Endocytic regulation of TGF- β signaling

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Transforming growth factor- β (TGF- β) signaling is tightly regulated to ensure its proper physiological functions in different cells and tissues. Like other cell surface receptors, TGF- β receptors are internalized into the cell, and this process plays an important regulatory role in TGF- β signaling. It is well documented that TGF- β receptors are endocytosed via clathrin-coated vesicles as TGF- β endocytosis can be blocked by potassium depletion and the GTPase-deficient dynamin K44A mutant. TGF- β receptors may also enter cells via cholesterol-rich membrane microdomain lipid rafts/caveolae and are found in caveolin-1-positive vesicles. Although receptor endocytosis is not essential for TGF- β signaling, clathrin-mediated endocytosis has been shown to promote TGF- β -induced Smad activation and transcriptional responses. Lipid rafts/caveolae are widely regarded as signaling centers for G protein-coupled receptors and tyrosine kinase receptors, but they are indicated to facilitate the degradation of TGF- β receptors and therefore turnoff of TGF- β signaling. This review summarizes current understanding of TGF- β receptor endocytosis, the possible mechanisms underlying this process, and the role of endocytosis in modulation of TGF- β signaling.

Keyword: TGF- β , endocytosis, clathrin, lipid rafts, endosome

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Introduction

Transforming growth factor- β (TGF- β) is a member of the structurally related growth factor family, which includes TGF- β , activin, Nodal, bone morphogenetic proteins (BMPs), myostatin and others. These factors play key roles in regulating a wide range of biological responses during development and tissue homeostasis in adults, and deregulation of their signal transduction has been associated with many human diseases, including cancer and tissue fibrosis [1-10]. TGF- β elicits its signaling by binding to its cell surface Ser/Thr kinase receptors, leading to the formation of the receptor heterocomplexes, in which the constitutively active type II receptor (T β RII) phosphorylates the type I receptor (T β RI). The phosphorylated T β RI adopts a conformation that facilitates the docking of receptor-regulated Smad proteins (R-Smad) and activates R-Smad through phosphorylation. Then R-Smad forms a complex with Co-Smad Smad4,

and together they are accumulated in the nucleus and regulate the expression of target genes [11-17].

Ligand binding to its receptor at the cell surface not only initiates signaling events but also triggers internalization of both ligand and receptors. Receptor-mediated endocytosis has both positive and negative functions in signal transduction [18-23]. It can sequester the receptors in intracellular compartments, target ligand-receptor complexes to lysosomes for degradation or increase the access of receptors to their intracellular substrates. Receptors internalized in endosomes can also be recycled back to the cell surface for re-use [24].

Two major endocytic pathways mediate internalization of cell surface receptors: clathrin-mediated endocytosis and non-clathrin-mediated endocytosis, the latter including lipid-raft- or caveolae-mediated endocytosis [19, 25, 26]. Clathrin-mediated endocytosis is utilized by many cell surface receptors such as G protein-coupled receptors, tyrosine kinase receptors (epidermal growth factor receptor, platelet-derived growth factor receptor and insulin receptor) and other non-kinase, single transmembrane receptors (low-density lipoprotein receptor, transferring receptor) [27], and is the best characterized pathway. The internalizing receptors are first concen-

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trated on the clathrin-coated pits, which are assembled on the cytoplasmic face of the plasma membrane by the recruitment of the adaptor complex AP2 (consisting of α , β 2, μ 2 and σ 2 subunits), triskelion clathrin (comprising three heavy chains and 3 light chains) and other accessory proteins such as Eps15, epsin, disabled-2, synaptotagmin and amphiphysin [28-30]. Then the pits undergo invagination and pinch off from the plasma membrane in a dynamin GTPase-dependent way [31]. After uncoating of the clathrin coat, the vesicle is finally fused with and the receptors are transported to early endosomes.

Besides clathrin-coated pits, cholesterol- and sphingolipids-enriched lipid rafts can also be found in the plasma membrane [32, 33]. These specialized detergent-insoluble, low-density membrane microdomains are called caveolae when the structural protein caveolin is associated. Lipid rafts can serve as signaling centers for nitric oxide, calcium, G protein-coupled receptors and protein tyrosine kinases, or as virus entrance [32, 34]. They also mediate the internalization of various proteins; cholera toxin, glycosylphosphatidylinositol (GPI)-anchored proteins, endothelin receptor and growth hormone receptor are some of the examples [34-36]. The internalized cargos are thought to be transported to not-well-characterized caveosomes and eventually to other organelles such as endosomes or lysosomes. Therefore, lipid rafts/caveolae regulate multiple cell signaling events.

Like most of the cell surface proteins, TGF- β receptors undergo endocytosis [37]. In this article, TGF- β receptor endocytosis, and the roles of endocytosis in TGF- β signaling, receptor degradation and signal inactivation will be reviewed. The role of endocytosis in establishment of the BMP morphogen gradient has been extensively reviewed [18, 38-40].

