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REVIEW

Nucleocytoplasmic shuttling of Smad proteins

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Nuclear accumulation of active Smad complexes is crucial for transduction of transforming growth factor β (TGF-β)-superfamily signals from transmembrane receptors into the nucleus. It is now clear that the nucleocytoplasmic distributions of Smads, in both the absence and the presence of a TGF-β-superfamily signal, are not static, but instead the Smads are continuously shuttling between the nucleus and the cytoplasm in both conditions. This article presents the evidence for continuous nucleocytoplasmic shuttling of Smads. It then reviews different mechanisms that have been proposed to mediate Smad nuclear import and export, and discusses how the Smad steady-state distributions in the absence and the presence of a TGF-β-superfamily signal are established. Finally, the biological relevance of continuous nucleocytoplasmic shuttling for signaling by TGF-β superfamily members is discussed.

***Keywords*:** Smad, nuclear import and export, TGF-β-superfamily signaling, karyopherin, nucleocytoplasmic shuttling

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Introduction

The transforming growth factor β (TGF-β) superfamily of ligands, which comprises TGF-βs, Activins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs), regulates many different cellular processes, including proliferation, apoptosis, adhesion, differentiation and specification of cell fate. These ligands play a crucial role in establishing and patterning the basic body plan during embryogenesis, and are also essential for tissue homeostasis, wound repair and immune regulation in adult organisms [1]. Both the ligands and components of the downstream signaling pathways are highly conserved from worm to man [2]. Receptors for these ligands are serine threonine kinases, and signaling occurs through heteromerization of type II receptors with type I receptors, which are brought together by the ligand. The constitutively active type II receptors activate the type I receptors by transphosphorylation. Activated type I receptors in turn phosphorylate their substrates, the best characterized of which are a subset of Smad proteins [3].

There are eight different Smad proteins, which fall into three functional classes. The first class are the receptor-regulated Smads or R-Smads (Smad1, 2, 3, 5 and 8), which are phosphorylated by the type I receptor kinases on an SXS motif at their extreme C-termini. In general Smad1, 5 and 8 are activated by BMP and GDF signals, whereas Smad2 and 3 are activated by TGF-β and Activin/Nodal signals. However, it has recently become clear that TGF-β can also activate Smad 1, 5 and 8 [4-6]. This is thought to be mediated by a receptor complex comprising the TGF-β type II receptor (TβRII), the TGF-β type I receptor (ALK5) and the tissue-specific type I receptor (ALK1) in endothelial cells [6], and through a receptor complex comprising TβRII, ALK5 and either ALK2 or ALK3 in epithelial cells [5]. Upon phosphorylation, the R-Smads form either homomeric or heteromeric complexes with Smad4, the only member in the second functional class of Smads, the common mediator Smads or co-Smads. Smad complexes then accumulate in the nucleus and regulate the transcription of target genes in conjunction with other transcription factors [7]. Smad4 only accumulates in the nucleus upon ligand activation in the context of R-Smad–Smad4 complexes and consequently Smad4 is dispensable for R-Smad nuclear accumulation in response to TGF-β, which occurs with the same efficiency in *Smad4* null cells [8-10]. The third functional class of Smads are the inhibitory Smads or I-Smads (Smad6 and 7) that regulate the pathway negatively by mediating receptor inactivation through recruit-

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ing either the E3 ubiquitin ligases Smurf1/2 [11, 12] or the catalytic subunit of protein phosphatase 1 to the receptors [13].

The R-Smads and Smad4 have a common structure [3] (Figure 1). In their N-termini there is a well-conserved domain called Mad-homology 1 (MH1) domain, which in Smad1, 3, 4, 5 and 8 binds to DNA directly. At the C-terminus there is another well-conserved domain, the MH2 domain, which is responsible for receptor–Smad and Smad–Smad interactions and also for many interactions with co-activators and co-repressors in the nucleus. The SXS motif that is phosphorylated in the R-Smads in response to ligand stimulation is at the extreme C-terminus of the MH2 domain and these phospho-serines are critical for stabilizing Smad–Smad complexes [14]. The MH1 and MH2 domains are separated by a less well-conserved proline-rich linker region. Smad6 and 7 contain a recognizable MH2 domain, but have only weak homology with the other Smads at their N-termini.

The initial view of the TGF-β-superfamily signaling pathways suggested that R-Smads and Smad4 would reside exclusively in the cytoplasm in the absence of a signal. Upon active signaling, they were thought to be released from the cytoplasm and translocate into the nucleus, where they would regulate gene transcription and be degraded subsequently [15]. More recent evidence, however, challenges this simple, linear view of the pathway. We now know that the Smads are continuously shuttling between the cytoplasm and the nucleus even in unstimulated cells. Moreover, there is compelling evidence that the bulk of the Smads that have accumulated in the nucleus in response to a signal are not degraded there, but rather engage in highly dynamic shuttling between the nucleus and the cytoplasm. The evidence suggests that such shuttling provides an elegant sensing mechanism to ensure that the concentration of active Smads in the nucleus reflects the actual signal strength at any given time.

This article reviews the evidence for Smad nucleocytoplasmic shuttling and outlines the different molecular transport mechanisms that have been proposed to account for nuclear import and export of Smads. It then discusses

