Activin/Nodal Signaling in Stem Cells

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Summary

Activin/Nodal growth factors control a broad range of biological processes including early cell fate decisions, organogenesis and adult tissue homeostasis. Here, we provide an overview of the mechanisms by which the Activin/Nodal signaling pathway governs stem cell function in these different stages of development and describe recent findings which associate Activin/Nodal signaling to pathological conditions, focusing on cancer stem cells in tumorigenesis and its potential as a target for therapies. Lastly, we will discuss future directions and questions that currently remain unanswered on the role of Activin/Nodal signaling in stem cell self-renewal, differentiation and proliferation.

Introduction

Activin and Nodal are members of the Transforming Growth Factor β (TGFβ) superfamily of morphogens, which comprises at least 42 members in humans and includes inhibins, TGFβs, bone morphogenetic proteins (BMPs), growth and differentiation factor (GDF), myostatin, Müllerian-inhibiting substance and others (Oshimori and Fuchs, 2012a). The TGFβ superfamily is found in metazoans and arose alongside multicellularity, with the Nodal, Activin and BMP families considered as the most evolutionary ancient family members (Pang et al., 2011). Nodal was identified in mouse through a retroviral insertion mutagenesis screen (Robertson et al., 1986) and indicated a distinct expression in the node region while causing a striking defect in gastrulation upon its disruption (Conlon et al., 1991; Conlon et al., 1994; Zhou et al., 1993). The Nodal subfamily is present in most metazoans except Drosophila and C. elegans (Rebagliati et al., 1998). Activin was discovered in the 1980s as a gonadal protein that induced Follicle Stimulating Hormone (FSH) release but since then has been found to be expressed in many different cell types at nearly all stages of development (Vale et al., 1986). Nodal and Activin ligands can both signal through the same receptors and effectors in order to regulate transcription. In many cases the effects of Nodal and Activin-mediated signalling are indistinguishable; hence they are referred to as the Activin/Nodal pathway. Similarly, as discussed further below, Nodal/Activin and TGFβ pathway share the downstream effectors Smad2 and Smad3. Thus, these pathways are often considered to have similar functions even though their tissue expression pattern is often different.

Nodal was one of the first genes knocked out in mice (Collignon et al., 1996; Zhou et al., 1993) and its function in early development has been broadly studied in different model organisms. Of particular relevance, genetic studies in the mouse have established

that Nodal signalling is necessary at the early epiblast stage during implantation where the pathway functions to maintain the expression of key pluripotency factors as well as regulate the differentiation of extra-embryonic tissue. Activins, dimers of different subtypes of Inhibin β, are also expressed in pre-implantation blastocyst but not in the primitive streak (Albano et al., 1993; Feijen et al., 1994). However, genetic studies have shown that Inhibins β are not necessary for early development in the mouse (Lau et al., 2000; Matzuk, 1995; Matzuk et al., 1995a; Matzuk et al., 1995b). Combined gradients of Nodal and BMP signalling within the primitive streak control endoderm and mesoderm germ layer specification and also their subsequent patterning whilst blocking neuroectoderm formation (Camus et al., 2006; Mesnard et al., 2006). Following implantation, a gradient of Nodal signalling defines the proximal–distal axis, which in turn establishes the anterior–posterior axis of the developing embryo (Arnold and Robertson, 2009). At later stages of embryogenesis, Nodal governs left–right axis asymmetry and further patterning of the neural and gut tubes (Brennan et al., 2002; Saijoh et al., 2003; Schier et al., 1997). In parallel, a vast number of studies have shown that Activin/Nodal morphogens regulate a range of cellular processes including cell cycle progression, progenitors proliferation/differentiation during organogenesis (Brennan et al., 2001; Feldman et al., 1998; Gritsman et al., 2000), as well as adult tissue homeostasis in some tissues (Strizzi et al., 2012). Of note, deregulation of TGFβ and Activin/Nodal signalling pathways also plays a prominent role in tumorigenesis and metastasis (Massague, 2008), which may be related to the function of these signalling pathways in embryonic development.

Consistent with its role in the epiblast stage, Nodal/Activin signalling has recently been shown to maintain pluripotency in human pluripotent stem cells (hPSCs) (Vallier et al., 2004) and also in mouse epiblast stem cells (mEpiSCs) (Brons et al., 2007). This

function is achieved through complex interactions with pluripotency factors including Nanog (Vallier et al., 2009a) and also by cross-talk with cell cycle related mechanisms (Pauklin and Vallier, 2013).

The near ubiquitous activity of Activin, Nodal and TGFβ during development and their function in tissues containing well established adult stem cells tentatively suggest that the function of Activin/Nodal signaling in self-renewal could be conserved across embryonic and tissue-specific adult stem cells.

In this review we discuss the role of Activin/Nodal signaling pathways in mediating pluripotency and early cell fate decisions, embryonic development, adult tissue homeostasis and tumorigenesis, with the aim to identify common stem cell related mechanisms. We also briefly discuss the function TGFβ signalling in these processes and the similarities with its sibling pathway Activin/Nodal.

Activin/Nodal signaling pathway

Ligands and Receptors

Nodal is synthesized as precursor, with a large pro-domain and a mature carboxy-terminal domain, which is cleaved by pro-protein convertases Spc1 and Spc4 (Constam and Robertson, 2000) to generate an active protein. Nodal forms homomeric dimers which are held together by disulphide bonds. There is only one Nodal gene in mouse, human and birds (Zhou et al., 1993), three in zebrafish (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998) and five in Xenopus (Jones et al., 1995; Joseph and Melton, 1997). In contrast, Activins are formed by homodimers or heterodimers of inhibin subunits (βa, βb, βc, βe) which are also held together by a disulphide bond. The combination of different inhibin subunits results in a diversity of Activins with Activin A (inhibin βa dimer), B (inhibin βb dimer) and AB (dimer of

inhibin βa and βb), being the most studied and the most evolutionary conserved. Genetic studies have shown that Inhibin βa and βb subunits have different functions in late development and adult tissues (Matzuk, 1995; Matzuk et al., 1995a; Matzuk et al., 1995b) while Inhibin βc and βe do not appear necessary for normal development and homeostasis (Lau et al., 2000).

Activins and Nodal exert their biological effects by interacting with two types of transmembrane receptors (types I and II), which have intrinsic serine/threonine kinase activities in their cytoplasmic domains (Figure 1 and Table 1) (Wrana et al., 1994). Activin/Nodal bind to type II Activin receptors (ActRII/IIB), leading to the recruitment, phosphorylation, and activation of type I Activin receptors (Activin receptor-like kinases – ALKs, including ALK1–7) and in particular ALK4, also known as ActRIB (Tsuchida et al., 2004). The serine/threonine kinase receptors ActRII/IIB and Alk4/7 then trigger the phosphorylation of the Smad transcription factors, discussed further below (Wrana et al., 1994). Of note, TGFβ members bind to a different set of receptors TGFBRI and TGFBRII (or Alk5) (Figure 1).

Activin/Nodal often act as morphogens (Box1) and their activity is regulated by multiple mechanisms including extracellular antagonists (Lefty1/2, Cerberus, Follistatin) and agonists (Cripto), processing enzymes (Spc1, Spc4), intracellular molecules (Smad6/7, TMEPA1) and co-regulators (FoxH1), as well as proteins involved in receptor trafficking and miRNAs (Schier, 2009) (Figure 1 and Table 1). These mechanisms coordinate the activity and tissue specificity of this important signaling pathway in different cellular and developmental contexts.

Smads and Smad-binding transcriptional regulators

The Activin/Nodal pathway exerts its effects by orchestrating transcriptional networks

controlling gene expression and downstream cellular processes. This is mediated by three classes of Smad proteins; the receptor-regulated R-Smads, the common-mediator Co-Smads and the inhibitory I-Smads. Smad1/5/8 signalling is activated by other TGFβ superfamily members such as BMP while Activin/Nodal and TGFβ signalling pathways are specifically mediated through Smad2 and Smad3 (R-Smads), Smad4 (Co-Smad) and Smad7 (I-Smad) (Figure 1, Table 2) (Shi and Massague, 2003). Smad2 and Smad3 form a complex (Smad2/3) in the cytoplasm, which interact with Smad4 after phosphorylation and then moves into the nucleus. R-Smads and Co-Smads contain a highly conserved amino-terminal Mad homology MH1 domain, a weakly conserved linker region, and the carboxyl-terminal MH2 domain (Figure 2)(Massague et al., 2005). The MH1 domain mediates the binding of Smads to DNA and their interaction with other transcription factors. The MH2 domain is involved in transcriptional activation, interaction between Smad proteins or its transmembrane receptors, as well as its binding to various transcription factors (Wrana, 2000). Phosphorylation of the linker region of Smads affects their stability and their movement to the nucleus - thus regulating the abundance of Smad proteins on the chromatin (Kretzschmar et al., 1999). The linker domain also mediates Smads proteasome-mediated degradation through interaction with Smurf proteins (Zhang et al., 2001). In addition, Smad2/3 is activated by receptor-mediated phosphorylation and inhibited by phosphatases such as PPM1A (Lin et al., 2006). Dephosphorylated Smad2/3 is then recognized by RanBP3 and exported out of the nucleus (Dai et al., 2009).