Internalization of TGF- β receptors

Receptor internalization

An early study that followed the turnover of 125 I-labelled TGF- β in BALB/c 3T3 mouse fibroblasts suggested that TGF- β is rapidly internalized via its receptors and degraded in lysosomes [41]. Using a similar approach, receptor-mediated TGF- β internalization has also been investigated in mink lung epithelial Mv1Lu cells and human embryonic kidney HEK293 cells, and both T β RI and T β RII were indicated to be required for optimal internalization of TGF- β [42]. However, by following ligand endocytosis, it is difficult to elucidate the contribution of individual receptors in this process and to investigate the role of endocytosis in TGF- β signaling, as there may be several TGF- β -binding proteins on the plasma membrane besides the signaling type I and type

II receptors [16, 43]. Therefore, it is important to directly investigate the internalization process of TGF- β receptors.

Endocytosis of TGF- β receptors and its role in TGF- β signaling have been studied extensively by multiple approaches. Owing to the lack of good antibodies for TGF- β receptors, it is difficult to specifically trace the trafficking of endogenous TGF- β receptors. To overcome this problem and to examine the role of the receptor kinase activity in modulating receptor endocytosis, Leof and colleagues have cleverly engineered artificial receptor constructs by fusing the extracellular domains of the granulocyte/macrophage colony-stimulating factor (GM-CSF) α and β receptors to the transmembrane and cytoplasmic domains of TGF- β type I and type II receptors and followed internalization of the GM-CSF ligand [44]. Using this system, they showed that both TGF- β type I and type II receptors are rapidly endocytosed [45], and that the kinase activity of T β RII, but not T β RI, is required for optimal internalization of the heteromeric receptor complexes [46]. Consistent with this, deletion of the kinase domain of T β RI had no effect on downregulation of the heteromeric receptor complexes [47].

Like most of the other cell surface receptors, TGF- β receptors are mainly internalized via clathrin-dependent endocytosis (Figure 1). Several lines of evidence support this notion. First, using GM-CSF receptor-TGF- β receptor chimeric constructs, Leof and colleagues found that TGF- β receptor endocytosis is mediated by clathrin-dependent pits as it is specifically dampened by potassium depletion, hypertonic medium and cytosol acidification in mouse fibroblast AKR-2B cells [45, 48]. Secondly, by following biotinylated cell surface T β RI, it has been documented that rapid internalization of T β RI can be blocked by the dynamin K44A mutant or by potassium depletion [49]. Thirdly, both T β RI and T β RII can associate with the β 2 subunit of the clathrin-associated adaptor complex AP2 [48]. The direct interaction of TGF- β receptors with β 2 adaptin is mediated by the intracellular domain of the receptors and the β 2 adaptin N-terminal trunk domain. This interaction is different from that of most other plasma membrane receptors that interact with the μ 2 subunit of AP2 [30].

However, TGF- β has also been reported to be endocytosed independently of the clathrin-mediated pathway as the internalization was blocked by phenylarsine oxide, a nonspecific inhibitor, but not affected by reagents which were thought to interfere with clathrin-mediated endocytosis, such as the primary amine monodansylcadaverine and the GTPase-deficient dynamin K44A mutant [42]. In addition, both TGF- β and BMP receptors have been shown to interact with caveolin [50-52] and can also be

Figure 1 TGF- β receptors are distributed in both lipid rafts/caveolae and nonraft membrane microdomains. Internalization of TGF- β receptors via clathrin-coated pits can enhance TGF- β signaling, whereas lipid rafts-mediated endocytosis of TGF- β receptors facilitates receptor degradation and thus the turnoff of signaling. The subcompartmental distribution of TGF- β receptors on the plasma membrane can be regulated: interleukin-6 and ADAM12 were shown to shift the receptors to the nonraft fractions, while heparan sulfate and hyaluronan-CD44 were shown to promote the lipid raft/caveolae localization of TGF- β receptors.

endocytosed in a lipid rafts/caveolae-dependent manner [51-53] (Figure 1), but the detailed mechanism remains to be elucidated.

Receptor endocytosis can take place constitutively or be activated by ligand, and the ligand-dependent endocytosis usually involves receptor phosphorylation or ubiquitination [30, 54]. Both type I and type II TGF- β receptors seem to constantly undergo internalization [49, 52, 55]. Once internalized into the cytoplasmic compartments, TGF- β receptor molecules seem to be recycled back to the cell surface in the absence of ligand [56]. The recycling of the internalized TGF- β receptors is impaired by dominant-negative Rab11 [57], suggesting that the recycling takes place in the perinuclear recycling endosomes [58] (Figure 2). As the activated receptors are more likely to be subjected to degradation, it is surprising to note that ligand treatment has no effect on the rates of internalization and recycling of TGF- β receptors [57].