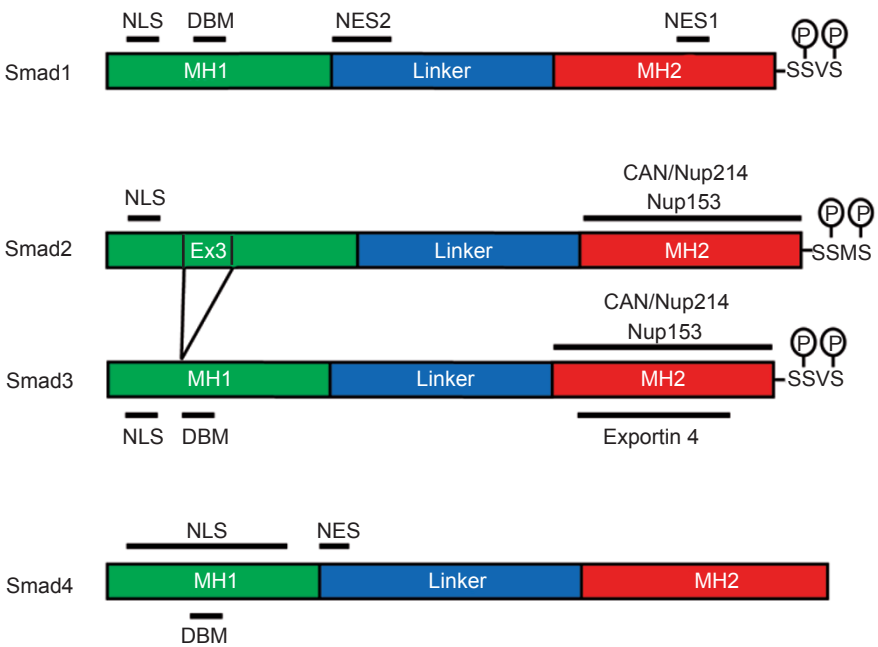

Figure 1 Smads have a conserved domain structure and harbor nuclear localization signals (NLS) and nuclear export signals (NES). R-Smads and Smad4 share two conserved domains, the N-terminal Mad-homology 1 (MH1) domain and the C-terminal MH2 domains. These domains are connected by a less well-conserved proline-rich linker region. In response to a TGF-β-superfamily ligand, the R-Smads, *e.g.* Smads1, 2 and 3, become phosphorylated at the C-terminal SXS motif, as indicated. The positions of NLSs and NESs as well as the DNA-binding motif (DBM) are indicated. An NLS is present in Smad2, but has not been shown to be functional. This is thought to be due to the presence of the additional exon 3 (Ex 3), which also interferes with DNA binding. A "hydrophobic corridor" in the MH2 domain of Smad2 and 3 is involved in binding to the nucleoporins CAN/Nup214 and Nup153, but full-length Smad4 seems to be required for interaction with CAN/Nup214 (not shown). The region of Smad3 that interacts with exportin 4 is also shown.

how the subcellular localization of Smads is regulated during signaling, and finally it evaluates the functional role of nucleocytoplasmic shuttling of Smads in TGF-β-superfamily signaling.

Evidence for Smad nucleocytoplasmic shuttling

The first evidence for nucleocytoplasmic shuttling of Smads came from studies on Smad4. In the absence of a TGF-β-superfamily ligand, Smad4 is distributed equally between the nucleus and the cytoplasm. Treatment of cells with leptomycin B (LMB), a specific inhibitor of the prototypic export receptor CRM1 or Exportin 1 [16], led to rapid nuclear accumulation of Smad4 [17, 18]. This simple experiment showed that CRM1 activity is required for nuclear export of Smad4, and demonstrated that Smad4 must be continuously shuttling between the nucleus and the cytoplasm in the absence of a TGF-β signal. The R-Smads in contrast were insensitive to treatment with LMB, indicating that CRM1 was not responsible for their nuclear export [18, 19]. This also meant that more sophisticated approaches were required to determine whether they also shuttle between the nucleus and the cytoplasm in unstimulated cells, and this was subsequently shown for Smad2 [19] using a quantitative nuclear transport reporter system [20]. The same study used a heterokaryon assay to demonstrate the shuttling ability of Smad2 and to map the export activity to a region comprising the linker and MH2 domain. Fluorescence perturbation and photoactivation experiments with GFP-fusions of Smad2 have, more recently, provided direct visual proof of the continuous exchange between the nuclear and the cytoplasmic pools of Smad2 both in tissue culture cells, in tissue explants of *Xenopus laevis* embryos, and in whole living zebrafish embryos [21-23]. Thus there is compelling evidence that Smad2 and 4 continuously shuttle between the cytoplasm and the nucleus in unstimulated cells.

TGF-β stimulation triggers a relatively slow nuclear accumulation of Smad2, 3 and 4, which reaches a maximum approximately 45 min after ligand stimulation [24]. Once accumulated, the bulk of Smads remain maximally nuclear for at least 4 to 5 h, after which they start to slowly relocalize to the cytoplasm [18]. The kinetics of Smad relocalization to the cytoplasm mirrors receptor inactivation and hence the slow fading of the signal. This has been demonstrated directly because if signaling is terminated prematurely by adding a specific type I receptor inhibitor SB-431542 [25, 26], nuclear accumulation of Smads begins to decrease immediately, causing the bulk of Smads to relocalize to the cytoplasm within 2 h [24]. How do the nuclear Smads "know", however, that the receptors have been inactivated? The most likely explanation is that continuous nucleocytoplasmic shuttling of Smads, even in actively signaling cells, which are in a state of maximal nuclear Smad accumulation, connects events at the plasma membrane with the nucleus. This notion has now been directly demonstrated by photobleaching and photoactivation experiments using GFP fusions of Smad2 and 4 [21-23].

The Smad2 that relocalizes to the cytoplasm upon inactivation of the signal is unphosphorylated, suggesting that nuclear export is accompanied by dephosphorylation [19, 24]. Furthermore, if cells are treated with TGF-β for 1 h to allow maximal nuclear accumulation of R-Smad–Smad4 complexes, and then treated with LMB to block CRM1 activity, as well as with the receptor inhibitor SB-431542 to block receptor activity, Smad4 as expected, stays trapped in the nucleus but Smad2 still redistributes to the cytoplasm [24]. As it is the C-terminal phosphorylated serines in the R-Smads that are responsible for stabilizing the R-Smad–Smad4 complexes [14], these results suggested that R-Smad–Smad4 complexes dissociate in the nucleus as a result of R-Smad dephosphorylation, and that monomeric R-Smads and Smad4 are then exported separately by distinct mechanisms. In agreement with this idea, CRM1-dependent Smad4 export is thought to be inhibited by complex formation of Smad4 with R-Smads, which physically prevents Smad4 from interaction with CRM1 [27]. Moreover, R-Smad C-terminal phosphatases have recently been identified. Smad1 phosphatases include pyruvate dehydrogenase phosphatase (PDP) and small C-terminal phosphatases (SCP1, 2 and 3) [28, 29], while PPM1A can dephosphorylate both subsets of R-Smads [30].