Smad4 and the R-Smads, with the exception of Smad2, bind directly to DNA although with low affinity and low specificity (Ross and Hill, 2008). Smad3 and Smad4 recognize a Smad-binding element (SBE), which consists of AGAC or its reverse complement. In order to achieve higher affinity and selectivity for DNA binding sites,

Smad proteins can also associate with various tissue-specific transcription factors (see Table 2) which mediate a range of processes including pluripotency (Mullen et al., 2011) (Suzuki et al., 2006; Vallier et al., 2009a), mesendoderm specification (Teo et al., 2011), muscle cell (Mullen et al., 2011) and hematopoietic differentiation (Trompouki et al., 2011). The Smad2/3 transcription factor complex can additionally recruit positive or negative regulators of transcription such as histone acetyltransferase CBP/p300 or histone deacetylases HDAC1-6, respectively. Smad2/3 can also cooperate with co-regulators SWI/SNF, MEDIATOR/ARC105 and NuRD in inducing or repressing the expression of various target loci (Ross and Hill, 2008). The resulting complexes (Smad-transcription factors-Cofactors) ensure a cell type-specific transcriptional response, either by activating or repressing transcription, which thereby enables Smad2/3 to control a range of mechanisms with sometimes opposing functions (Mullen et al., 2011).

Activin/Nodal signaling in embryonic stem cells

Activin/Nodal signaling in pluripotency

The function of Activin/Nodal signalling in germ layer specification has been broadly studied in model organisms: first in the mouse embryo (Conlon et al., 1991; Zhou et al., 1993) and later in Xenopus (Jones et al., 1995), and Zebrafish (Feldman et al., 1998). It was initially found that Activin/Nodal signalling was necessary for endoderm specification (Jones et al., 1995; Zhou et al., 1993) and this view remained unchallenged until *in vitro* studies in human embryonic stem cells (hESCs) suggested that Activin/Nodal signalling was necessary and sufficient to maintain the pluripotent status of the post-implantation epiblast (Vallier et al., 2004). This initial report was followed by *in vivo* studies in mouse showing that the absence of Nodal signalling results in the loss of pluripotency markers and the gain of ectopic neuroectoderm marker expression

in the epiblast immediately following implantation (Camus et al., 2006; Mesnard et al., 2006) (Figure 4). Therefore, Activin/Nodal signalling appears to operate via similar mechanisms during both in the mouse epiblast and in hESCs grown *in vitro*. This hypothesis was confirmed by the derivation of epiblast stem cells (EpiSCs) from post-implantation mouse embryo using defined culture media containing Activin and FGF (Brons et al., 2007). Similarly to hESCs, EpiSCs rely on Activin/Nodal signalling to undergo self-renewal, while chemical inhibition of the Alk4/7 receptors drive their differentiation toward the neuroectoderm pathway. Furthermore, the same culture conditions can be used to induce differentiation of mouse EpiSCs, hESCs and human induced pluripotent stem cells (hiPSCs) into derivatives of the three germ layers, confirming that these pluripotent cells rely on the same set of signalling pathways, including Activin/Nodal, to control their cell fate decisions (Vallier et al., 2009b; Vallier et al., 2009c). Taken together, these reports lead to the conclusion that hPSCs and EpiSCs share a similar pluripotency state characterised by their dependency on Activin/Nodal signalling.

Nevertheless, hESCs and EpiSCs are not strictly identical: contrary to EpiSCs, hESCs express pre-implantation markers such as Rex1 (Chan et al., 2009) and not post-implantation markers such as FGF5 (Vallier et al., 2004), and also can exhibit X activation, indicative of pre-implantation stages (Lengner et al., 2010; Tomoda et al., 2012). These observations could underline species divergence. Indeed, human and mouse seem to use different signalling pathways during their early development (Nichols et al., 2001). Therefore, Activin/Nodal signalling could have an early function in human pre-implantation embryos, which could be masked by redundant mechanisms in the mouse embryo between TGFβs and Activin/Nodal signalling (Sato et al., 2003). Basic studies on human embryo using recent advances in single cell gene expression

profiling could be advantageous to confirm this hypothesis. These results would be essential to develop new culture systems for the derivation of “Inner Cell Mass” (ICM)-like ESCs and help to dissipate the controversy concerning the existence of ground state hESCs (Gafni et al., 2013).

FGF is also necessary to maintain the expression of pluripotency markers in hESCs (Levenstein et al., 2006). Nevertheless, chemical inhibition of FGF receptors can be rescued by increasing the quantity of exogenous Activin whereas absence of Activin signalling cannot be reversed by high dose of FGF (Vallier et al., 2005). Therefore, FGF signalling appears to synergise with Activin to regulate pluripotency rather than to act independently (Figure 4). This mechanism could involve Sox2 since this gene is regulated in hPSCs by ERK2, an effector of the FGF signalling (Yu et al., 2011). Thus, FGF may support the function of Activin/Nodal signalling in hESCs by activating a complementary transcriptional network (Goke et al., 2013).

Although Nodal/Activin signalling is crucial to maintain pluripotency in the murine epiblast and derived cells, the function of this signalling pathway in mouse ESCs remains unclear. Indeed, overexpression of Smad6/7 in mESCs grown in foetal calf serum only decreases their proliferation suggesting that TGFβ/Activin/Nodal are not required for their pluripotency (Ogawa et al., 2007). Furthermore, genetic studies in the mouse have not revealed any function for Activin/Nodal signalling in embryos at pre-implantation stages. Despite these observations, some evidence suggests a possible role for Activin/Nodal signalling in mouse ESCs. Chromatin immunoprecipitation analyses combined with deep sequencing (ChIP-Seq) analyses showed binding of the Smad2/3 complex to Oct4 locus in mESCs grown in the absence of serum, while chemical inhibition of ALK4/7 induces differentiation toward trophectoderm (Lee et al., 2011).

Further investigations are therefore necessary to define more clearly the importance of Activin/Nodal signalling in mESCs.

Interestingly, mESCs appear to rely on fundamentally different mechanisms of self-renewal when compared to hESCs. A popular model for this implies that mESC pluripotency does not require an inductive signalling pathway but rather, that it is the result of a passive balance between different signalling pathways repressing differentiation (i.e. LIF blocks mesendoderm while BMP4 blocks neuroectoderm) or the total absence of inductive signals of differentiation (2i + LIF system) (Ying et al., 2003; Ying et al., 2008). Accordingly, mESCs self-renewal can be stabilised by chemically inhibiting GSK3β and the ERK kinase pathway in the absence of exogenous growth factors, confirming that extra-cellular stimuli are not required for pluripotency in mESCs.

The situation is fundamentally different in hESCs where Activin plays a direct and inductive role not only in blocking neuroectoderm differentiation but also in maintaining the expression of key pluripotency factors such as Oct4, and Nanog (Vallier et al., 2009a). Smad2/3 also directly interact with Oct4 and Nanog across a range of promoters and may be necessary for the activity of these factors. Consequently, the Smad2/3 complex is fully integrated into the transcriptional network characterising hESCs and loss of Smad2/3 transcriptional activity consistently results in differentiation (Vallier et al., 2009a). The use of chemical inhibitors remains largely inadequate to maintain pluripotency in hESCs underscoring once again that pluripotency state of hESCs and mESCs are conceptually different. Interestingly, the attempts to generate ground state-like hESCs directly from embryos either include an exogenous source of TGFβ and/or chemical inhibition of Alk-4/7 induces differentiation of the resulting pluripotent stem cells (Gafni et al., 2013; Theunissen et al., 2014). This suggests that the

role of TGFβ in the pre-implantation human embryo has been underestimated and deserves further investigation.