Internalization signals of TGF- β receptors

Internalization of most cell surface receptors is mediated by short specific sequences in their cytoplasmic domains [29, 30]. Two types of such internalization signals with distinct consensus sequences mediate clathrin-dependent endocytosis: one with tyrosine-containing sequences such as NPXY and YXX ϕ (X is any amino acid and ϕ is a hydrophobic amino acid) and the other known

as di-leucine-based motifs [DE]XXXL[LI]. These sequences can directly bind to the endocytic machinery: all of them are recognized by the μ 2 subunit of AP2; NPXY can also interact with clathrin and the phosphotyrosine-binding domain of the adaptor protein disabled-2; and di-leucine-based signal can also associate with the β 2 subunit of AP2 [29, 30]. Other variable internalization signals have also been identified, such as acidic clusters [59] and ubiquitination [54, 60], and shown to be essential for the internalization of the respective receptors. The conjugated ubiquitin can be recognized by ubiquitin-binding motifs in the accessory proteins, such as eps15 and epsin [30, 54]. Therefore, these internalization signals play an important role in cargo enrichment on the clathrin-coated pits as well as in vesicle formation.

Such internalization signals have also been identified in TGF- β receptors. By following the endocytosis of truncation, deletion and substitution mutants of T β RII from the cell surface, Ehrlich *et al.* [55] have found that a di-leucine-like signal in the cytoplasmic region proximal to the transmembrane domain, Ile₂₁₈Ile₂₁₉Leu₂₂₀, is necessary for T β RII endocytosis. In accordance with the recognition of the di-leucine signal by the clathrin endocytic machinery, T β RII is constitutively internalized in a clathrin-dependent manner [55]. Furthermore, both T β RI and T β RII can interact with the β 2 subunit of AP2 via their intracellular domains [48]. However, it is currently

Figure 2 Subcellular localization of receptors regulates TGF- β signaling. TGF- β receptors are endocytosed constitutively in the absence of ligand. After entering the early endosomes, they are sorted to recycling endosomes and recycled back to the plasma membrane. Upon ligand binding, the receptors can initiate signaling by activating the Smad pathway. The ligand-bound receptors can continue and even maximize their signaling activity in early endosomes, where the signaling-promoting factors such as SARA, Hrs and endofin reside. Some of the ligand-bound receptors are recycled back to the plasma membrane for re-use, or sorted to late endosomes, where they interact with Dapper2 and are targeted to lysosomes for degradation. The GDP-bound form of Rab5, Rab5 S34N, which interferes with endosome fusion, has been shown to enhance ligand-independent Smad signaling.

unclear whether the di-leucine-like signal of T β RII is directly involved in this interaction.

The internalization signal of T β RI is distinct from the classical consensus sequences. There are two putative tyrosine-based YXX Φ motifs in the intracellular domain of T β RI: Y₁₈₂DMT and Y₂₄₉QTV. However, mutation of either tyrosine residue had no effect on T β RI endocytosis [56]. Instead, deletion and substitution mutation studies have identified a motif (R₄₈₂LTALRIKKTL₄₉₂) close to the C-terminal tail that is important for T β RI downregulation [47]. This motif was referred as the NANDOR (no activating non-down-regulating) Box. Although this motif is highly conserved among all the type I receptors of the TGF- β family, it does not belong to any of the two types of well-characterized internalization signals. Ala replacement of any single amino acid in this box had no effect on ALK4 internalization, indicating that cooperation between the amino acid residues or certain conformation of the NANDOR Box is important for mediating

receptor endocytosis/downregulation [61], but mutation of two to four amino acids blocked receptor downregulation [47]. Interestingly, this motif seems to be essential for T β RI activation as its deletion abolished T β RI phosphorylation by T β RII and the ability of T β RI to mediate TGF- β signaling [47]. As the kinase activity of T β RI may not be necessary for receptor internalization [46], the function of this motif in coupling receptor activation and internalization/downregulation needs further investigation. Supporting the importance of the COOH-terminus of type I receptors in mediating receptor endocytosis, a study with the type I activin receptor ALK4 showed that a conserved Trp, six amino acids upstream of the NANDOR Box (W₄₇₇ in ALK4), is essential for ALK4 internalization, and substitution of this Trp with Ala blocked ligand-induced endocytosis of ALK4 [61].