Thus Smad shuttling during active signaling involves continuous (but low level) R-Smad dephosphorylation, which leads to dissociation of Smad complexes in the nucleus and export of monomeric Smads to the cytoplasm. If the receptors are still active the R-Smads are rephosphorylated, form complexes and return to the nucleus. If the receptors are no longer active, the Smads will reaccumulate in the cytoplasm [24].

General principles of nucleocytoplasmic transport

Nucleocytoplasmic transport occurs through the nuclear pore complex (NPC), which forms a hydrophobic channel through the nuclear envelope. In mammalian cells, NPCs are made up of multiple copies of approximately 30 different nucleoporins [31]. Only relatively small molecules can pass through the NPC by diffusion and thus proteins larger than ~20–30 kD need to be actively transported through the nuclear pore. Interaction

of transport substrates with the NPC and their subsequent translocation are frequently mediated by transport receptors, karyopherins [32]. These proteins bind cargo molecules and can interact with repeats of FG-dipeptides, which are a common structural motif found in the majority of nucleoporins. Such interactions enable the

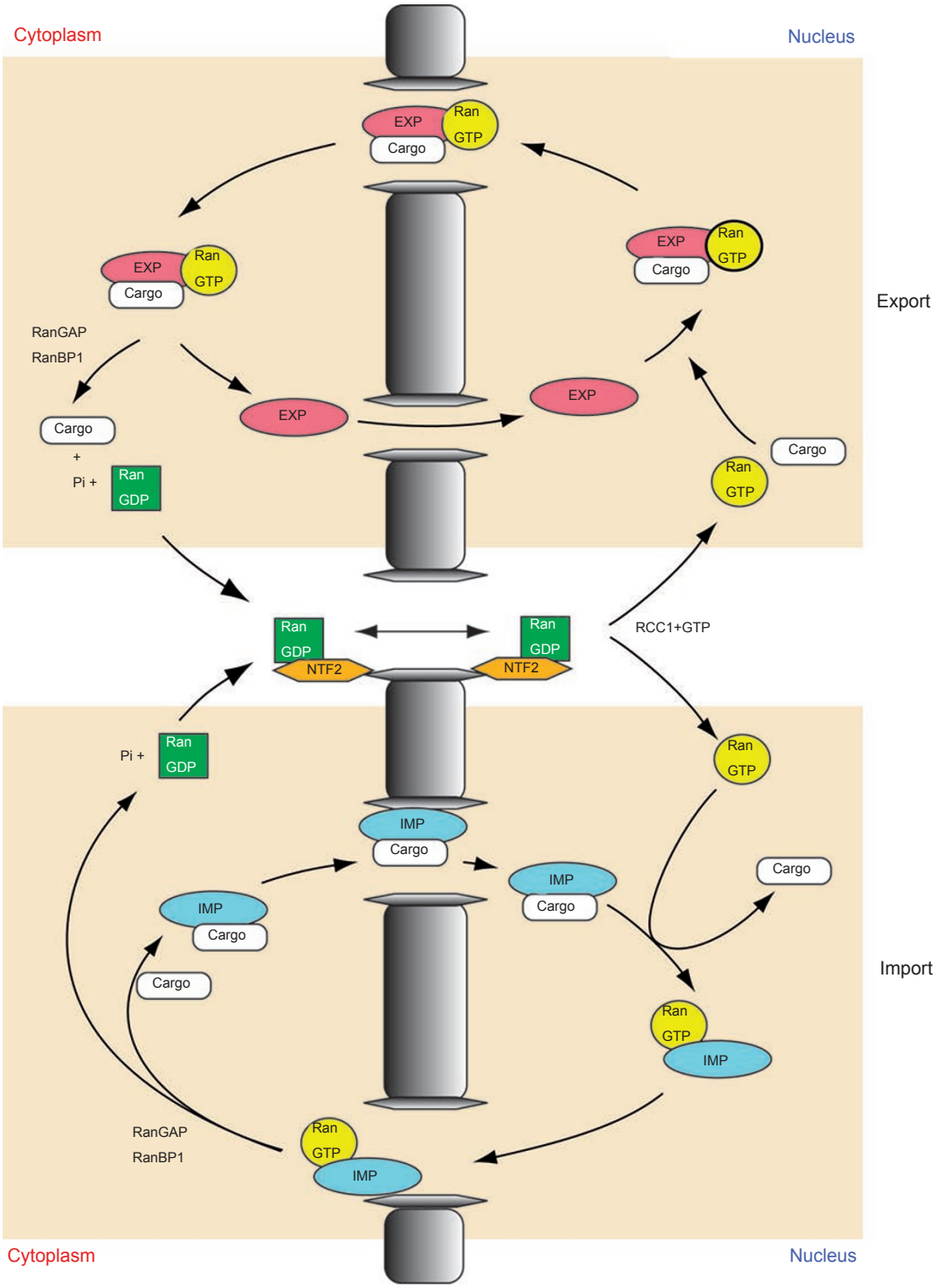

Figure 2 Nuclear import and export cycles and the involvement of the RanGTPase system. For details see text. Adapted from [32].

transporter-cargo complexes to pass through the nuclear pore. According to the transport direction, karyopherins are classified as importins or exportins [32].

The directionality of nucleocytoplasmic transport is established by the RanGTP gradient [32] (Figure 2). Ran is a small GTPase, which is mainly in its GTP-bound state in the nucleus, but in its GDP-bound state in the cytoplasm. This is because RanGAP resides exclusively in the cytoplasm, which, together with RanBP1, catalyzes cytoplasmic RanGTP hydrolysis. Conversely, the GDP-exchange factor RCC1 is bound to chromatin and thus resides exclusively in the nucleus. Importins bind their cargo in the cytoplasm without the need for RanGTP. In the classical pathway, the cargo is bound by one of the several variants of importin α through a basic nuclear localization signal (NLS), which in turn recruits the actual transport receptor, importin β, forming a trimeric import complex. After transport into the nucleus, RanGTP disrupts the import complex and the cargo protein is released into the nucleoplasm (Figure 2). Exportins in contrast bind their cargo only in the presence of RanGTP (i.e. in the nucleus) by forming a heterotrimeric exportin-cargo-RanGTP complex. After translocation of the export complex into the cytoplasm, RanGTP hydrolyzes its GTP with the help of RanGAP1 and RanBP1, causing dissociation and release of the exported cargo into the cytoplasm [32] (Figure 2). In addition to the karyopherin-dependent transport, some proteins have been reported to contact the NPC directly and enter or exit the nucleus without the need for transport receptors [33].

