# MARFAN SYNDROME-RELATED MUTATIONS LEAD TO ABNORMAL

# TRAFFICKING OF TGF-β FAMILY RECEPTORS

By

JING LIN

A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Cell and Developmental Biology

Written under the direction of

Dr. Richard Padgett

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

May 2019

**ABSTRACT OF THE DISSERTATION**

**MARFAN SYNDROME-RELATED MUTATIONS LEAD TO ABNORMAL**

**TRAFFICKING OF TGF-β FAMILY RECEPTORS**

**By JING LIN**

**Dissertation Director:**

**Dr. Richard Padgett**

The transforming growth factor-β (TGF-β) family includes a wide range of secreted, soluble proteins that are structurally similar growth factors; they play pivotal roles in various developmental processes. TGF-β ligand binding to TGF-β type I and type II receptors, which are located on the cell membrane, trigger the whole signaling cascade. The activated type I and type II receptors bind with the ligand to form a receptor complex, which further activates downstream signal transducer SMADs by phosphorylation. Eventually the activated pSMADs translocate to the nucleus to regulate specific gene expression.

The mis-regulation of TGF-β signaling is associated with several genetic diseases, including Marfan syndrome (MFS). MFS is an autosomal dominant genetic disorder of connective tissue that affects the ocular, skeletal, cardiovascular and pulmonary systems, often leads to death in early adult life. The cause of MFS can be directly attributed to germ line mutations in fibrillins. As

shown by several recent studies, fibrillin mutations lead to excessive levels of bioactive TGF-β in the tissue microenvironment.

Other components in the TGF-β pathway can also be disrupted to result in related disorders; usually these show milder manifestations of the phenotypes seen in MFS. Most of these MFS-like disorders can be attributed to heterozygous missense germ line mutations in either TGF-β type II or type I receptor genes, and most of these mutations fall into the C-terminal domain in TGF-β receptors. Sequences in the C-terminal domain of the receptors are important for proper trafficking. The C-terminus tail in type II receptor regulates its endocytosis, via what is defined as the LTA motif. Many MFS-like associated amino acids reside in LTA motif, which lead to the hypothesis that these disease mutations may disrupt the normal trafficking route of TGF-β receptors.

I tested this hypothesis by engineering MFS-like mutations into the nematode type II receptor and testing resulting its activity and trafficking. Based on my observations using the model organism *C. elegans*, I found that some of these MFS-like phenotypes cause mis-trafficking of TGF-β receptors.

## ACKNOWLEGEMENTS

I would like to first acknowledge part of this dissertation (Chapter II) has been submitted to the journal *PlosOne*.

I would like to give many thanks to my wonderful adviser, Dr. Richard W. Padgett. It was really lucky for me to join his lab, and he was always helpful and kind. I really appreciate that his door was always open when I had scientific questions. His dedication to science, enthusiasm for research and inspiration of sparkling ideas enlighten my research.

I particularly appreciate my committee members: Dr. Monica Driscoll, Dr. Nancy Walworth and Dr. Andrew Singson. Thanks for the guidance and time they offered on my research. My committee always gave me honest advice and innovative insights into my research. I am also very grateful for great suggestions about my career path.

I am very grateful to every member in Padgett lab. First I want to express my gratuity to our awesome postdoctoral research fellow, Dr. Mehul Vora. He not only guided me on my research, taught me new laboratory skills and facilitated my scientific writing, but also contributed to many whimsy comments and jokes. Our great lab manager, Nanci Kane, did most of the injection work and proofreading for my paper. She also taught me about injection techniques and cataloged my reagents which helped me a lot in my project. Our previous

iv

**TABLE OF CONTENTS**

# LIST OF TABLES

# LIST OF ILLUSTRATIONS

# Chapter I: Introduction

Transforming growth factor-β (TGF-β), which is the prototype of the TGF-β super family, plays an important role in cellular proliferation, differentiation and apoptosis. The abnormal functioning of the TGF-β signaling pathway causes several types of cancers and Marfan syndrome-like diseases. Below I will briefly introduce TGF-β signaling, the mechanisms and theories of TGF-β related diseases and the current state of research in the field.