Regulation of receptor internalization

Few studies have touched on how internalization of

TGF- β receptors is regulated. The FK506-interacting protein FKBP12 can directly associate with T β RI through a hydrophobic interaction with the Leu-Leu motif in the GS domain of the receptor [62], and has been proposed to inhibit the basal activity of TGF- β receptors resulting from randomly encountered receptor interactions [63]. FKBP12 may also function as a negative regulator of T β RI internalization as rapamycin, which disrupts the T β RI-FKBP12 interaction, can enhance the internalization of wild-type T β RI, but not the mutant deficient in FKBP12 binding [64]. Internalization of endoglin and T β RII has been shown to be controlled by thrombin in human endothelial cells. Thrombin and a peptide agonist of its receptor PAR1 promote endocytosis of endoglin and T β RII, but not T β RI; and the endocytosis, which negatively regulates TGF- β signaling, is dependent on protein kinase C- ζ [65]. Whether other mechanisms are involved in regulation of TGF- β receptor endocytosis is unclear. For instance, ubiquitination has been shown to be important in regulating endocytosis of receptor tyrosine kinases [66], but the possibility of its involvement in TGF- β receptor internalization remains to be investigated.

Cell type-specific receptor internalization

Internalization of TGF- β receptors has been suggested to be cell-specifically regulated. Although the heteromeric receptor complexes of type I and type II receptors are efficiently internalized and downregulated in both Mv1Lu epithelial cells and AKR-2B fibroblasts, the homomeric type I or type II receptors are endocytosed differently [45, 67]. In AKR-2B cells, the homomeric receptors exhibit a similar internalization rate as the heteromeric receptor complexes, whereas in Mv1Lu cells, the homomeric receptors are internalized less efficiently in comparison to the heteromeric receptor complexes [67]. In both cell lines, the homomeric receptor complexes seem to be recycled back to the cell surface as they do not undergo downregulation. However, the underlying mechanism is presently unknown.

Contrary to what is observed in fibroblasts [46], the kinase activity of T β RII is not essential for downregulation of heteromeric TGF- β receptor complexes in epithelial cells [56]. In addition to this, a signaling-incompetent mutation of T β RI – T200V – abolishes endocytosis/receptor downregulation in fibroblasts, whereas it has no such effect in epithelial cells [56].

Promoting effect of endocytosis on TGF- β signaling

Promotion of TGF- β signaling by endocytosis

Receptor internalization can play either a positive or

a negative role in signal transduction. By reducing the number of cell surface receptors and inducing receptor degradation, endocytosis regulates signaling negatively, whereas endocytosis can also increase the proximity of the internalized receptors to their substrates or other signaling intermediaries in the cytoplasm [18, 20-22, 37]. Several studies have investigated the role of endocytosis in TGF- β signaling and yielded different conclusions.

By using the GM-CSFR-TGF- β receptor fusion system in combination with endogenous TGF- β receptors, Penheiter *et al.* [68] found that TGF- β receptors can form a complex with SARA (Smad Anchor for Receptor Activation) and Smad, and T β RI phosphorylation can take place even when clathrin-coated vesicle formation is blocked by low-temperature treatment, potassium depletion or the dominant-negative K44A dynamin mutant, while the receptor-mediated phosphorylation and thus activation of Smad2/3 require receptor internalization, suggesting that receptor endocytosis plays an essential role in TGF- β signaling. However, it is unclear how clathrin-mediated endocytosis initiates Smad2/3 phosphorylation in the receptor-SARA-Smad complex, which has been pre-assembled presumably at the plasma membrane. Although clathrin-dependent endocytosis has also been suggested to be essential for TGF- β /Smad signaling in human kidney mesangial cells, a different mechanism was proposed [69]. Runyan *et al.* [69] found that inhibition of clathrin-mediated endocytosis only slightly affects TGF- β -induced Smad2 phosphorylation and Smad2-Smad4 association, but decreases the nuclear accumulation of Smad2 and therefore attenuates Smad-mediated transcriptional responses. It was proposed that endocytosis is possibly required to release Smad2 from the SARA complex as inhibition of clathrin-dependent endocytosis by potassium depletion prolonged the SARA-Smad2 complex formation. The positive role of clathrin-mediated endocytosis in TGF- β signaling is supported by immunofluorescence and cell fractionation studies [52]. TGF- β receptors can be internalized via both clathrin-coated pits and cholesterol-rich lipid rafts/caveolae as they are found in both EEA1-positive early endosomes and caveolin-1-positive compartments. Disruption of clathrin-coated pits or clathrin-mediated endocytosis by potassium depletion, dominant-negative mutants of Eps15 or dynamin attenuates TGF- β -induced Smad2 phosphorylation, whereas disruption of lipid rafts by the cholesterol-depleting agent Nystatin can shift TGF- β receptors to nonraft compartments and enhances TGF- β /Smad signaling. These data suggest that clathrin-mediated endocytosis promotes, whereas caveolae-mediated internalization turns off, TGF- β /Smad signaling (see below). Similar results showing the localization of TGF- β receptors in EEA1-

positive vesicles and the stimulatory role of clathrin-dependent endocytosis in TGF- β /Smad signaling were also reported by Hayes *et al.* [70].

