discovered that one of the Smad-interacting transcriptional repressor proteins, SIP1, is capable of repressing Smad2/3-driven mesendodermal differentiation via direct interaction with the said Smads at the target genes' promoters (Verschuere et al 1999).

Interestingly, BMP signalling (albeit possibly at different intensity levels) is thought to play at least one similar role in both mouse and human ESC systems. That role is repression of the neuroectodermal differentiation via Smad-mediated up-regulation of factors of the *Id* (*inhibitors of differentiation*) family, antagonising the activity of bHLH (basic helix-loop-helix) factors promoting neural differentiation (Ying et al 2003).

Some recent evidence, however, highlights an omnipresent complexity of the effects of Smad signalling on ESCs. Knockdown of Smad4, the co-Smad commonly required by both Smad2/3 and Smad1/5/8 signalling branches, in human ESCs appears to indicate dispensability of the Smad-mediated TGF β /activin signalling for the maintenance of pluripotency (Avery et al 2010). The most logical explanation of such an effect is that the sole purpose of enhanced Smad2/3 signalling is the suppression of the signalling by Smads1/5/8, as SMAD4 knockdown will negate both. Consistent with the earlier mentioned role for BMP signalling in ESCs, cells with the diminished Smad4 activity displayed an up-regulation of genes associated with neural differentiation (*Pax6*, *NeuroD1*, *HASH1*). Significantly, in the absence of Smad4 activity, inactivation of the TGF β /activin signalling using small molecule receptor kinase inhibitor SB431542 did not result in differentiation, strengthening the possibility that human ESC differentiation is driven by the canonical Smad-mediated BMP signalling. Simultaneous dramatic up-regulation of the BMP

target genes (*e.g.* *Msx1*) and down-regulation of Smad2/3 targets (*LeftyA/B*) were observed in human ESCs during SB431542-induced differentiation. However, when SMAD2/3 and SMAD4 signalling was inactivated simultaneously, the expression of pluripotency genes *OCT4* (*POU5F1*) and *NANOG* was not significantly decreased, challenging the postulate that expression of those genes requires active canonical SMAD2/3, or for that matter any SMAD, signalling (Xu et al 2008). Further experimentation involving specific inactivation of the BMP SMAD1/5/8 signalling, either by receptor- or gene-specific inactivation approaches, will be required for further clarification of the interplay of the signalling by the two branches in the maintenance of pluripotency and early differentiation choices of human and, probably, other ESCs. Another important question to be addressed concerns the possible role of SMAD-independent signalling, as the differentiation is induced by inhibition of the type I receptor kinase activity, known to signal through the pathways not involving the Smads.

Consistent with the idea that BMP signalling is not directly involved in the maintenance of the pluripotency-controlling network, genome-wide mapping of Smad1 and Smad4 promoter occupancy indicates direct involvement in the regulation of genes responsible for lineage commitments rather than the maintenance of pluripotency (Fei et al 2010). Interestingly, the Smad1/4 binding mapped predominantly to the genes enriched in bivalent histone methylation marks (H3K27^{me3} and H3K4^{me3}) typically repressed in the pluripotent state but primed for quick induction upon differentiation. Described in the study by Fei et al. lack of the change in the pluripotency gene (*NANOG* and *OCT4/POU5F1*) expression as a result of the knockdown of *Smad4* and (to lesser degree) *Smad1* points once again to the dispensable nature of the function of Smads for regulation of key genes in the pluripotency network.

5. Smads as regulators of cellular behaviour

5.1 Context-dependent signalling by the Smad transcriptional complexes

Smads bind DNA via their N-terminal MH1 with a relatively low affinity and, unlike most other transcription factors, are thought to require association with DNA-binding co-factors, either repressors or activators for recruitment of the transcription initiation machinery (Massague et al 2005, Ross & Hill 2008). Most of the interactions with those co-factors, as well as other proteins, are thought to be mediated by the MH2 domain, and are tightly regulated by posttranslational modifications, including phosphorylation, sumoylation and, in case of inhibitory Smads, acetylation (see Figure 1B). It also has to be remembered that the majority of *Smad2* gene product contains a DNA binding-disrupting insert in the MH1 domain, and thus is likely to act as an auxiliary adaptor protein (Massague et al 2005).