Mechanism of Smad transport between the cytoplasm and the nucleus

Smad import into the nucleus

A number of studies over the past years have addressed the translocation mechanism of Smad nuclear import and the ideas about how this process works have changed over the past 8 years. In fact both karyopherin-dependent import with the major import activity residing in the MH1 domain, and karyopherin-independent import mediated by direct contacts between nucleoporins and the MH2 domain, have been proposed.

For Smad3, a karyopherin-dependent translocation into the nucleus was described independently by two groups. One group identified a lysine-rich NLS-like sequence in the MH1 domain of Smad3 (^{40}KKLKK^{44}), which is conserved in all R-Smads [34]. In Smad3, this motif was proposed to mediate karyopherin-dependent nuclear import of Smad3 by binding to importin β1 directly [35]. Similarly, another study demonstrated that Smad3 interacts with importin β1, and is imported into the nucleus in a Ran- and energy-dependent manner [36]. Both groups found that nuclear import of the isolated MH1 domain was much stronger than import of the full-length protein in an *in vitro* import assay, and that importin β1 interacts much more strongly with the isolated MH1 domain than with the full-length protein in GST-pulldowns. In the same assays, TGF-β-dependent phosphorylation of Smad3 seemed to enhance both its interaction with importin β1 and its nuclear import [36]. The conserved NLS-like motif is non-functional in Smad2, perhaps because of an insertion encoded by exon 3 of Smad2, which is close to the NLS-like motif and might impair its accessibility for importin β1 binding [37] (Figure 1). The proposed mechanism deviates from the classical import mechanism by avoiding the need for one of the several importin α proteins as a bridging factor between importin β1 and the cargo. An NLS-like motif in the MH1 domain of Smad1 has also been shown to be important for nuclear import of this BMP-responsive R-Smad [38].

In contrast to Smad3, Smad4 has been proposed to use the classical pathway for nuclear import. An extended, bipartite NLS has been identified in Smad4 (amino acids 45-110), which overlaps with the corresponding sequence motif responsible for Smad1 and 3 import, but additionally extends into the DNA-binding region of the Smad4 MH1 domain. The isolated Smad4 MH1 domain interacts with importin α1 through this motif [39].

In contrast to this karyopherin-dependent nuclear import of Smad3 and 4, a very different mechanism for the import of Smad3, 4 and also 2 has also been described [19]. Using mainly *in vitro* experiments, these authors demonstrated that the main import activity for Smad2 and 3 resided in the linker-MH2 domain and import seemed entirely independent of karyopherins [19, 40]. Rather, these Smads were thought to interact directly with nuclear pore components, mainly with the nucleoporin CAN/Nup214, which is located at the cytoplasmic side of the nuclear pore and Nup153, which is located at the nucleoplasmic side [19]. An identical mechanism was proposed to be responsible for Smad4 import, except that in this case the full-length protein was required for import [40]. A further follow-up study, aimed at assessing the import mechanism by which TGF-β-induced homomeric Smad3 and heteromeric Smad3–Smad4 complexes are imported, demonstrated karyopherin-independent import for such complexes in an *in vitro* import assay [27].

This view of Smad nuclear import has been revised again more recently by some of the same authors as a result of a screen carried out in *Drosophila* cells looking for the importins required for nuclear accumulation of Mothers against DPP (Mad), which is the *Drosophila*

homolog of mammalian Smad1, in response to expression of BMP receptors [41]. This screen identified the *Drosophila* protein mole-skin (Msk) as being important for the nuclear import of phosphorylated Mad. These authors also showed that knockdown of the mammalian orthologs of Msk, importin 7 and 8 (Imp7 and Imp8) inhibited signal-induced nuclear accumulation of Smad1 and Smad2/3 in mammalian tissue culture cells [41]. Interestingly, knockdown of Imp7 and 8 either individually or in combination had no effect on the subcellular localization of Smad2/3 in unstimulated HeLa cells, leading the authors to suggest that monomeric Smads are imported into the nucleus by a distinct mechanism from complexed Smads in ligand-induced cells, and that Smad monomers might be imported in a karyopherin-independent manner as had been earlier suggested [19, 40]. Very recently, further work has investigated the possible mechanism of Imp7/8-mediated Smad import. Nuclear import of Smad4, both in the presence and absence of signal has been shown to require Imp7 and 8; and a KKLK motif in the MH1 domain, which is part of the originally identified NLS in Smad4 [39], was shown to be required for Smad4 import, although not directly for binding to Imp8.

Taken together it seems clear that in the context of Smad complexes after ligand stimulation, all the Smads require karyopherins to import them into the nucleus. For Smad1, 3 and 4, the basic NLS-like motifs in the N-terminal MH1 domains are required, although the situation is less clear for Smad2. Knockdown experiments strongly implicate Imp7 and 8, but it will also be important now to reassess the role of importin α and β. More work is needed to resolve the issue of whether R-Smad import in the basal state is dependent on karyopherins, or independent.

Smad export from the nucleus

The mechanism of Smad4 export from the nucleus was identified in 2000 and was shown to be mediated by CRM1 [17, 18]. As mentioned above these experiments made use of the specific CRM1 inhibitor, LMB [16]. Treatment of unstimulated cells with LMB triggered nuclear accumulation of Smad4, demonstrating that Smad4 export is CRM1-dependent. A canonical nuclear export signal (NES) was identified in the N-terminal part of the linker region of Smad4 (^{142}DLSGLTLQ^{149}), and deletion or mutation of critical residues in this NES enhanced nuclear localization of Smad4 [17, 18]. Interestingly, a variant of Smad4, XSmad4β that exists only in *X. laevis* and in no other vertebrates, lacks this NES and is constitutively nuclear [42]. Hence, this NES seems to be necessary to mediate nuclear export of Smad4.