Importantly, the Smad2/3 complex can be found on a range of mesendoderm genes, even in undifferentiated hESCs or in EpiSCs, and this binding could explain why transcripts of differentiation markers can be detected either by Q-PCR or gene expression array in these cell types (Brown et al., 2011). Indeed, the presence of the Smad2/3 complex on these promoters could result in transcriptional leakiness, producing significant amounts of these transcripts that are usually associated with differentiated cells. This phenomenon has little or no phenotypic effects since the proteins of the corresponding genes cannot be detected. Interestingly, a broad number of these genes display bivalent histone marks (positive and negative), which have been shown to prime transcription in stem cells (Pan et al., 2007). Activin/Nodal signalling via Smad2/3 could therefore maintain pluripotency but also enable hESCs to prime the expression of tissue-specific differentiation genes, thus allowing rapid cell fate choices. This supports the concept that hESCs may represent a primed state of pluripotency as opposed to the ground state observed in mESCs. However, mESCs grown in serum are also "primed" to differentiate toward extra-embryonic tissues (Niwa et al., 2005) or to progress toward the epiblast stage (Toyooka et al., 2008). Thus, "priming" could be a common mechanism between stem cells since the main objective of this cell type *in vivo* is not to self-renew but to generate the necessary cells for normal development and organogenesis.

Activin/Nodal signaling as inducer of endoderm differentiation

Despite its essential activity in maintaining pluripotency, Activin/Nodal signaling is also absolutely required for endoderm differentiation (Arnold and Robertson, 2009;

D'Amour et al., 2005; Kubo et al., 2004). Accordingly, inhibition of Activin/Nodal signaling blocks the expression of endoderm markers and promotes the expression of mesoderm markers in the presence of BMP4 *in vitro* (Kubo et al., 2004) and in a broad number of species (Chen and Schier, 2001).

Activin/Nodal signalling achieves this function by interacting with other key signaling pathways, especially BMP and WNT (Tam and Loebel, 2007). The molecular mechanisms involved in this cross-talk among pathways have been particularly well studied in amphibian and fish where it has been shown that BMP-Smad1/5/8 interact with mesoderm regulators such as Brachyury to repress endoderm markers, induced by Nodal-Smad2/3 (Garnett et al., 2009; Messenger et al., 2005; Morley et al., 2009). At the same time, WNT signaling also plays an essential function in mesendoderm specification by controlling the expression of Nodal and its co-receptor Cripto during gastrulation (Tam and Loebel, 2007).

Importantly, genome wide analyses performed on hESCs differentiating into endoderm have shown that the Smad2/3 complex directly controls the transcriptional activity of a broad number of endoderm genes (Brown et al., 2011). Thus, the transcriptional network driving endoderm specification is ultimately orchestrated by Smad2/3 and its partners (Table 2, Figure 4). BMP and WNT could be required only to initiate and to stabilize this network, respectively. Accordingly, BMP plays a crucial role *in vitro* to block the protective activity of Activin/Nodal signalling on pluripotency and to promote the induction of endoderm specification (Sakaki-Yumoto et al., 2013). Furthermore, WNT/β-catenin interacts with Smad2/3 target genes such as Sox17 to activate the expression of other genes such as FoxA2 (Sinner et al., 2004), which are essential for endoderm pattering and organogenesis. Further genome analyses detailing the target

genes downstream of Smad1/5/8 and β-catenin could help to further uncover the nature of the molecular cross talk between Activin/Nodal, WNT and BMP.

The mechanisms by which Activin/Nodal signalling maintains pluripotency while inducing endoderm differentiation also remain to be fully elucidated and several studies have started to reveal some important regulatory mechanisms. ChIP-Seq analyses showed that the location of Smad2/3 binding in the genome changes upon endoderm differentiation, suggesting that the specificity of Activin/Nodal signalling might be defined by the genomic location of its binding partners (Brown et al., 2011). Interestingly, the transcriptional networks downstream of Smad2/3 and Nanog, as well as Oct4, significantly overlap in hESCs, which further points to a potential interaction between these factors. Co-immunoprecipitation analyses have shown that Smad2/3 and Nanog could be part of the same protein complex in hESCs, and that they cooperate to orchestrate the transcriptional network characterizing hPSCs (Vallier et al., 2009a). Further studies have also revealed an interaction between Smad2/3 and Eomes upon mesendoderm specification (Teo et al., 2011). Therefore, it is possible to consider a model in which Smad2/3 switch binding partners during differentiation, allowing a cell type specific outcome of the Activin/Nodal signaling.

The model proposed above also suggests that inhibition of Nanog expression is necessary to enable Smad2/3 to interact with Eomes and thus, to redirect the activity of Activin/Nodal signaling towards endoderm formation. This inhibition is likely to be induced by one or more signaling pathways, which could be considered as the true inducer of differentiation. WNT and BMP signaling are the most likely candidates: their function in endoderm and mesoderm specification has been studied in amphibians, fish and mouse and they are often included in the cocktail of growth factors used to generate endoderm from hPSCs *in vitro*. Importantly, Activin/Nodal signaling cannot fulfill this

function alone, since even high doses of exogenous Activin/Nodal only reinforce the expression of pluripotent markers in hPSCs (Vallier et al., 2005).

In addition to its role in primitive streak and mesoderm induction, BMP4 has also been shown to induce the differentiation of hESCs toward extra-embryonic tissue and this effect can be blocked by Activin/Nodal (Vallier et al., 2009c). Furthermore, Smad1/5/8 and Smad2/3, which mediate BMP and Activin/Nodal signaling, respectively, bind to the same region of the Nanog promoter, suggesting that BMP4 and Activin/Nodal may compete to modulate the expression of key pluripotency markers (Xu et al., 2008). BMP4 can also induce differentiation by activating the expression of Eomes, which then feeds into the Smad2/3 transcriptional network and ultimately repress the expression of Nanog (Teo et al., 2011).

WNT signaling also plays a key function in controlling Activin/Nodal signaling since blocking the PI3K/Erk pathway and thereby inhibiting GSK3β are sufficient to induce endoderm differentiation of hESCs (Singh et al., 2012). Furthermore, β-catenin and the Smad2/3 complex have been shown to converge on mesendoderm genes to activate their transcriptional activity (Bernardo et al., 2011). Considered together, these reports support the role of WNT in modulating the activity of Activin/Nodal signaling. Nevertheless, the molecular mechanisms by which this synergy takes place remains unknown and further molecular analyses are necessary to fully understand the cross-talk between Smad2/3 and GSK3β/β-catenin.

Finally, a recent study showed that the Hippo pathway can repress Smad2/3 transcriptional activity on endoderm genes in hESCs and thus, maintain the pluripotent state and block mesendoderm induction (Beyer et al., 2013). However, this mechanism seems to be limited to primitive streak genes such as Brachyury and is cell culture

dependent, suggesting the existence of additional mechanisms involving inductive signals of differentiation.

Considered together, these observations illustrate how Activin/Nodal signaling is interconnected with other signaling pathways, enabling Smad2/3 to have divergent functions in different cell types (self-renewal versus differentiation) and to control a diversity of biological process within the same cell type. However, the precise function and specificity of Smad2/3 in all these cellular processes remains unclear. Indeed, the model proposed above explains in part that the tissue-specific activity of Smad2/3 is dictated by tissue specific transcription factors, however, it does not provide the molecular mechanism by which Smad2/3 can interact with so many factors while retaining its specificity of action. Proteomic studies in combination with DNA pull down methods could help to identify the partners that co-operate with Smad proteins to enact different processes within the same cell, such as the induction of p21 for cell cycle regulation or Sox17 for endoderm specification. These experiments could indicate if Smad2/3 has a generic function in transcriptional regulation such as recruiting epigenetic regulators or if its function varies in the context of different genes and protein complexes.