Smads responsive to different branches of signalling, TGF β /activin/nodal vs. BMP/GDF, tend to depend on different co-factor sets for recruitment to the promoters of regulated genes. Smads 2 and 3, transmitting the TGF β /activin signals, tend to rely on members of the forkhead transcription factors (FoxH/FAST) and of the Mix transcription factor (Bix, Mixer) families. The partners of BMP Smads1/5/8 are less characterised, with notable exceptions of the large zinc-finger-containing Schnurri protein in *Drosophila* and Runx1-3 Runt-domain factors in mammals (Ross & Hill 2008). A common theme with all Smad-dependent transcription complexes is the requirement for the recruitment of chromatin-remodelling factors such as p300/CBP and Swi/SNF complex (Ross et al 2006). Significantly, core Smad transcriptional complexes can exist as either heterodimers or heterotrimers, depending on

the promoter and cellular state (Massague et al 2005). Some of the better studied examples include activation of the ARE (activin response element) promoters by the Smad2-Smad4 heterotrimeric activating complex (Ross et al 2006). In these cases, p300 and Swi/SNF recruitment was accompanied by the nucleosome remodelling via acetylation of histone H3, allowing for initiation of transcription by the Pol II machinery. In the case of repression of gene expression by the Smad3-Smad4 heterodimer, recruitment of HDAC4/5 by the bound Smad-containing complex lead to deacetylation of histone H4 and formation of the restrictive chromatin on the promoter region, rendering it transcriptionally inactive (Kang et al 2005).

Specificity of the promoter binding by the Smad complexes depends on many factors, including availability and identity of the transcriptional co-factors recruited to the Smad complex (Massague et al 2005). For instance, Smad complexes recruiting coactivators often contain the before-mentioned FoxH and Mix and related factors, as well as Runx, OAZ and few others. Corepressor-recruiting complexes start with E2F factors 4 or 5, Runx2 and Nkx3.2 homeobox factors (Massague et al 2005). As eluded to earlier, the chromatin-remodelling coactivators normally are p300/CBP, P/CAF, and corepressors include HDACs (1,4,5) or p107.

5.2 Smad signalling and cytoskeletal dynamics

A few lines of evidence point to the possibility that Smad signalling might be regulated by and itself regulates the changes in cytoskeletal dynamics. For instance, Smad protein levels were found to be induced in osteocytes under mechanical loading, and integrin signalling was proposed to interact with that signalling from the BMP receptors (Jadlowiec et al 2006, Rath et al 2008). BMPs are also involved in chemosensing and axon guidance by a number of cell types, and dynamics of the distribution of BMP type I and II receptors is a tightly controlled process, including by the BMPs themselves (Liu et al 2003, Nishita et al 2006, Sammar et al 2009).

5.3 Smad signalling and the cell cycle

Some of the original roles attributed to the TGF β 1-3 molecules were cytostatic and pro-apoptotic functions (Ten Dijke et al 2002). Most of these effects are mediated by regulation of the TGF β target genes with anti-proliferative or pro-apoptotic functions. One of the best-known examples is activation of the promoter of p15INK4b gene, a potent cell cycle inhibitor (Gomis et al 2006). Regulation of this promoter also represents an interesting example of attenuation of the Smad activity, with normally present C/EBP β transcriptional co-activator's function inhibited by its regulated alternatively spliced form (LIP), acting as a dominant-negative form (Gomis et al 2006). Some of the recently uncovered mechanisms for regulation of the cell cycle progression via regulation of microRNA function are discussed in the next section.

5.4 Smads and microRNAs

MicroRNAs (miRNAs) have recently been identified as omnipresent and versatile post-transcriptional regulators of gene expression, with established roles in induction and maintenance of pluripotency (Viswanathan & Daley 2010). They are thought to exert their effects by targeting specific mRNAs, typically in their 3' untranslated regions, in complexes with proteins of the Argonaute (Ago) family (Bartel 2009). This normally results in

translational repression or degradation of the targeted mRNA. One of the well-established mechanisms is the pluripotency-promoting factor Lin28, often used in the generation of induced pluripotent stem cells (iPSCs), a known RNA-binding protein found to specifically degrade the miRNA let-7 (Viswanathan & Daley 2010).