## Introduction to the TGF-β pathway

### Overview of the TGF-β family

The large number of TGF-β polypeptides is often divided into two or more subfamilies. The first subfamily consists of the TGF-β subfamily and the second subfamily includes bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins (ACTs), inhibins (INHs), and glial-derived neurotrophic factors (GDNFs). Outside these classical subfamilies, some proteins such as Müllerian inhibiting substance (MIS), which is also known as anti-Müllerian hormone (AMH), left-right determination factor (Lefty) and nodal growth differentiation factor (Nodal) are also members of the TGF-β family, whose origins are less clear (Poniatowski et al., 2015). These proteins have their specific receptors and function on a cellular level (Fig 1).
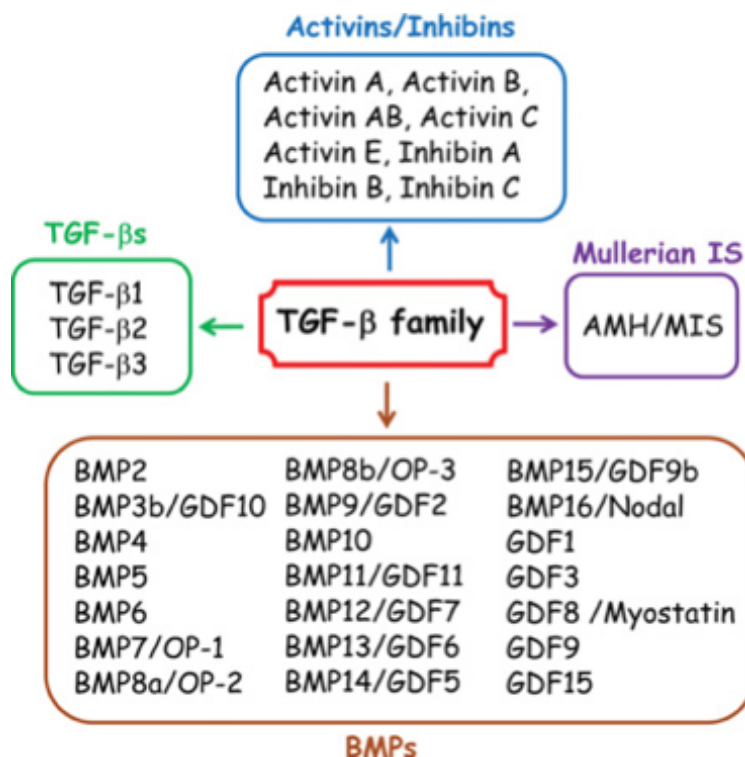
Figure 1. Schematic representation of the mammalian TGF-β family (Santibanez et al., 2011).

Members of the TGF-β family are dimeric, multifunctional regulators playing important roles in embryonic and developmental processes in animals from invertebrates to mammals (Massague, 1998). Members of this super family are implicated in regulating fundamental biological processes that include wound healing, morphogenesis, motility, cell proliferation, cell differentiation, cell-cell adhesion, cell migration, cell death, cytoskeletal organization, and immune responses (Massague, 1998).The malfunction of TGF-β signaling is involved in specific cancer patients (Massague, 1998). TGF-β ligands play pivotal roles in regulation of the above developmental processes in multiple tissues and organisms.

The function of TGF-βs will be discussed in detail later in this thesis. The following paragraphs will give a general overview on the functions of BMPs, ACTs and GDFs that are involved in various biological processes. BMPs play important roles in bone formation and are responsible for inducing mesenchymal stem cells to differentiate into bone. BMPs also have diverse functions in multiple organ systems and thus are considered to be body morphogenetic proteins. For example, BMPs are involved in maintenance of mature tissue homeostasis (maintaining the integrity of joints), initiating bone fracture healing, and regulating vascular system remodeling (Wang et al., 2002). There is evidence that BMPs are required in body axis formation and patterning (Gordon and Blobe, 2008).

Activins (ACTs) are required for cell differentiation and craniofacial development. ACTs also play roles in the regulation of spine formation, neurogenesis, and the maintenance of long-term memory in transgenic mouse (Ageta and Tsuchida, 2011). Polypeptide growth and differentiation factors (GDFs) are important for osteogenesis, cementogenesis, and connective tissue formation by their functions in the regulation of several key cellular processes. GDFs are also instrumental in the induction and regulation of wound healing processes (Hogan et al., 1994).

The TGF-β signaling pathway was discovered over 30 years ago and has been the subject of many studies by several groups; however, there is still an

abundance of unanswered questions. This work will provide novel insights into

TGF-β related disease Marfan syndrome (MFS) and its MFS-like diseases.

**TGF-β receptors**

TGF-β signal transduction functions through protein receptors. This receptor

family, like the TGF-β ligand family, can be cataloged into subfamilies that have

structural similarities (Massague, 1998). Based on their roles in signal

transduction, TGF-β receptors are subdivided into three classes: type I, II and III