Activin/Nodal signaling in adult tissue stem cells

Many organs harbor stem cells that function in tissue maintenance and injury repair. These stem cells replenish specialized cell types throughout development and adult life either by constant cell divisions (e.g. intestinal stem cells) (Li and Clevers, 2010) or by transient activation when needed (e.g. hematopoietic system, hair follicles, mammary gland)(Fuchs, 2009; Lange and Calegari, 2010) (Orford and Scadden, 2008). The TGFβ superfamily is involved in self-renewal of adult stem cells in many of these tissues. At

high levels, TGFβ usually inhibits cell proliferation in a reversible manner and this might be particularly relevant for the regulation of quiescent state and re-entry of adult stem cells into cell cycle (Massague, 2012; Tumbar et al., 2004). The mechanism by which TGFβ regulates the cell cycle is described further below. However, the function of Activin/Nodal signalling in the self-renewal or differentiation of adult stem cells is less clear, despite several recent reports suggesting a key role (Cambray et al., 2012; Dunphy et al., 2011; Kadaja et al., 2014). Indeed, the expression of Nodal seems to be limited to certain tissues which undergo considerable remodeling such as endometrium, placenta and lactating mammary gland (Quail et al., 2013; Strizzi et al., 2012), which suggests that Nodal might not be involved in the maintenance and specification of many adult stem cells, in contrast to TGFβ, more widely expressed. , Activin transcripts can be detected in a diversity of tissues including the pituitary gland, the spleen, the bone marrow and specific parts of the brain (Luisi et al., 2001), but functions in cell cycle are yet to be fully investigated. Due to difficulties in the reliable detection of Activins and Nodal expression with the possibility of alternative splice variants for the latter (Strizzi et al., 2012), further studies are needed to generalize these observations to a broader range of adult tissues and sub-populations of cells in each tissue.

Here, we discuss key examples where Activin/Nodal signalling is known to be important in adult stem cells, and where the related TGFβ pathway plays roles that share parallels with, or may shed light on, the functions of Activin/Nodal in these stem cell systems.

Hair follicle stem cells.

In adult tissues, there are instances where Activin signalling has a specific role in maintaining cell "stemness" while the function of TGFβ seems to be less important.

Indeed, the absence of TGFβ receptor II in mouse skin epithelium does not induce large changes during normal homeostasis (Guasch et al., 2007; Oshimori and Fuchs, 2012b). In contrast, conditional ablation of the Activin receptor type 1B (*Alk4 or Acvr1b*) causes the degeneration of hair follicles and the formation of cysts with keratinaceous debris. Therefore, despite their similarities and common effectors, TGFβ and Activin signalling appear to control different mechanisms in skin stem cells (Qiu et al., 2011). Accordingly, the self-renewal of hair follicle stem cells and the suppression of epidermal differentiation involves Activin B and several other genes that are known to be involved in enhancing Activin signalling such as *Wwp2*, *S100A4*, *Sulf2*, and *Inhbb* (Kadaja et al., 2014). The expression of these genes is also controlled by Sox9, a central regulator of hair follicle stem cells. In turn, administration of Activin B can partially compensate for the loss of Sox9 in the hair follicle niche by blocking the premature differentiation of hair follicle stem cells (Kadaja et al., 2014). It would be interesting to determine if the switch between quiescent and active states of hair follicle stem cells involves specific cell cycle regulators of the INK4 and KIP/CIP family such as p15 or p21, which are known to be regulated by the cytostatic response of TGFβ signalling in various cells. In addition, the precise effect of Smad2/3 inhibition on cell fate decision in skin stem cells could also reveal novel functions of Activin signalling in their self-renewal and their capacity of differentiation.

Hematopoietic Stem Cells

In most adult stem cells such as hematopoietic stem cells or neural stem cells where TGFβ signalling plays an important role, the involvement of Activin or Nodal remains unclear. Nevertheless, as TGFβ and Nodal/Activin pathways share receptors and

transduction proteins, we will briefly summarise the function of TGFβ signalling in these stem cells and draw parallels between these two closely related pathways.

Adult Hematopoietic Stem Cells (HSCs) reside in the bone marrow among progenitors at different stages of the hematopoietic lineage (Orkin and Zon, 2008; Zhang et al., 2008). TGFβ signaling pathway has long been implicated in regulating HSC quiescence (Fortunel et al., 2000; Yamazaki et al., 2006). It functions by upregulating the transcription of the cyclin-dependent kinase (CDK) inhibitor p57 and suppressing PI3K/Akt signaling, thus preventing HSC re-entry into the cell cycle (Yamazaki et al., 2006). The latent TGFβ present in the bone marrow seems to be activated by non-myelinating Schwann cells (Yamazaki et al., 2011) and the TGFβ response is mediated by TβRII receptors leading to Smad2/3 phosphorylation.

Of note, HSCs are not a homogenous population of cells but instead can be divided into at least two distinct subtypes which have unique self-renewal properties and exhibit biased differentiation towards different mature hematopoietic lineages (Dykstra et al., 2007; Lemischka et al., 1986; Sieburg et al., 2006). Interestingly, these HSC subpopulations have distinct cellular responses to TGFβ signalling, which affects their cell cycle state (discussed below) and thus their proliferation capacities (Challen et al., 2010). Therefore, the switch model proposed for pluripotency/endoderm differentiation for Activin/Nodal-Smad2/3 in PSCs appears to be applicable to BMP4-Smad1/5/8 in HSCs. R-Smads could therefore operate in a similar manner in HSCs hematopoietic specification and in hPSCs early germ layer differentiation.

Overall, TGFβ signaling exhibits an essential function in controlling the self-renewal of stem cells in various adult tissues such as the skin, the hematopoietic system and the central nervous system. Interestingly, Activin/Nodal signaling could have complementary functions in self-renewal and differentiation of adult stem cells. Further

investigations including tissue specific gene knock out for Nodal and the Inhibins could help to further understand the specificity of each of these growth factors in organ homeostasis and tissue repair.

Activin/Nodal signaling in cancer and metastasis

Cancer stem cells (CSCs) can give rise to a new tumor which shows similar features to its parental tumor. CSCs have been identified in various cancer types including pancreatic cancer, melanoma, glioma, chronic myeloid leukemia and malignant squamous cell carcinoma (Chen et al., 2008; Driessens et al., 2012; Schepers et al., 2012; Schober and Fuchs, 2011). A number of mutations leading to cancer affect genes involved in the Activin/Nodal/TGFβ signaling pathways including ACVRI, TGFBRI/II, Smad2 and Smad4 (Massague, 2008). These mutations tend to accumulate in tissue-specific stem cells due to their longevity (Lobo et al., 2007). Interestingly, the role of Activin/Nodal signalling in tumorigenesis and cancer stem cells often reflects the function of this pathway in embryonic development or in adult tissue homeostasis. Indeed, the Activin/Nodal pathway regulates self-renewal and differentiation of cancer stem cells, and increases the plasticity and metastatic potential of tumour cells (Lonardo et al., 2011; Spiller et al., 2012; Topczewska et al., 2006). Accordingly, the mutation of the inhibin a subunit (an Activin inhibitor) in the mouse gonad results in stromal/granulosa tumour suggesting that Activin signalling could be tumorigenic if not tightly controlled (Matzuk et al., 1992). Similarly, Nodal is expressed in a diversity of tumors including melanoma, prostate, breast and testicular cancer (Hardy et al., 2010; Lawrence et al., 2011; Lonardo et al., 2011; Spiller et al., 2012; Strizzi et al., 2012; Topczewska et al., 2006) which degree of malignancy correlates with the amount of secreted Nodal (Spiller et al., 2012). In addition, the Nodal co-receptor Cripto is widely

overexpressed in tumor cells from many different origins and correlates with invasiveness and poor prognosis in melanoma, pancreatic cancer, breast cancer and testicular cancer (Lonardo et al., 2011; Postovit et al., 2008).

In melanoma, Nodal signalling also promotes the vascularisation of the tissue surrounding the tumor, which might be responsible for the malignancy and high incidence of metastases due to increased invasiveness in these cancers (Hardy et al., 2010; Seftor et al., 2012). A similar vascularisation-promoting effect has been noted for breast cancers: Nodal signalling leads to the upregulation of pro-angiogenic factors in the tissue surrounding the tumor cells (Quail et al., 2013). Based on these observations, it is tempting to hypothesise that abnormally high activation of Nodal signalling in adult stem cells, combined with genetic mutations could result in increased proliferation but also resistance to differentiation, thereby mimicking the mechanisms maintaining pluripotency of hPSCs. Future investigations including Smad2/3 ChIP-Seq and proteomic analyses in cancer stem cells will be useful to compare the mechanisms involving Activin/Nodal signalling in self-renewal and differentiation during embryonic development and tumorigenesis.