The situation is more complicated for the R-Smads. A CRM1-dependent export mechanism identical to that of Smad4 has been suggested for the BMP-responsive Smad1. First, a potential NES, termed NES1 was identified in the C-terminal region of the Smad1 MH2 domain. Mutation of this motif caused Smad1 to localize predominantly to the nucleus [38]. In addition, a second NES, termed NES2 and located in the linker adjacent to the MH1 domain, was described [43]. NES2 partly overlaps with the functional NES found in the corresponding region of Smad4 (Figure 1). Smad1 NES1 and NES2 are absolutely conserved among all the R-Smads, and although they seem to mediate CRM1-dependent export in the case of Smad1, they have no such activity in Smad2 and 3, whose export is clearly CRM1-independent. There is currently no explanation as to why the motifs are functional in Smad1 but not in Smad2 and 3.

Nuclear export of Smad2 and 3 is clearly independent of CRM1, in both the absence and the presence of a TGF-β signal [18, 19, 24, 27]. For Smad2, a karyopherin-independent export mechanism mediated by direct binding to nucleoporins has been suggested [19], similar to what was originally proposed for Smad2/3 import. However, Smad3 has recently been shown to require a karyopherin for nuclear export, in this case exportin 4 [44], suggesting that Smad2 export is also likely to be karyopherin-dependent.

Mechanisms underlying TGF-β-induced nuclear accumulation of Smads

Irrespective of the exact mechanism by which Smads translocate through the NPC, it has been of great interest to determine how the steady-state distribution of Smads in both the resting state and the presence of a TGF-β superfamily ligand is established.

It is generally accepted that, in the absence of a TGF-β signal, Smad4 is distributed equally between the nucleus and the cytoplasm [18]. Such distribution is thought to be established by the constitutive nuclear import and export of Smad4, which are thought to be of comparable rates. In contrast, the R-Smads reside predominantly in the cytoplasm in the absence of a signal, although they are by no means excluded from the nucleus. This has been most thoroughly studied for Smad2. Given the continuous exchange of R-Smads between the two compartments, their nucleocytoplasmic distribution directly reflects their mean residence times in both compartments. The mean residence times are determined by the rates of nuclear import and nuclear export, and by possible interactions with retention factors that could cause temporary sequestration of Smads from the transport machinery. The Smad-interacting FYVE-domain protein SARA [45] has

been suggested to act as a retention factor for Smad2 in the absence of a signal [46]. This was on the basis of the observation that addition of the recombinant Smad-binding domain of SARA in a nuclear import assay inhibited nuclear import of Smad2 *in vitro*, and overexpression of SARA targeted Smad2 to early endosomes *in vivo* [46]. However, in cell lines expressing low levels of GFP-Smad2 no localization to early endosomes is observed and GFP-Smad2 shows a rather diffuse cytoplasmic distribution [22], as does endogenous Smad2 [18]. Both findings together indicate that the amount of SARA is limiting and is unlikely to bring about quantitative cytoplasmic sequestration of Smad2. Alternatively, the predominantly cytoplasmic localization of R-Smads could result from R-Smad import rates simply being weaker than the export rates. Quantitative kinetic studies on the import and export of GFP-Smad2 have proven that this is indeed the case [23].

How does ligand stimulation then lead to nuclear accumulation of active Smad complexes? Four distinct mechanisms could theoretically account for the nuclear accumulation of a continuously shuttling, predominantly cytoplasmic protein in response to ligand stimulation. Ligand induction could lead to a release from cytoplasmic anchoring or to the establishment of nuclear anchoring. Alternatively, the presence of a signal could increase the rate of nuclear import or decrease the rate of nuclear export, or both. All four mechanisms could lead to a change in mean residence times in either compartment or in both compartments, and thus cause a predominantly nuclear steady-state distribution.

As Smad4 is dispensable for nuclear accumulation of R-Smads and its own nuclear accumulation is strictly dependent on R-Smad accumulation [8, 27, 37], the mechanism of R-Smad nuclear accumulation is of most interest. An anchor-release model was originally suggested for Smad2 based on the finding that overexpression of SARA sequesters Smad2 into clusters in the cytoplasm [45, 46], and that overexpression of the Smad2-interacting nuclear protein FAST-1/FoxH1 traps Smad2 in the nucleus even in uninduced cells [19]. In this model, phosphorylation-dependent disruption of the Smad2–SARA interaction [45] enables phosphorylated Smad2 to move into the nucleus, where it is retained by means of phosphorylation-dependent interactions with the transcription factor FoxH1/FAST-1 or related proteins [19]. However, no loss-of-function experiments have been performed to show that SARA and FoxH1/FAST-1 actually perform these functions when present at their endogenous levels.

More recently, a detailed kinetic analysis of Smad nucleocytoplasmic shuttling has led to a different view [23] (Figure 3). FRAP mobility measurements in the cytoplasm did not indicate mobilization of GFP-Smad2 in response to TGF-β, as would be expected for an anchor-release mechanism. Nuclear mobilities of GFP-Smad2 and GFP-Smad4, however, are slightly diminished in TGF-β-induced cells, pointing to possible nuclear tethering of active Smad complexes [23]. Compartment-specific photoactivation revealed that export rates of GFP-Smad2 are strongly decreased in TGF-β-induced cells when compared with uninduced cells, whereas import rates are apparently unchanged (but see below). These data suggest that neither increased import nor release from cytoplasmic retention plays a predominant role in TGF-β-induced R-Smad nuclear accumulation *in vivo*. Rather, selective trapping of active Smad complexes in the nucleus from a cycling pool of monomeric Smads is likely to be the most important mechanism [23]. The nuclear trapping may be due to binding of Smad complexes to nuclear structures, which would explain the observed decrease in mobility. For example, this could reflect weak binding to DNA, where Smad complexes scan for their optimal binding sites in promoters of target genes. Alternatively, Smad complexes may not be exported simply because they are not recognized by the export machinery as has already been shown for Smad4 [27]. Interestingly, the transcriptional regulator TAZ has very recently been shown to be involved in retaining Smad complexes in the nucleus. When TAZ is knocked down Smad2/3–Smad4 complexes form, but fail to accumulate in the nucleus [47], and TAZ appears to be able to bind with active Smad complexes to promoter elements. Whether binding to TAZ in the nucleus is sufficient to account for the measured decrease in nuclear export rate of Smad complexes relative to monomeric Smads remains to be investigated.