Although the above-described studies showed an important role of clathrin-mediated endocytosis in TGF- β signaling, several lines of evidence indicate that TGF- β signaling can take place on the plasma membrane. First, it was reported that blocking clathrin-dependent internalization of TGF- β receptors by potassium depletion or mutant dynamin (K44A) had no effect on the phosphorylation or nuclear accumulation of Smad2 and Smad-mediated transcriptional response [49]. Consistent with this, Smad2 can associate with TGF- β receptors under conditions of potassium depletion. It is still unclear why different results were obtained from similar experimental systems. Secondly, a dispensable role of clathrin-mediated endocytosis in BMP-induced Smad1/5 activation has been suggested [51]. Both type I and type II BMP receptors undergo internalization via the clathrin-coated pits, but only type II BMP receptor can be endocytosed via caveolae. BMP-induced phosphorylation of Smad1/5 was not influenced by dynamin K44A or by chlorpromazine, a disruptor of clathrin-coated pits, although prolonged treatment of chlorpromazine decreased the transcriptional responses of BMP. Interestingly, BMP-induced alkaline phosphatase production seems to need intact lipid rafts as decrease of cholesterol level specifically blocked alkaline phosphatase production [51]. Thirdly, activin-induced Smad2 phosphorylation is not dependent on receptor endocytosis, as ALK4 W477A, which is unable to undergo ligand-dependent internalization, can still mediate activin-stimulated Smad2 phosphorylation and transcriptional responses even under the condition of potassium depletion [61].

TGF- β signaling in endosomes

Endosomes are important signaling centers for TGF- β signal transduction. Several positive regulators of TGF- β signaling are localized in endosomes. For instance, three FYVE (Fab1, YOTB/ZK632.12, Vac1, and EEA1) domain-containing proteins, SARA, endofin and Hrs/Hgs ((hepatocyte growth factor-regulated tyrosine kinase substrate), have been suggested to promote TGF- β , activin and decapentaplegic (Dpp) signaling [71-75]. The FYVE domain is a zinc-binding motif known to bind phosphatidylinositol 3-phosphate, a lipid enriched in early endosomes, and can specifically recruit the FYVE-domain-containing proteins to early endosomes [76]. The FYVE domain is essential for the stimulatory effect of these proteins on TGF- β signaling [71, 73, 75, 77], indicating that their localization in early endosomes is important to fulfill their functions. It was shown that SARA

functions as a scaffold protein by specifically interacting with Smad2/3 and TGF- β receptors to facilitate Smad2/3 phosphorylation by the TGF- β receptor [71]. Similarly, Hrs can interact with Smad2 and cooperate with SARA to promote activin-mediated activation of Smad2 [72]. Endofin can also facilitate TGF- β signaling. However, unlike SARA or Hrs, endofin binds to Smad4 and, in cooperation with SARA, may assist the heterocomplex formation between receptor-phosphorylated Smad2/3 and Smad4 [75] (Figure 3). Endofin has also been suggested to promote BMP signaling by helping recruit Smad1 to BMP receptors [78]. In this regard, endofin acts very similarly as SARA to function as a Smad anchor for receptor activation.

Different from its positive role in TGF- β signaling, SARA and Hrs have also been shown to negatively regulate Dpp signaling in *Drosophila*. SARA can bring the catalytic subunit of protein phosphatase 1 to and dephosphorylate the Dpp receptor Thickveins (Tkv) [79], whereas loss of Hrs leads to enhanced Dpp signaling and an increased level of Tkv [80]. Interestingly, endofin has been suggested to recruit protein phosphatase 1 to dephosphorylate BMP type I receptors and therefore attenuate BMP signaling [78]. It is currently unknown how the opposite functions of endofin in BMP signaling are controlled.

Another function of SARA in *Drosophila* developing wing epithelial cells is to target molecules of Dpp signaling, such as its receptor Tkv, to endosomes associated with the spindle machinery to ensure appropriate distribution of the signaling molecules in the two daughter cells after mitosis [74]. By doing so, SARA ensures the maintenance of Dpp signaling levels across mitosis.