Cell cycle regulation

Although TGFβ is a know cytostatic factor (which inhibits cell growth and proliferation) (Massague, 2004) . Accordingly, TGFβ pathway has a prominent role in regulating cell cycle progression in many cell types and it acts as a central pathway for mediating cytostatic responses. In most cases, it triggers potent anti-proliferative effects by inducing the expression of cyclin dependent kinase inhibitors (CDKIs) of the INK4 (p14, p15, p16, p18, p19) or KIP/CIP (p21, p27, p57) protein family (Massague, 2008). These cell cycle inhibitors usually cause the cells to reversibly arrest in G1 phase but they can

also lead to terminal differentiation (Evans et al., 2003) or programmed cell death. Of note, this function of the TGFβ signaling pathway could be inhibited by SNON or SKI in hPSCs (Tsuneyoshi et al., 2012) since these genes are known to limit the transcriptional activity of Smad2/3 and especially to block induction of CDKi such as p21 (Zhu et al., 2007). Importantly, Activins are known to control cell cycle by similar CDKIs dependent mechanisms (Chen et al., 2002) thereby suggesting an overlapping function between TGFbeta and Activin signaling in proliferation control. Nodal function in cell cycle control remain to be fully investigated especially since Nodal seems to potentiate the plasticity and metastatic capacity of CSC.

Moreover, the interplays between Activin/Nodal/TGFbeta signaling and cell cycle regulations are certainly more complex especially in the context of stem cells. Indeed, our group and others have shown that Activin signalling in human pluripotent stem cells could be directly controlled by Cyclin D / CDK complexes which can limit the shuttling of Smad2/3 into the nucleus. Thus, these cycle regulators restrain the inductive effects of Activin/Nodal signaling on endoderm differentiation (Pauklin and Vallier, 2013). This mechanism enables cell cycle specific regulation of cell fate choice in hESCs. Endoderm differentiation can only be induced in early G1 phase when Cyclin Ds are expressed at low levels while neuroectoderm specification can only be induced in late G1 phase when Cyclin Ds are highly expressed. These mechanisms could also be important for a number of somatic stem cells since functional studies have demonstrated that loss of function of Cyclin D/CDK results in the lengthening of G1 phase in neuronal stem cells in vivo while increasing their differentiation into neurons (Lange and Calegari, 2010; Lange et al., 2009). Similarly, absence of Cyclin Ds or CDK4/6 results in premature differentiation of Hematopoietic Stem Cells (Lange and Calegari, 2010). Considered together, these studies highlight a complex relationship between cell

cycle and TGFβ/Activin/Nodal signalling pathways and how these mechanisms could be essential to synchronize proliferation and cell fate choice in stem cells.

In addition to TFGβ-mediated regulation of CDKIs, there is also evidence for TGFβ controlling cell cycle progression via other routes. Specifically, TGFβ can inhibit expression of c-myc, and also relieve inhibition of Rb expression; both these activities would repress proliferation and promote differentiation. These observations underline once again the intrinsic relationship between cell cycle regulators and cell fate choice and the essential role played by TGFβ, and potentially by Activin/Nodal, in this process. It would be tempting to suggest that aberrant regulations of these mechanisms could be part of the process leading to the emergence of cancer stem cells. This hypothesis could be explored further by studying the role of cell cycle related factors in the inhibition of cancer stem cells differentiation.

Perspectives

Activin/Nodal signaling has been shown to control various mechanisms in different model organisms and in a diversity of cell types. The function of this pathway in pluripotent stem cells remains relatively recent and opens new perspectives to understand the cross talk between cell cycle, cell fate decisions, and epigenetic regulation. It is of course tempting to hypothesize that these mechanisms could be conserved in adult stem cells and ultimately constitute the central unit defining "stemness". Indeed, Activin, Nodal or TGFβ growth factors are found in various tissues and their activity is essential for a number of cell types. However, mechanistic insight on the function of these signaling molecules in self-renewal/cell fate decisions is still

lacking. The technical challenge to perform tissue specific genetic studies in animal models explains in part this situation. The importance of other signaling pathways such as Wnt might have also obscured the role of Activin/Nodal/TGFβ in these mechanisms. Finally, the dominant function of TGFβ in cell cycle regulation might mask its role in in regulating cell fate decisions in multipotent stem cells. Indeed, gain or loss of function of TGFβ signaling members often results in uncontrolled proliferation or quiescence, both of which indirectly affect cell fate decisions, thereby masking any potential role for these factors in differentiation. The availability of new culture systems to maintain somatic stem cells in vitro such as the 3D organoid approach (Sato and Clevers, 2013), associated with efficient genome editing methods such as CRISPR (Cong et al., 2013; Mali et al., 2013) could provide new opportunities to delineate the contribution of Activin/Nodal/TGFβ in the self-renewal of adult stem cells and their differentiation toward functional cell types during organ homeostasis.

In parallel, human pluripotent stem cells represent a unique opportunity to study the molecular mechanisms controlling Activin/Nodal functional activity and cellular specificity in self-renewal and differentiation. Indeed, Activin/Nodal signaling pathway seems to be constantly controlling opposite cellular mechanisms such as proliferation vs quiescence, self-renewal vs differentiation and tumorigenesis vs apoptosis. Furthermore, a large number of genes with apparently divergent functions have been identified as targets for Activin/Nodal-Smad2/3 signalling, for example Nanog (pluripotency) and Sox17 (endoderm differentiation). Although this was initially counter-intuitive, it is now evident that Activin/Nodal signaling activity is mediated by lineage specific transcription factors that help targetting the Smad2/3 complex and co-regulator complexes to specific loci in a context-dependent manner. However, such models also raise questions concerning the molecular function of Smad2/3 in these

protein complexes, which control conflicting aspects of cellular biology. Indeed, it remains to be uncovered if the Smad2/3 complex is only necessary to build transcriptional complexes controlling the expression of different set of genes or if it has more direct function by controlling the activity of key transcription factors and epigenetic modifiers. Furthermore, the number of Smad2/3 partners continues to increase with very little overlap between different cell types. Proteome-wide analyses are yet to reveal the full list of Smad2/3 binding partners, and thus the complexity and diversity of protein-protein interactions involving Smad2/3 are likely to be underestimated. Identification of Smad2/3 binding partners at various developmental stages and in stem cells will help clarifying why Smad2/3 has so many apparently distinct functions in different developmental contexts and how this diversity is mechanistically achieved.

To conclude, TGFβ/Activin/Nodal pathways function not only in cell fate choice during embryogenesis but also in cell cycle regulation and adult tissue homeostasis. Since cross-talk between cell cycle regulation, self-renewal and differentiation is essential for controlling the function of stem cells during development and in adult organs, the TGFβ/Activin/Nodal pathways may function as a direct link between these fundamental processes. Further research will be necessary to demonstrate the importance of these mechanisms in normal regenerative process and in the formation of cancer stem cells. Thus, a more complete picture of the mechanistic aspects of Activin/Nodal signaling in stem cells could help to develop new regenerative approaches and unveil novel therapeutic targets for the treatment of cancer.

Box 1. Spatio-temporal effects of Activin/Nodal concentration gradients. Activin

and Nodal ligands have short-range effects on nearby cells as well as long-range effects during development (Smith et al., 2008). As an example of short-range effect, Nodal is positively autoregulated by Smad2/3 via an asymmetric enhancer located in its first intron (Adachi et al., 1999) and via an upstream left-side specific enhancer (Saijoh et al., 2003). For long-range effects, Nodal is secreted by node cells and can activate its target genes in distant cells of the lateral plate mesoderm (Oki et al., 2007). The Nodal effect is also dose-dependent since low levels of Nodal are sufficient to induce target genes such as Brachyury/T, whereas Goosecoid is only activated by high levels of Nodal during mesoderm and endoderm patterning (Gurdon and Bourillot, 2001; Schier and Talbot, 2005). Nodal forms concentration and activity gradients during development that provides positional information, which ultimately directs the cell fate decision of the target cells (Brennan et al., 2001). This is particularly evident in the formation of vegetal-animal axis (Faure et al., 2000; Gritsman et al., 2000; Harvey and Smith, 2009). The time of Nodal signaling is also important for cell fate decisions since duration of Nodal signaling has different effects and results in the generation of different cell types (Hagos and Dougan, 2007). Of note, the spatiotemporal effects of Activin/Nodal concentration gradients have yet to be taken into account *in vitro*. Indeed, protocols of differentiation use large doses of Activin and 2D culture system, which are likely to bypass the regulation of gradient formation. This could result in the absence of positional information during *in vitro* specification and represent one of the challenges for generating specific cell types from hPSCs.

Table 1. Main components of the Activin/Nodal signaling pathway.