Fluorescence perturbation experiments in conjunction with siRNA knockdown of different components, inhibition of signaling by small molecules and protein interaction studies *in vitro* can lead to a hypothesis of how ligand stimulation leads to nuclear accumulation of active Smad complexes. However, experimentally it is impossible to determine whether such a hypothesis is consistent with the observed kinetics of nuclear accumulation of activated Smad complexes and their behavior after receptor inactivation, as many critical reactions within this complex signaling network are not amenable to experimental investigation. To overcome these problems a mathematical model was developed [48] based on the kinetic measurements that had been made in epithelial cells, stably expressing GFP-tagged Smad2 [23], using the network topology outlined in Figure 3. The model simultaneously fitted four independent datasets with excellent accuracy and moreover fitted a dataset not used in

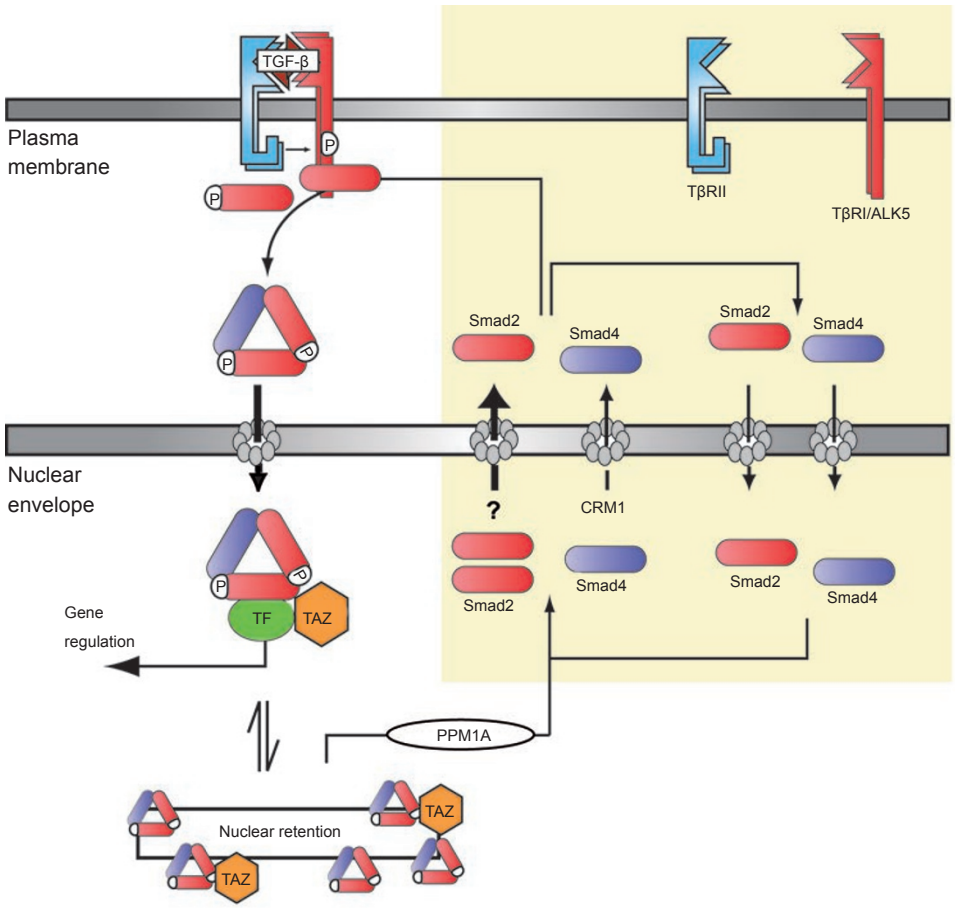

Figure 3 A model for nucleocytoplasmic shuttling of Smads in uninduced and in TGF-β-stimulated cells. The shaded portion represents Smad shuttling in unstimulated cells. Receptor activation by TGF-β ligand binding leads to R-Smad phosphorylation and complex formation with Smad4. In the absence of signaling, Smad2 appears cytoplasmic because its export rate is faster than its import rate. In these conditions Smad4 is distributed approximately equally between the cytoplasm and nucleus. Upon ligand induction Smad complexes accumulate in the nucleus, as a result of their import being faster than that of monomeric Smads and their export being inhibited, possibly as a result of nuclear retention. The transcriptional regulator TAZ has been shown to be required for ligand-induced Smad nuclear accumulation. Smad complexes regulate transcription in conjunction with other transcription factors (TF). The phosphatase, PPM1A has been proposed to be the Smad2 C-terminal phosphatase. Adapted from [23]. For further details, see text.

its construction [48]. In doing so, it verifies the plausibility and mechanistic relevance of the underlying network topology. Most interestingly, the mathematical model has led to additional mechanistic insights. First, it was evident that although inhibition of Smad export on TGF-β stimulation was essential, it was not sufficient to cause the observed kinetics of Smad nuclear accumulation on TGF-β signaling. The mathematical model requires that the import of Smad complexes into the nucleus should be approximately 5 times faster than the import of monomeric Smads [48]. Although this increase in Smad import rate was not measureable *in vivo* [23], it was earlier shown using *in vitro* experiments that phosphorylated Smad3 interacts more efficiently with importin β1 than unphosphorylated Smad3, and is imported into the nucleus more efficiently [36]. The mathematical modeling also indicated that the C-terminal Smad phosphatase must reside in the nucleus.