The small GTPase Rab5 is a crucial regulator of early endosome dynamics [58]. Like all other GTPases, it cycles between the inactive GDP-binding form and the active GTP-binding form. It was shown that Rab5 S34N, a GDP-bound dominant-negative Rab5 mutant, can stimulate the expression of a Smad3-responsive reporter in a ligand-independent manner, whereas the GTP-bound Rab5 Q79L mutant attenuates the transcriptional activity induced by activin [77]. As Rab5 S34N inhibits clathrin-mediated recycling and early endosome fusion [81], it was suggested that the stimulatory effect of Rab5 S34N on Smad-dependent signaling could be a consequence of decreased degradative or recycling trafficking of TGF- β /activin receptors, which results in their accumulation in early endosomes, where other TGF- β /activin signaling-promoting factors SARA, endofin and Hrs reside [77] (Figure 2). However, this hypothesis remains to be tested as RN-tre, a specific Rab5 GTPase-activating protein that facilitates GTP hydrolysis, does not increase the expres-

Figure 3 Early endosomes function as a signaling organelle to promote TGF- β signaling. Early endosomes are enriched with phosphatidylinositol-3 phosphate, which recruits to the membrane the FYVE domain-containing proteins, such as SARA, Hrs and endofin. SARA and Hrs have been shown to function as scaffold proteins to bring Smad2/3 to TGF- β /activin receptors and facilitate receptor-mediated Smad phosphorylation. Endofin associates with Smad4 as well as TGF- β receptors to promote Smad activation in collaboration with SARA.

sion of the Smad-responsive reporter [77]. Nevertheless, these data strongly indicate that early endosomes are a signaling organelle for the TGF- β /Smad pathway.

Endocytosis and turnoff of TGF- β signaling

Receptor degradation via the endocytic pathway

TGF- β receptor activity is tightly regulated by a set of their interacting proteins [7, 43, 82]. Endocytosis, which has long been regarded as a means to turn off signaling by mediating receptor degradation, plays an important role in controlling the amount of receptors on the plasma membrane. Several studies have examined the half-life of TGF- β receptors and yielded different results. Using the 125 I-TGF- β 1-binding assay, Centrella *et al.* [83] found that the half-life of cell surface T β RI and T β RII is about 2 h in primary osteoblasts. However, the half-life of the newly synthesized mature (endoglycosidase H-resistant) form of T β RII in Mv1Lu lung epithelial cells, as analyzed by 35 S-Met metabolic labeling, varied from about 1 h [84] to 2.5 h [85]. TGF- β 1 treatment reduced the half-life of T β RII to 45 min [84] or 1.7 h [85]. Interestingly, the half-life of the newly synthesized mature T β RI seems to be longer than that of T β RII and is also reduced by li-

gand treatment [85]. When the constitutively active T β RI (caT β RI) was overexpressed in HEK293T cells, the half-life of the cell surface caT β RI is about 4 h [86]. Nonetheless, these studies together suggest that TGF- β receptors are constantly turned over and that their downregulation is enhanced by ligand treatment.

Although the formation of T β RI/T β RII complexes is required for TGF- β signal transduction, downregulation of either T β RI or T β RII was observed when they were expressed separately [45, 55, 86]. Together with the observation that these receptors may have different turnover dynamics, it is likely that either receptor alone can undergo downregulation. However, cooperation between receptors or receptor complex formation is needed for effective receptor downregulation. Using receptor-deficient cell lines in combination with an ectopic expression system, it was shown that downregulation of T β RII and betaglycan (a TGF- β type III receptor) needs T β RI [87]. Additional evidence that effective receptor downregulation may require the functional interaction between type I and type II receptors was provided by the GM-CSF-TGF- β receptor chimeric constructs: both homomeric and heteromeric TGF- β receptors undergo fast internalization, but only the heteromeric receptors are downregu-

lated [45, 67].

The turnover of cell surface TGF- β receptors is subject to their regulation by other molecules. For instance, H-8, an inhibitor of cAMP-dependent protein kinase and cGMP-dependent protein kinase, and staurosporine, a potent protein kinase C inhibitor, have been shown to enhance TGF- β binding to its receptors, whereas phosphatase inhibitors okadaic acid, vanadate and fluoride decrease the binding [83], indicating that the turnover of TGF- β receptors is modulated by other signaling events. The oncogene H-ras may also modulate the downregulation of cell surface TGF- β receptors [88].

Turnoff of TGF- β signaling in lipid rafts/caveolae

Lipid rafts/caveolae are generally regarded as signaling centers for nitric oxide, calcium, G protein-coupled receptors and protein tyrosine kinases [32, 34, 89]. However, several lines of evidence suggest that these membrane microdomains negatively regulate TGF- β signaling. Caveolin-1, a critical component of caveolae, has been shown to attenuate TGF- β signaling [50]. It physically associates with T β RI and interferes with TGF- β -induced Smad2 phosphorylation and subsequent downstream events.