Pathway Component	**Signalling Pathway**	**Gene name**	**Binding partners**	**Function**
Ligands	Nodal	*Nodal* (human, mouse, bird), *cyclops, squint, southpaw* (fish), *×nr1, ×nr2, ×nr4, ×nr5, ×nr6* (frog)	Nodal pathway inhibitors	Nodal-related TGFβ ligands, activate signalling
		Gdf1 (mouse)		Ligand, activates signalling
		Gdf3 (mouse)		
		Vg1 (frog, fish, bird)		
	Activin	*Activin bA, bB, bC, bE (human)*	Follistatin	Ligand, activates signalling
Receptors	Nodal	*ALK4, ALK7*	ActRII, ActRIIB, Co-	Type I serine-threonine kinase receptor

			receptors	
		ActRII, ActRIIB	ALK4, ALK7	Type II serine-threonine kinase receptors
	Activin	*ALK4*	*ActRII*	Type I serine-threonine kinase receptor
		ActRII	ALK4	Type II serine-threonine kinase receptors
Co-receptors	*Nodal*	*Cripto (human), Cryptic* (mouse), *one-eyed pinhead* (fish), *FRL-1/×CR1, ×CR2, ×CR3* (frog)	ALK4	EGF-CFC co-receptors, necessary for activating Nodal signalling but inhibits Activin signalling
Inhibitors	*Nodal*	*Lefty1, Lefty2*	Nodal pathway ligands	Inhibit Nodal signalling by interacting with Nodal ligands and EGF-CFC co-receptors.
		Cer1, Cer2, Gremlin		Cerberus/DAN family members; Inhibit signalling by interacting with Nodal ligands
	Activin	*Follistatin*	Activin	Inhibits signalling by binding to Activins
Intracellular transduction proteins	Nodal, Activin	*Smad2*	Smad3, Smad4	Receptor-Smads; Regulate gene transcription and cell cycle (hPSCs, endoderm differentiation, reproductive tissues)
		Smad3	Smad2, Smad4	
		Smad4	Smad2, Smad3	Co-Smad, helps transporting Smad2 and Smad3 into the nucleus
		Smad7	Smad2, Smad3	Inhibitory-Smad, blocks the activity of Smad2 and Smad3

Table 2. Known binding partners of Smad2/3 and their function. Tissue specificity is indicated where known.

Signalling pathway	**Smad family member**	**Tissue type**	**Interacting protein**	**Function**	**Target loci**
Activin/Nodal	Smad2, Smad3	Human pluripotent stem cell	Nanog	Maintenance of pluripotency	Pluripotency genes (Oct4, Nanog)
	Smad2, Smad3		Oct4		
	Smad2, Smad3		P300	Transcriptional activation by histone acetylation	

	Smad2, Smad3		SnoN	Inhibition of differentiation	Endoderm genes
	Smad2, Smad3	Mesendoderm	EOMES	Induction of endoderm	Endoderm genes
	Smad2, Smad4		FOXH1		GSC (Smad2 activates, Smad3 represses)
	Smad2, Smad3		GSC		Endoderm genes
	Smad2, Smad3	Mesoderm	Mixer	Mesoderm induction	Mesoderm genes
TGFβ	Smad3	Myotube	MyoD1	Myocyte identity	Myocyte genes
	Smad3	Pro-B-cell	Pu.1	Pro-B-cell maturation	B-cell specific genes
	Smad2, Smad3, Smad4	Keratinocyte	FOXO3	Cell cycle inhibition	CDK inhibitors p15Ink4b, p21Cip1
	Smad2, Smad3, Smad4	Keratinocyte	E2F4/5		Myc
	Smad2, Smad3, Smad4	Epithelial cells	C/EBPbeta		CDK inhibitor p15Ink4b
	Smad2, Smad3, Smad4	Epithelial cells	Sp1		p15Ink4b
	Smad2	Epithelial cells	ATF3		Id1

Figure Legends.

F**igure 1. Components of Activin/Nodal.** Extracellular ligands Activin or Nodal bind to type I (ACVRIIA/IIB) and type II transmembrane receptors (Alk4/7) while TGFβ growth factors bind to TGFBRI and TGFBRII/Alk5. Nodal requires the additional binding of the transmembrane co-receptor CRIPTO to form an activated receptor complex with type I and type II receptors. The activated receptor complex (both for Activin/Nodal and TGFβ pathways) phosphorylates Smad2 and Smad3 proteins, which enter the nucleus in

complex with Smad4. Smad proteins are targeted to distinct loci by sequence specific transcription factors which are often expressed in a cell type-dependent manner. Smad proteins act as transcriptional regulators and are able to induce or repress the transcription of their target loci by recruiting epigenetic modifiers, which will further modulate the accessibility of the surrounding chromatin by inducing epigenetic modifications on histones or DNA. In some cells types, Smad2 and Smad3 proteins can interact with Smad1, Smad5 or Smad8, which usually mediate Bmp4 signalling, thus mediating the crosstalk between Activin/Nodal and Bmp signaling pathway.

Figure 2. The functional domains and sites of post-translational modifications on Smad2/3 and Smad4 proteins. Smad proteins contain three distinct functional domains: the N-terminal MH1 domain (blue), the middle linker domain (green) and C-terminal MH2 domain (pink). Smad2/3 and Smad4 proteins not only act as important effectors for Activin/Nodal signalling pathway but interconnect various other signalling pathways which induce post-translational modifications on specific residues of Smad proteins. Colored circle - phosphorylation site, colored star – PIAS ubiquitinylation site, colored square - ubiquitinylation site, colored diamond - p300 interaction site. NLS – nuclear localisation signal, NES - nuclear export signal, DNA - DNA binding region.

Figure 3. Signalling pathways maintaining the self-renewal of hESCs. Self-renewal of hESCs is maintained by Activin/Nodal and FGF2 signalling. Self-renewal signals from Activin/Nodal signalling are mediated by Smad2/3 proteins which upon phosphorylation bind to Oct4 and Nanog proteins and coregulate a broad number of

genes involved in maintenance of pluripotency. These pluripotency factors including Oct-4, Nanog and Sox2, in turn block the differentiation to mesendoderm and neuroectoderm while coordinating the self-renewal of pluripotent stem cells.

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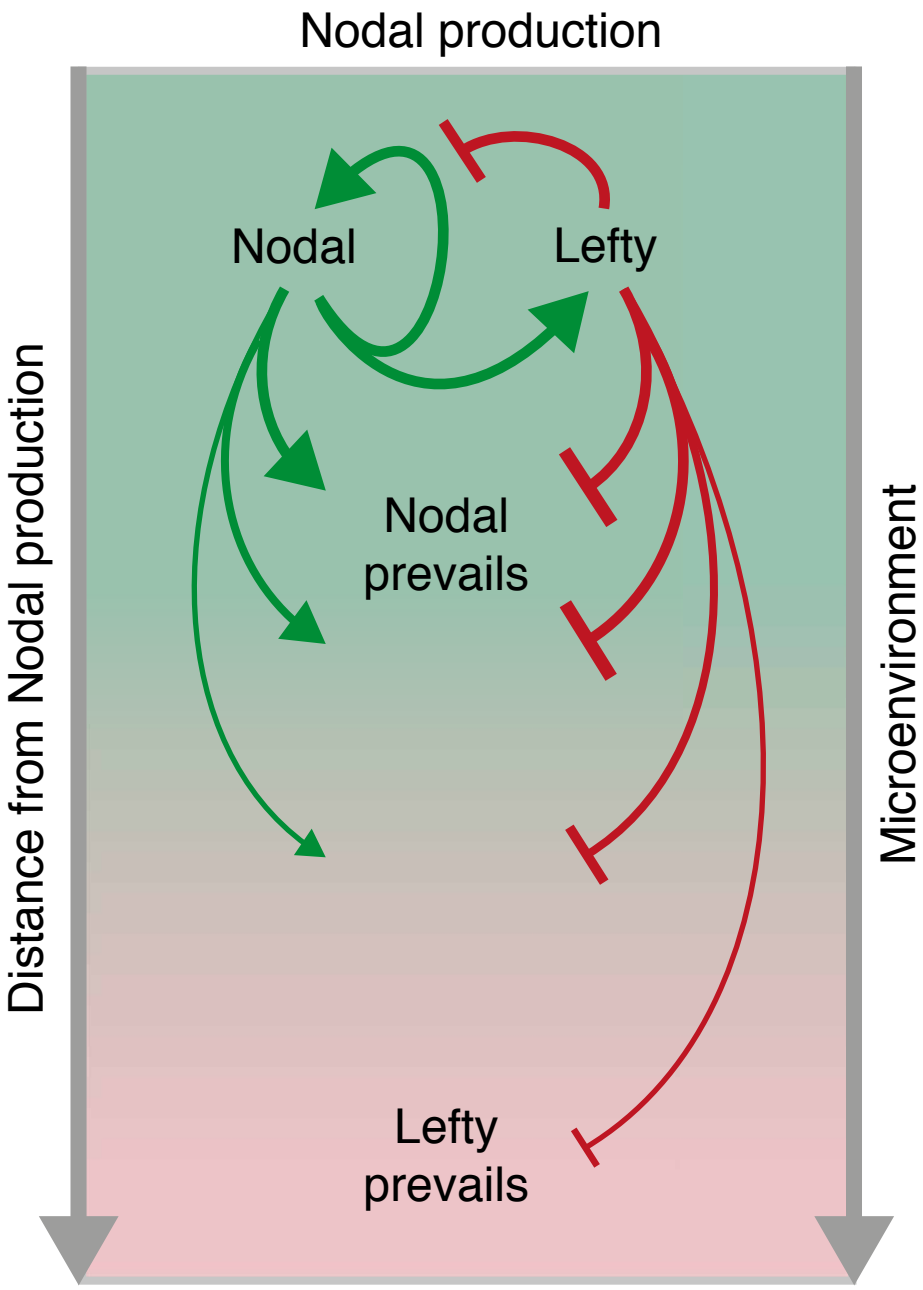
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Figure 1. Pauklin et al. Development