The biological function of Smad nucleocytoplasmic shuttling

Appropriate reaction of cells to external stimuli requires exact timing of both the initiation and the duration of the specific response. It is also important that the strength of the response in terms of levels of active

nuclear Smads matches the level of receptor activation at all times. Activation of Smads by TGF-β in epithelial cells proceeds relatively slowly and is maintained over extended time periods. Maximal activation is reached after about 45 min of active signaling in cell culture [18, 24] and is characterized by plateauing levels of phosphorylated R-Smads and maximal nuclear accumulation of Smads. Both features are maintained for several hours. It is not until the signal ceases, i.e. the actively signaling receptors have been inactivated by either dephosphorylation or degradation after 4-5 h, that the bulk of the Smads redistribute into the cytoplasm. In some pancreatic cancer cells the duration of signaling was found to be attenuated, and this correlated with lower receptor levels in these cells [10]. These observations, together with the finding that premature termination of active signaling by receptor inhibition causes the Smads to redistribute into the cytoplasm, led to the idea that nucleocytoplasmic Smad shuttling provides a sensing mechanism whereby the Smads themselves directly monitor receptor activity [24]. Thus, as the receptors are inactivated, the signal fades and the pool of active Smads in the nucleus diminishes. In contrast, if the receptors remain highly active for a long period of time, a pool of active nuclear Smads will be maintained. The quantitative relationship between nuclear Smad2–Smad4 complexes (system output) and receptor activity (system input) was recently tested using the mathematical model of the TGF-β/Smad pathway described above [48]. The system's response in terms of levels of nuclear Smad2–Smad4 complexes to a theoretical two-step activation of receptors followed by receptor deactivation was simulated. The simulation demonstrated that Smad nucleocytoplasmic shuttling indeed couples the amount of nuclear Smad2–Smad4 complexes to receptor activity, although with a time delay. This delay acts to filter noise, and thus transient fluctuations in receptor activity, whose duration is short compared with the characteristic activation time, are dampened and do not cause a corresponding fluctuation in the concentration of nuclear Smad2–Smad4 complexes. Therefore, nucleocytoplasmic shuttling of Smads enables the cell to continuously monitor signal strength and provides the flexibility that is needed to react appropriately to changing signal intensities. This may be especially important during early embryonic development when TGF-β-superfamily signals are highly dynamic [49].

Concluding remarks

The shuttling of the Smads between the cytoplasm and nucleus in both unstimulated cells and in cells stimulated with TGF-β is now well established. The biological significance of Smad nucleocytoplasmic shuttling in the presence of signal is that it provides a mechanism whereby the intracellular transducers of the signal can continuously monitor receptor activity, an essential requirement of signaling in multicellular organisms. It is now apparent that this is a common mechanism employed also by other signaling pathways. Since the original Smad nucleocytoplasmic shuttling hypothesis was proposed [24], a very similar model has been suggested for the JAK/STAT pathway and for serum-induced signaling via the serum response factor and its co-activator Mal [50-52].

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References

1 Massague J. TGFβ in cancer. *Cell* 2008; **134**:215-230.
2 Massague J. TGF-β signal transduction. *Annu Rev Biochem* 1998; **67**:753-791.
3 Shi Y, Massague J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell* 2003; **113**:685-700.
4 Bharathy S, Xie W, Yingling JM, Reiss M. Cancer-associated transforming growth factor β type II receptor gene mutant causes activation of bone morphogenic protein-Smads and invasive phenotype. *Cancer Res* 2008; **68**:1656-1666.
5 Daly AC, Randall RA, Hill CS. Transforming growth factor β-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth. *Mol Cell Biol* 2008; **28**:6889-6902.
6 Goumans MJ, Valdimarsdottir G, Itoh S, *et al*. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. *Mol Cell* 2003; **12**:817-828.
7 Ross S, Hill CS. How the Smads regulate transcription. *Int J Biochem Cell Biol* 2008; **40**:383-408.
8 De Bosscher K, Hill CS, Nicolas FJ. Molecular and functional consequences of Smad4 C-terminal missense mutations in colorectal tumour cells. *Biochem J* 2004; **379**:209-216.
9 Liu F, Pouponnot C, Massague J. Dual role of the Smad4/DPC4 tumor suppressor in TGFβ-inducible transcriptional complexes. *Genes Dev* 1997; **11**:3157-3167.
10 Nicolas FJ, Hill CS. Attenuation of the TGF-β-Smad signaling pathway in pancreatic tumor cells confers resistance to TGF-β-induced growth arrest. *Oncogene* 2003; **22**:3698-3711.
11 Ebisawa T, Fukuchi M, Murakami G, *et al*. Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* 2001; **276**:12477-12480.