The inhibitory Smad Smad7 inhibits TGF- β signaling via multiple mechanisms, acting as a decoy substrate to form a stable complex with receptors to prevent recruitment of R-Smads [90, 91], engaging protein phosphatase 1 to dephosphorylate receptors [92], and functioning in the nucleus to recruit HDAC to repress transcription [93], or disrupt the functional Smad-DNA complex formation [94]. Another way in which Smad7 can inhibit TGF- β signaling is by recruiting E3 ubiquitin ligases Smurf1 and Smurf2 and inducing ubiquitination and degradation of type I receptors [95, 96]. The interaction of Smad7 and Smurf proteins with TGF- β receptors targets the complexes to lipid rafts/caveolae, and caveolin-dependent internalization of TGF- β receptors could promote receptor turnover and signaling termination [52]. Consistent with this, cholesterol has been suggested to inhibit Smad2 activation, promote TGF- β receptor degradation, and therefore inhibit TGF- β signaling; and this effect may result from the shifted localization of TGF- β receptors from nonraft to lipid raft microdomains in the plasma membrane [53].

As lipid rafts-mediated internalization of TGF- β receptors promotes receptor degradation, modulation of the receptor distribution in lipid rafts vs nonraft microdomains is a mechanism to regulate TGF- β signaling. Interleukin (IL)-6 enhances TGF- β /Smad signaling in kidney tubular epithelial cells, and this enhancement was shown to be because of IL-6-induced partitioning of TGF- β

receptors to nonraft microdomains and thus decreased degradation of TGF- β receptors [97] (Figure 1). In support of this, potassium depletion attenuates the stimulatory effect of IL-6 on TGF- β /Smad signaling, whereas Nystatin increases it. ADAM12 (a disintegrin and metalloproteinase 12) is a glycoprotein with zinc protease and integrin-binding activities in its extracellular domain and has been implicated in the control of membrane fusion, cytokine and growth factor shedding, and cell migration [98]. A recent study reported that it can interact with the extracellular domain of T β RII and enhance TGF- β signaling [99]. Interestingly, neither its protease activity nor the intracellular domain is required for this stimulatory effect. Rather, it induces the accumulation of T β RII in EEA1-positive early endosomes and stabilizes the T β RII protein probably by preventing the association between T β RII and Smad7 and the internalization of T β RII into caveolin1-positive vesicles.

Two polysaccharides have been shown to negatively influence TGF- β /Smad signaling by shifting the receptors to lipid rafts/caveolae fractions. Heparan sulfate, a polysaccharide that is a component of proteoglycans, could stimulate lipid rafts-mediated endocytosis and rapid degradation of TGF- β receptors [100]. Consistent with this, more receptors were found in nonraft fractions in heparan sulfate-deficient cells than in wild-type cells. Similarly, hyaluronan functions through its receptor CD44 to partition TGF- β receptors to caveolin-1-positive caveolae fractions and enhance the interaction of caveolin-1 with Smad7, leading to attenuation of TGF- β signaling [101]. These observations suggest another layer of regulation of TGF- β signaling by other pathways; however, the mechanistic details remain to be established, such as how IL-6 and ADAM12 divert the trafficking of TGF- β receptors from caveolin-dependent endocytosis to clathrin-dependent endocytosis, and how the polysaccharides shift the receptors to lipid raft fractions.

Lipid rafts/caveolae may not only serve as a place to promote Smad7-mediated degradation of TGF- β receptors, but are also involved in some of the specific TGF- β signal pathways. For instance, a recent study showed that ALK1, a TGF- β type I receptor that signals via Smad1/5 in endothelial cells [102], is localized in caveolae, and caveolin-1 associates with ALK1 and promotes TGF- β /ALK1 signaling [103]. Caveolin-1 can also enhance TGF- β -induced expression of type I procollagen via the PI3K/Akt pathway although it inhibits Smad3 activation [104]. In addition, association of TGF- β receptors with endothelial nitric oxide synthase (eNOS) in lipid rafts/caveolae fractions is important for the activation of eNOS as TGF- β -induced dissociation of TGF- β receptors from eNOS resulted in a decrease of eNOS phosphorylation

and its enzymatic activity [105]. Lipid rafts may also be important for ligand-induced heterocomplex formation of TGF- β receptors [106].

It should be noted that in contrast to the model that clathrin-mediated endocytosis of TGF- β receptors promotes TGF- β signaling whereas caveolae/lipid raft-mediated internalization is required for receptor degradation [52], data from the studies with GM-CSF receptor-TGF- β receptor chimeric constructs as well as endogenous TGF- β receptors suggested that TGF- β receptor degradation may be mediated by clathrin-dependent endocytosis, but does not require the cholesterol-rich caveolae/lipid rafts as receptor turnover was blocked by potassium depletion, but not by the cholesterol-depleting agent Nystatin [57].

The mechanisms regulating the stability of TGF- β receptors and Smad proteins are reviewed in detail by Lönn *et al.* [107].