Remarkably, Smads have recently been implicated in the regulation, in a ligand-dependent manner, of miRNA activation via processing (Davis 2008, 2010). Smads were originally found to control miRNA maturation via an effect on Drosha, an RNase III enzyme, miRNA-processing complex (Davis et al 2008). It has recently been established that this processing is highly miRNA sequence-dependent, with Smads binding specifically to the SBE-like sequence in a double-stranded stem region in most cases, thus mimicking the normal DNA sequence recognised by the Smads. Some target miRNAs contained a more GC-rich consensus, resembling the recognition site for the BMP Smads1/5/8 (Davis et al 2010).

Of particular interest amongst the microRNA targets of regulation by Smads is miR-21, known to be specifically involved in the maintenance of pluripotency. In mouse ESCs, it was found that a transcription factor REST/NRST (RE-1 silencing transcription factor, a neuronal repressor) acts to promote maintenance of pluripotency via suppression of the miR-21 (Singh et al 2008). miR-21 was shown to specifically suppress the self-renewal of the mouse ESCs, and decrease the levels of expression of core pluripotency markers *Oct4/Pou5f1*, *Nanog* and *Sox2*.

Amongst other Smad-regulated microRNAs are miR-105, shown to regulate the cell cycle and apoptosis by affecting expression of PCNA and cyclin B1, and p53, respectively (Sirotkin et al 2010). miR-214 was found to regulate the polycomb group genes, in particular *Ezh2*, known to mediate epigenetic gene silencing, including in ESCs (Juan et al 2009). miR-215, another BMP/Smad-regulated microRNA, has a well-established role in regulation of cell cycle progression and checkpoint (Georges et al 2008).

Of the microRNAs containing the GC-rich Smad-binding consensus, of particular relevance is miR-23b, which is known to feed back and regulate Smads themselves in an adult stem cell setting (Rogler et al 2009). It is particularly interesting as this microRNA was found to be induced by BMP/TGF β signal themselves, suggesting the possibility of a novel negative feedback mechanism.

5.5 Role of the inhibitory Smads

Inhibitory Smads exist in all organisms, and as the name indicates act to antagonise canonical Smad signalling. In mammals, Smad6 is believed to largely target the BMP signalling, while Smad7 seems to inhibit both BMP and TGF β /activin branches (Massague et al 2005). So far, four major mechanisms have been identified: competition with R-Smads for type I receptor binding, targeting type I receptors for degradation, competing against R-Smad for the interaction with co-Smad, and recently proposed role in regulation of the nuclear Smad complex formation (Itoh & ten Dijke 2007, Massague et al 2005). Being themselves targets of TGF β /BMP signalling-induced up-regulation, inhibitory Smads are believed thus to constitute an important part of the feedback mechanism, acting to limit strength and amplitude of the TGF β /BMP signal (Massague et al 2005).

6. Conclusion and future directions

In this review, we have tried to exemplify the wide range of processes regulated by Smads and regulating them in return, as well as the major mechanisms by which that regulation is achieved.

One of the important considerations for the field that we want to bring forward is the need for a careful interpretation of the phenotypes elicited by ligand stimulation, receptor inhibition and Smad manipulation. The ambiguity in interpretations stems from the promiscuous nature of the receptors (towards both ligands, receptor partners and even R-Smads transmitting the signal), limited specificity of the small molecule inhibitors, and lastly, but very significantly, substantial amount of non-canonical, Smad-independent signalling from the receptors.

It does appear that Smads indeed act as the intracellular “hubs” of the signalling, as they integrate inputs from all major cellular pathways, including the TGF β /BMP signalling itself, the mitogen/receptor tyrosine kinase/MAPK pathway, JAK/Stat mediated pathways and the Wnt signalling pathway. It is possible then that Smads themselves can act as the key transcriptional switches. Indeed, the Smad promoter occupancy studies have identified a limited number of gene targets, many of which are the “master” regulators, positioned high in the hierarchy of transcriptional networks responsible for activation of distinct developmental programs. In particular in a naïve pluripotent stem cell it appears to be possible to manipulate lineage and cell-fate decisions by altering states of the two branches of the Smad signalling, TGF β /activin and BMP (e.g. Chng et al 2010). It is particularly interesting that maintenance of the balance of the signalling through the two branches appears not to significantly disturb the maintenance of pluripotency, supporting the idea of embryonic stem cell’s “ground state”, while the disbalance causes differentiation with a strong and consistent slant in lineage choices (Figure 2, Avery et al 2010, Ying et al 2008).