12 Kavsak P, Rasmussen RK, Causing CG, *et al*. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF β receptor for degradation. *Mol Cell* 2000; **6**:1365-1375.
13 Shi W, Sun C, He B, *et al*. GADD34-PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor. *J Cell Biol* 2004; **164**:291-300.
14 Wu JW, Hu M, Chai J, *et al*. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-β signaling. *Mol Cell* 2001; **8**:1277-1289.
15 Lo RS, Massague J. Ubiquitin-dependent degradation of TGF-β-activated smad2. *Nat Cell Biol* 1999; **1**:472-478.
16 Fornerod M, Ohno M, Yoshida M, Mattaj IW. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 1997; **90**:1051-1060.
17 Watanabe M, Masuyama N, Fukuda M, Nishida E. Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. *EMBO Rep* 2000; **1**:176-182.
18 Pierreux CE, Nicolas FJ, Hill CS. Transforming growth factor β-independent shuttling of Smad4 between the cytoplasm and nucleus. *Mol Cell Biol* 2000; **20**:9041-9054.
19 Xu L, Kang Y, Col S, Massague J. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFβ signaling complexes in the cytoplasm and nucleus. *Mol Cell* 2002; **10**:271-282.
20 Coburn GA, Wiegand HL, Kang Y, *et al*. Using viral species specificity to define a critical protein/RNA interaction surface. *Genes Dev* 2001; **15**:1194-1205.
21 Batut J, Howell M, Hill CS. Kinesin-mediated transport of Smad2 is required for signaling in response to TGF-β ligands. *Dev Cell* 2007; **12**:261-274.
22 Nicolás FJ, De Bosscher K, Schmierer B, Hill CS. Analysis of Smad nucleocytoplasmic shuttling in living cells. *J Cell Sci* 2004; **117**:4113-4125.
23 Schmierer B, Hill CS. Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor β-dependent nuclear accumulation of Smads. *Mol Cell Biol* 2005; **25**:9845-9858.
24 Inman GJ, Nicolás FJ, Hill CS. Nucleocytoplasmic shuttling of Smads 2, 3 and 4 permits sensing of TGF-β receptor activity. *Mol Cell* 2002; **10**:283-294.
25 Inman GJ, Nicolás FJ, Callahan JF, *et al*. SB-431542 is a potent and specific inhibitor of Transforming Growth Factor-β superfamily type I activin receptor-like kinase receptors, ALK4, ALK5 and ALK7. *Mol Pharmacol* 2002; **62**:65-72.
26 Laping NJ, Grygielko E, Mathur A, *et al*. Inhibition of transforming growth factor (TGF)-β1-induced extracellular matrix with a novel inhibitor of the TGF-β type I receptor kinase activity. *Mol Pharmacol* 2002; **62**:58-64.
27 Chen HB, Rud JG, Lin K, Xu L. Nuclear targeting of transforming growth factor-β-activated Smad complexes. *J Biol Chem* 2005; **280**:21329-21336.
28 Chen HB, Shen J, Ip YT, Xu L. Identification of phosphatases for Smad in the BMP/DPP pathway. *Genes Dev* 2006; **20**:648-653.
29 Knockaert M, Sapkota G, Alarcon C, Massague J, Brivanlou AH. Unique players in the BMP pathway: small C-terminal domain phosphatases dephosphorylate Smad1 to attenuate BMP signaling. *Proc Natl Acad Sci USA* 2006; **103**:11940-11945.
30 Lin X, Duan X, Liang YY, *et al*. PPM1A functions as a Smad phosphatase to terminate TGFβ signaling. *Cell* 2006; **125**:915-928.
31 Hetzer MW, Walther TC, Mattaj IW. Pushing the envelope: structure, function, and dynamics of the nuclear periphery. *Annu Rev Cell Dev Biol* 2005; **21**:347-380.
32 Gorlich D, Kutay U. Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* 1999; **15**:607-660.
33 Xu L, Massague J. Nucleocytoplasmic shuttling of signal transducers. *Nat Rev Mol Cell Biol* 2004; **5**:209-219.
34 Xiao Z, Liu X, Henis YI, Lodish HF. A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation. *Proc Natl Acad Sci USA* 2000; **97**:7853-7858.
35 Xiao Z, Liu X, Lodish HF. Importin β mediates nuclear translocation of Smad 3. *J Biol Chem* 2000; **275**:23425-23428.
36 Kurisaki A, Kose S, Yoneda Y, Heldin CH, Moustakas A. Transforming growth factor-β induces nuclear import of Smad3 in an importin-β1 and Ran-dependent manner. *Mol Biol Cell* 2001; **12**:1079-1091.
37 Reguly T, Wrana JL. In or out? The dynamics of Smad nucleocytoplasmic shuttling. *Trends Cell Biol* 2003; **13**:216-220.
38 Xiao Z, Watson N, Rodriguez C, Lodish HF. Nucleocytoplasmic shuttling of Smad1 conferred by its nuclear localization and nuclear export signals. *J Biol Chem* 2001; **276**:39404-39410.
39 Xiao Z, Latek R, Lodish HF. An extended bipartite nuclear localization signal in Smad4 is required for its nuclear import and transcriptional activity. *Oncogene* 2003; **22**:1057-1069.
40 Xu L, Alarcon C, Col S, Massague J. Distinct domain utilization by Smad3 and Smad4 for nucleoporin interaction and nuclear import. *J Biol Chem* 2003; **278**:42569-42577.
41 Xu L, Yao X, Chen X, et al. Msk is required for nuclear import of TGF-β/BMP-activated Smads. *J Cell Biol* 2007; **178**:981-994.
42 Masuyama N, Hanafusa H, Kusakabe M, Shibuya H, Nishida E. Identification of two Smad4 proteins in Xenopus. Their common and distinct properties. *J Biol Chem* 1999; **274**:12163-12170.
43 Xiao Z, Brownawell AM, Macara IG, Lodish HF. A novel nuclear export signal in Smad1 is essential for its signaling activity. *J Biol Chem* 2003; **278**:34245-34252.
44 Kurisaki A, Kurisaki K, Kowanetz M, *et al.* The mechanism of nuclear export of Smad3 involves exportin 4 and Ran. *Mol Cell Biol* 2006; **26**:1318-1332.
45 Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor. *Cell* 1998; **95**:779-791.
46 Xu L, Chen YG, Massague J. The nuclear import function of Smad2 is masked by SARA and unmasked by TGFβ-dependent phosphorylation. *Nat Cell Biol* 2000; **2**:559-562.
47 Varelas X, Sakuma R, Samavarchi-Tehrani P, *et al.* TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nat Cell Biol* 2008; **10**:837-848.
48 Schmierer B, Tournier AL, Bates PA, Hill CS. Mathematical modeling identifies Smad nucleocytoplasmic shuttling as a dynamic signal-interpreting system. *Proc Natl Acad Sci USA*

2008; **105**:6608-6613.
49 Schmierer B, Hill CS. TGFβ-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 2007; **8**:970-982.
50 Meyer T, Vinkemeier U. Nucleocytoplasmic shuttling of STAT transcription factors. *Eur J Biochem* 2004; **271**:4606-4612.
51 Vartiainen MK, Guettler S, Larijani B, Treisman R. Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL. *Science* 2007; **316**:1749-1752.
52 Vinkemeier U. Getting the message across, STAT! Design principles of a molecular signaling circuit. *J Cell Biol* 2004; **167**:197-201.