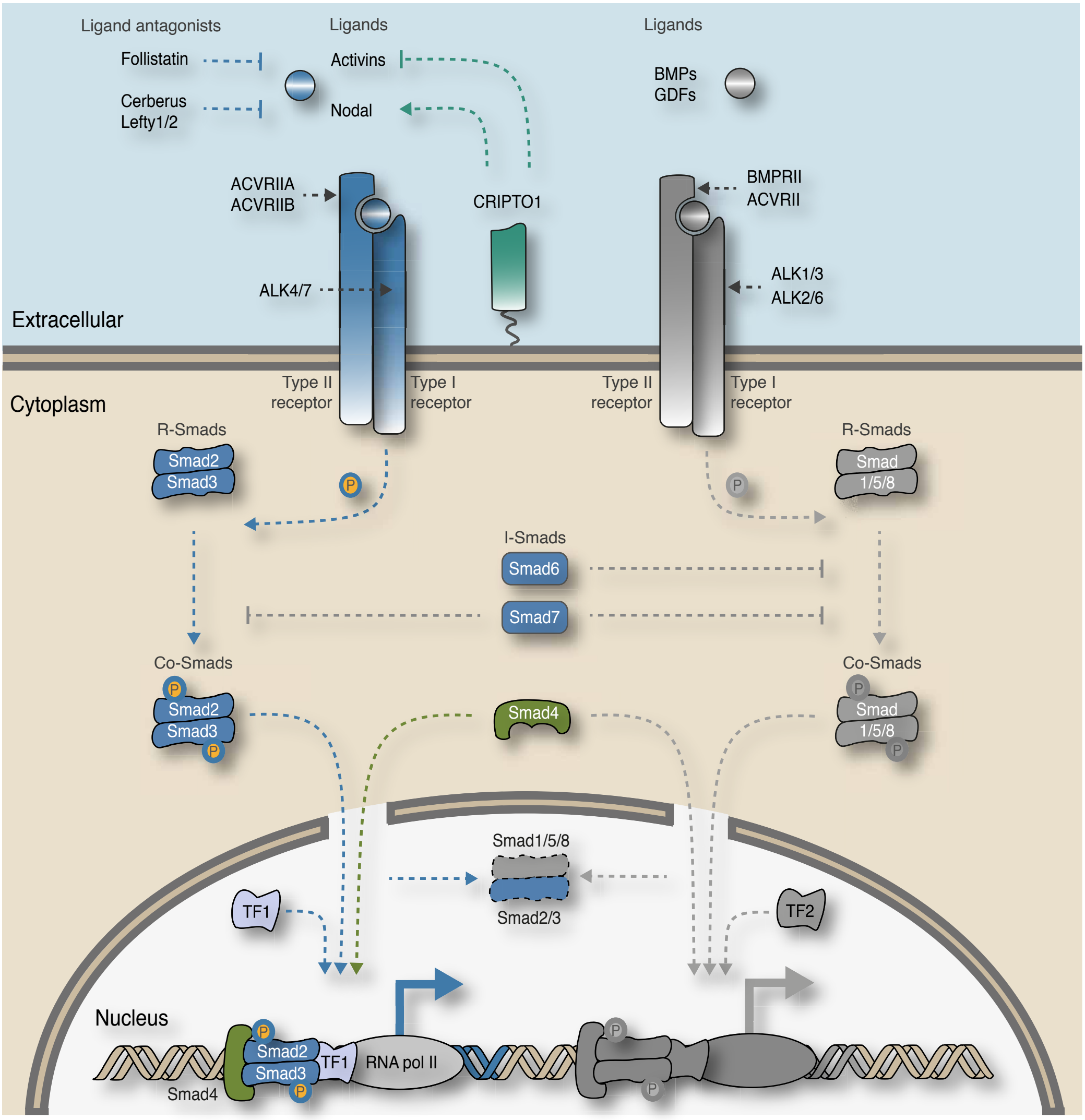
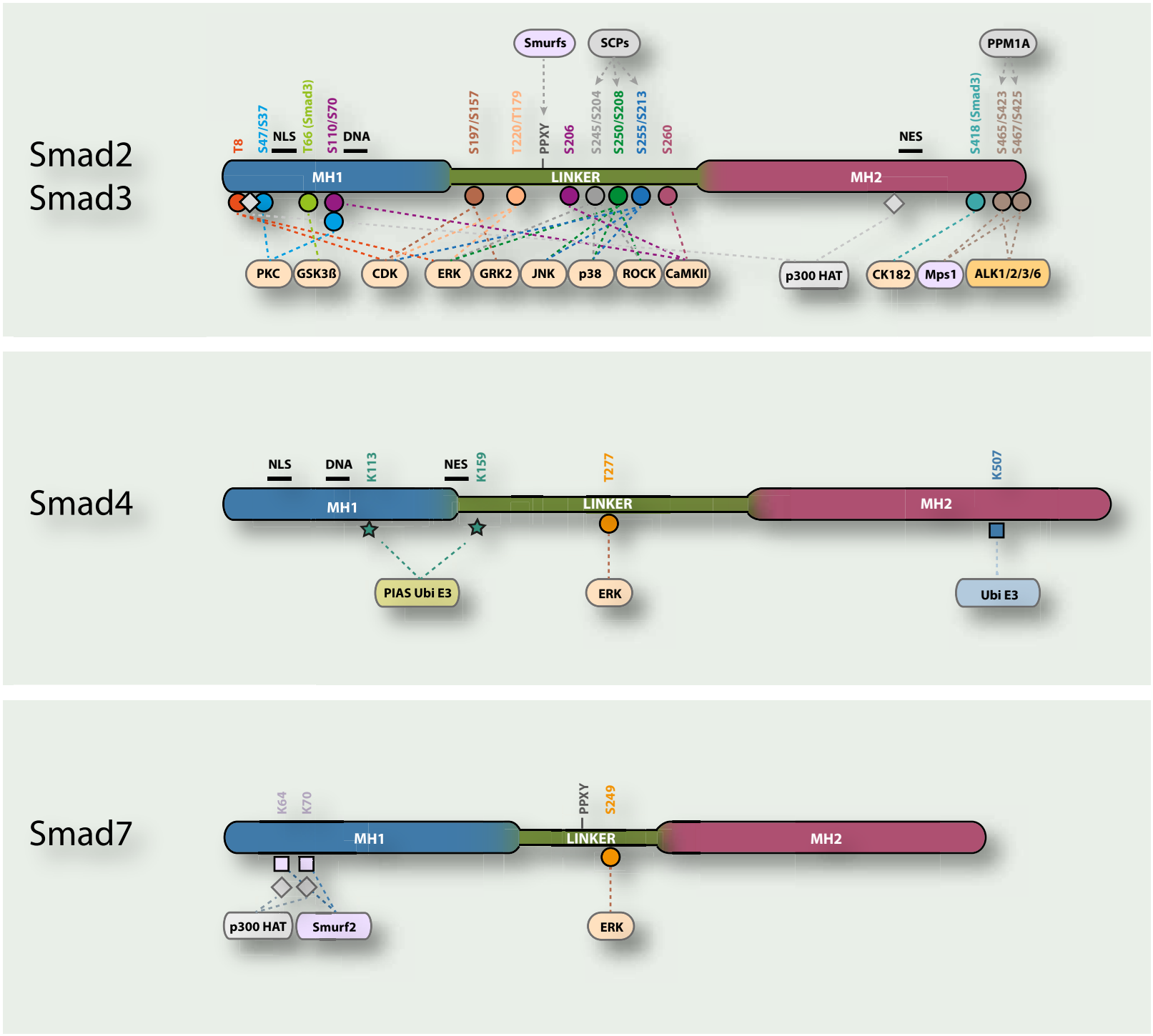