Dapper2

Dapper was initially identified as an interacting protein of Dishevelled (Dvl) and functions as a general antagonist to block both the canonical β -catenin-mediated and noncanonical JNK-mediated Wnt signaling in *Xenopus* [108]. Its fish ortholog Dapper2, which shares several conserved domains with the *Xenopus* Dapper (Dapper1), can attenuate TGF- β /activin/Nodal signaling [86]. Overexpression of Dapper2 in zebrafish embryos led to phenotypes mimicking the ones in the loss-of-function mutants of the Nodal co-receptor oep, pinhead and eye fusion; and knockdown of its expression by morpholino-antisense oligonucleotides resulted in expanded mesoderm formation, suggesting that Dapper2 modulates mesoderm formation by acting as a negative regulator of activin/Nodal signaling. Consistent with its function in zebrafish embryos, fish Dapper2 could also attenuate TGF- β and activin signaling in mammalian cells, as shown by Smad phosphorylation and reporter assays [86]. Biochemical analysis further revealed that Dapper2 can interact with T β RI and the activin/Nodal type I receptor ALK4 and exhibit a high binding affinity to the activated receptors, and overexpression of Dapper2 led to lysosomal degradation of the cell surface T β RI. As Dapper2 protein was found in late endosomes, it was proposed that Dapper2 modulates the levels of cell surface ALK4 or T β RI by targeting the endocytosed activated receptors for lysosomal degradation (Figure 2). The mouse Dapper2 has been shown to attenuate TGF- β /activin signaling [109]. Thus, although Dapper2 has been suggested to regulate Wnt signaling in embryos [110], the inhibitory function of Dapper2 in TGF- β /activin/Nodal signaling is evolutionally conserved from fish to mouse. In agree-

ment with its function in TGF- β /activin/Nodal signaling, Dapper2 expression depends on Nodal signals in fish embryos [86].

β -Arrestin 2

β -Arrestins bind to G protein-coupled receptors and function to attenuate their signaling activities, to act as scaffold proteins to connect to other signaling pathways such as the mitogen-activated protein kinase (MAPK) cascades and to facilitate clathrin-mediated internalization of cell surface receptors [111]. β -Arrestin 2 can bind to the type III TGF- β receptor betaglycan, and this binding is triggered by T β RII-mediated phosphorylation on the intracellular domain of betaglycan [112]. More importantly, the association of betaglycan with β -arrestin 2 leads to the internalization of both T β RII and betaglycan and thus downregulation of TGF- β signaling. However, it is unclear whether the downregulation of TGF- β signaling is due to the intracellular sequestration or increased turnover of receptors.

ARIP2

Internalization of the type II activin receptor (ActRII) was shown to be promoted by activin receptor-interacting protein 2 (ARIP2) [113]. Overexpression of ARIP2 attenuated the activin-induced reporter expression, indicating that internalization of ActRII negatively regulates activin signaling. ARIP2 associates with ActRII via its NH2-terminal PDZ domain and with Ral-binding protein 1 (RalBP1) via its COOH-terminal region. RalBP1 has been shown, as an effector of the GTPase Ral, to regulate endocytosis of epidermal growth factor and insulin receptors [114]. Interestingly, unlike ARIP2, which contains only one PDZ domain, ARIP1, which has five PDZ domains and two WW domains, was reported to promote activin signaling by acting as a scaffold protein to bring ActRIIA and Smad3 proteins together [115].

Concluding remarks

Although endocytosis of TGF- β receptors has been extensively investigated, many important questions still remain unsolved. The current prevailing view is that lipid rafts/caveolae negatively modulate TGF- β signaling by promoting TGF- β receptor turnover, probably via Smad7-mediated recruitment of Smurf, which catalyzes receptor ubiquitination [52]. But it is unclear how Smad7 can target the activated TGF- β receptors to lipid rafts/caveolae, or, alternatively, how the TGF- β receptors-Smad7 interaction preferentially occurs in lipid rafts/caveolae. It seems that Smad7-Smurf-mediated degradation of TGF- β receptors depends on both proteosomal and

lysosomal activities [96]. Then, how does ubiquitination promote lysosomal turnover of TGF- β receptors? Does ubiquitination play any role in TGF- β receptor endocytosis? Degradation of TGF- β receptors occurs not only via caveolae-mediated endocytosis, but also via clathrin-dependent internalization to early endosomes and then to late endosomes. It is totally unknown how the endosomal sorting of TGF- β receptors to different destinations (plasma membrane for re-use or late endosomes/lysosome for degradation) is regulated. Lipid rafts/caveolae are signaling centers for membrane-attached tyrosine kinases, while they play a negative role in TGF- β signaling by promoting receptor degradation. In addition to the canonical Smad pathway, TGF- β can also activate other signaling molecules such as small GTPases (Ras, Rac, Rho and CDC42), MAPKs, PI3K/Akt, PAK and others in a cell type-dependent manner [8, 116]. Whether the activation of any of these molecules is dependent on compartment-specific localization of TGF- β receptors is another important subject of future investigation.

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