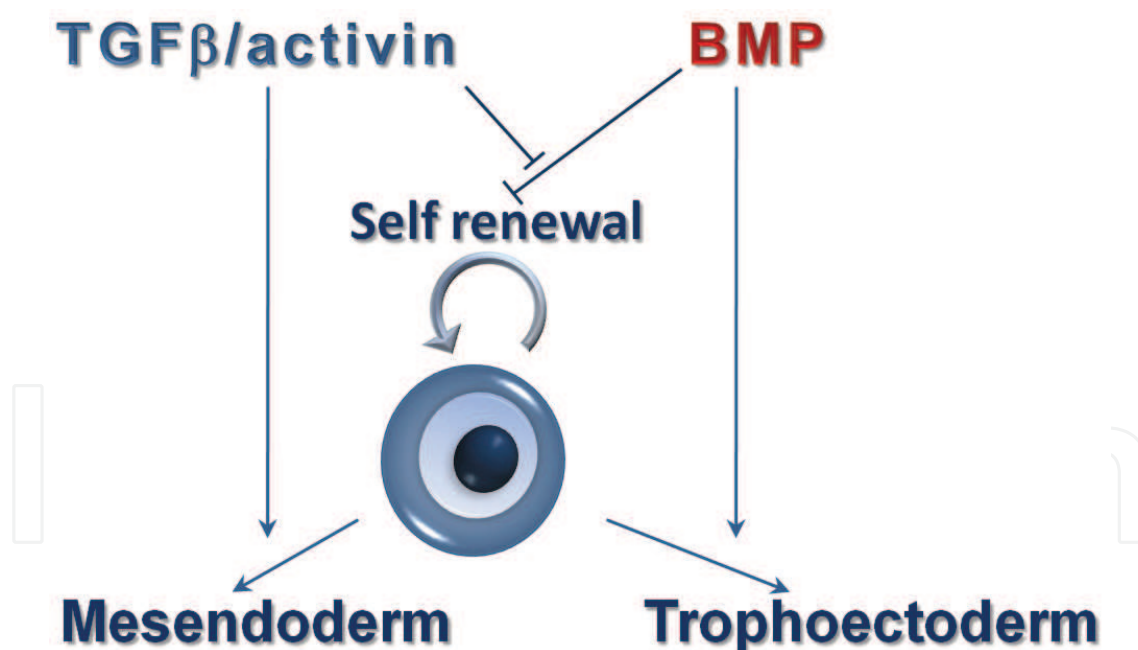


Fig. 2. Recent evidence suggests that Smad-mediated signalling (from either TGF β /activin or BMPs) is dispensable for maintenance of the pluripotent “ground state” of the embryonic stem cell. See text for discussion.

Some of the most intriguing questions include dissection of the “Smad code”, i.e. different levels of the TGF β /activin and BMP Smad activities, for the various lineage-specification programs for early lineage commitment and downstream cell-type differentiation decisions. It is possible, however, that a significant input from the non-canonical, Smad-independent

signalling is required in establishment and execution of those differentiation programs. It is also important to perform a genome-wide occupancy assessment for the TGF β /activin and BMP Smads at various levels of inputs from other interfering pathways, such as mitogen/ERK, Wnt and JAK/Stat-mediated signals.

To properly understand the nature of Smad activity in particular tissues, a comprehensive analysis of alternative transcript abundance might prove fruitful. For instance, the question about the biological role of the major, exon 3-retaining form of *Smad2* remains open, as it is believed this form does not bind DNA. The recent availability of microarrays containing probes for various significantly represented transcript forms will prove extremely useful in that task.

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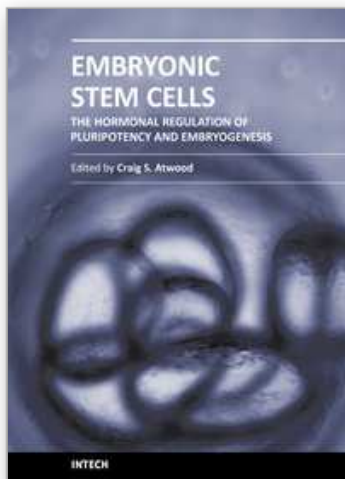
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