Figure 2. Pauklin et al. Development



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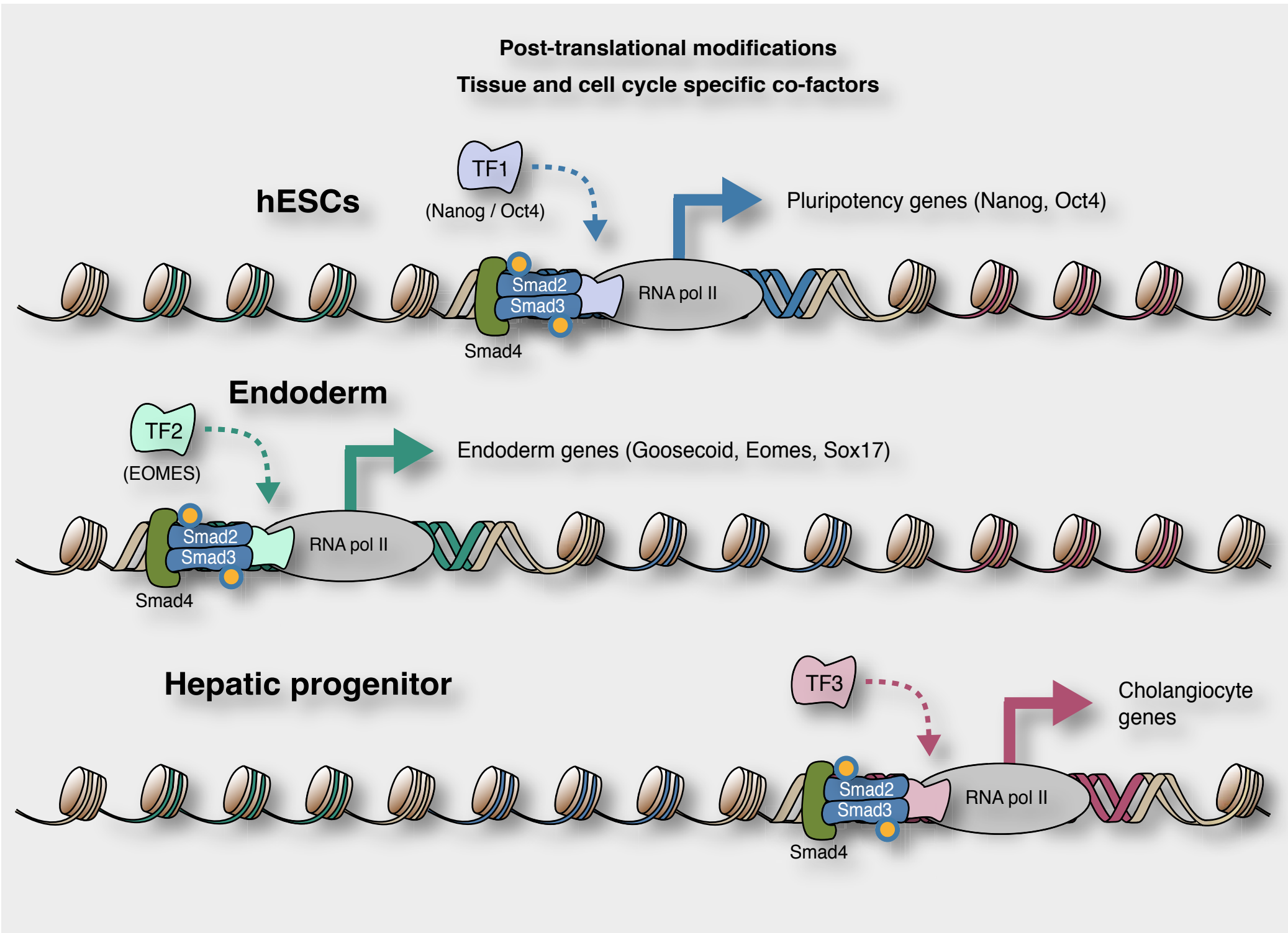

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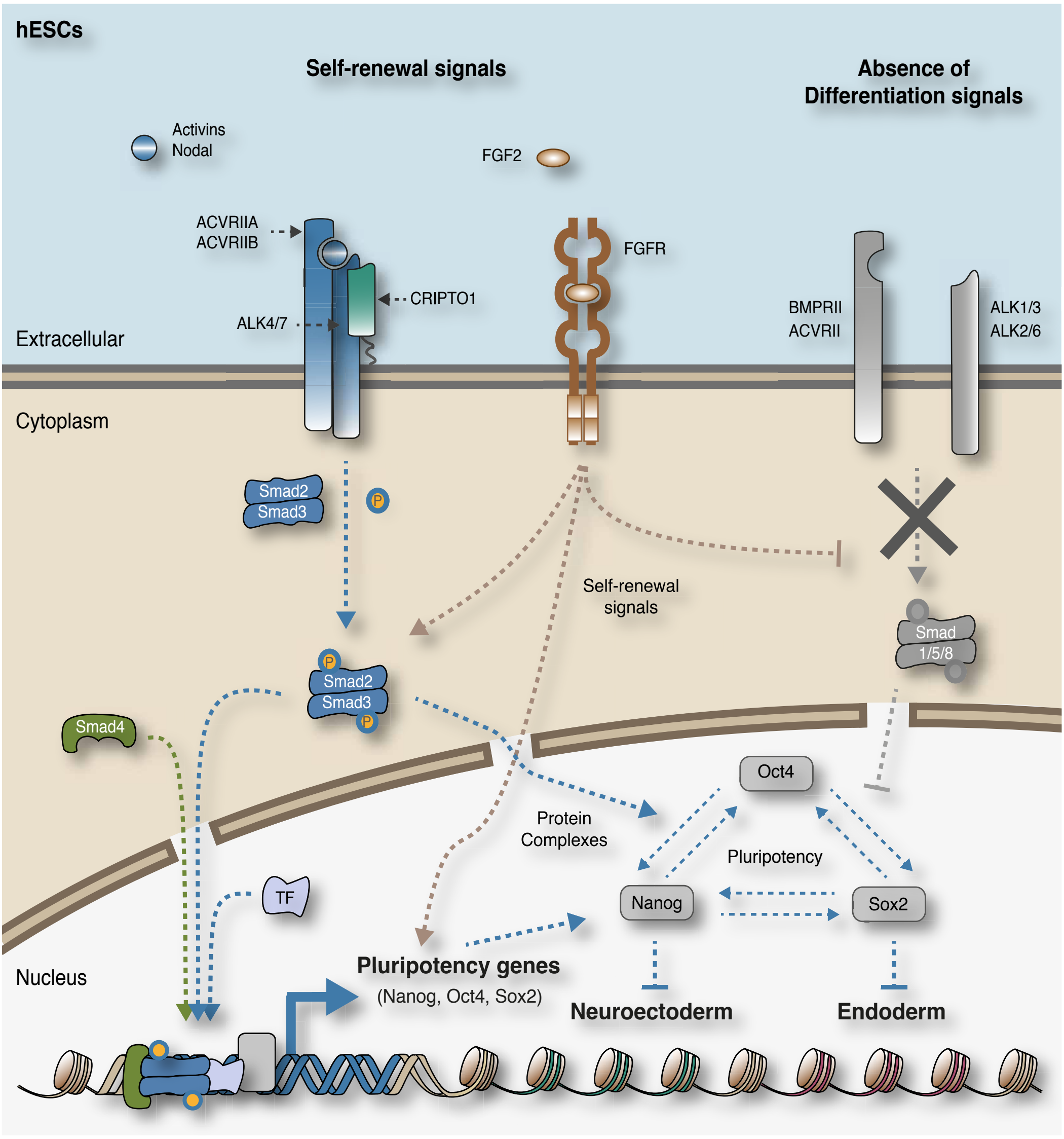

Figure 5. Pauklin et al. Development