(Massague et al., 2000). Type I and type II receptors are the major functioning

receptors interacting with specific ligands, while type III TGF-β receptors rarely

participate in direct signal transduction, in most cases they act as the auxiliary

proteins in TGF-β signaling (Massague et al., 2000) (Fig 2).
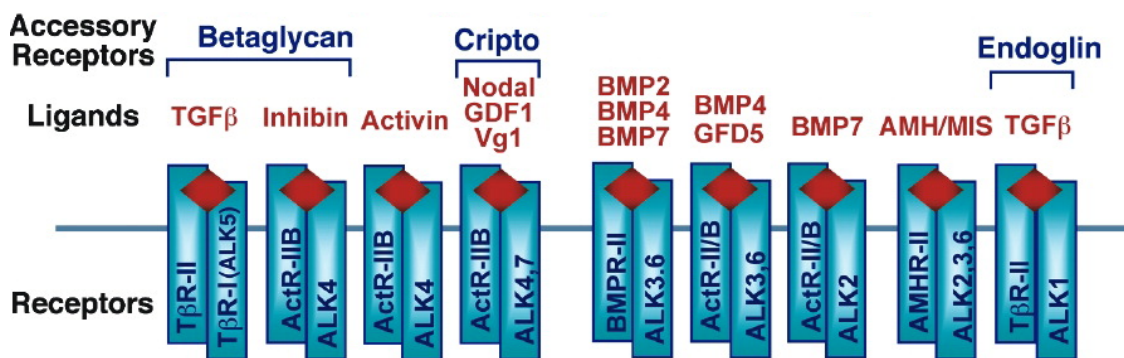


Figure 2. The schematic representation members of TGF-β super family. Both ligands and receptors are shown; Accessory receptors are labeled out (Shi and Massague, 2003).

From Fig 2, it is clear that not only do different TGF-β ligands interact with their

specific TGF-β receptors, but different subcategory TGF-β type I and type II

receptors are associated with their own subcategories. Both type I and type II

receptors are ~500 amino acid long and are hetero-dimeric, transmembrane

serine/threonine kinases that consist of an extracellular domain, a short trans-

membrane domain and an intracellular domain (Shi and Massague, 2003). Type I

and type II receptors also have sequence homologies including cysteine-rich

regions for ligand binding in the extracellular domain at the N-terminus of both

receptors (Shi and Massague, 2003). The kinase domain, which is the key

component in TGF-β receptor function, occupies a major portion of the

cytoplasmic domain C-terminus (Wrana and Attisano, 1996). However, there is a

short and unique sequence in the intracellular domain that is adjacent to the

trans-membrane region in type I receptor. This short sequence is composed

primarily of glycine and serine residues and is highly conserved (*C. elegans* to

mammals) (Shi and Massague, 2003). This region is termed as the GS domain

and is required for type I receptor activation (Massague et al., 2000). The domain

arrangements of TGF-β receptors are shown in Fig 3.
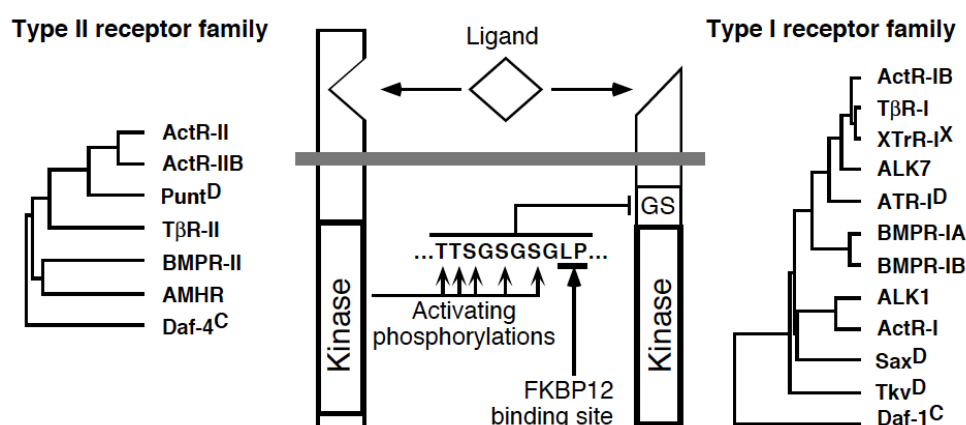


Figure 3. The domain organizations of TGF-β type I and type II receptors on the cell membrane. Members of type I and type II receptors are listed on both sides. The GS region in type I receptor is highlighted and part of the amino acid sequence in GS domain is shown. FKBP12 is the inhibitor to type I receptor binding to the GS domain (Massague, 1998).

**Ligand-receptor interactions**

The interaction between TGF-β receptors and their ligands is a prerequisite for the signaling pathway activation. There are two general modes of ligand binding to receptor. The first ligand binding model is typical for the BMP subfamily while the other is characteristic of TGF-βs and activins (Shi and Massague, 2003). A BMP ligand selectively binds a BMP type I receptor due to greater affinity toward type I receptor compared to a type II receptor. In contrast, the BMP ligand-type I receptor complex has a high affinity for the type II receptor (Shi and Massague, 2003).

Unlike the BMP subfamily, TGF-β and activin ligands have a high affinity to type II receptors and very weak interactions with the isolated type I receptors (Wrana et al., 1994). Ligand binding to the extracellular domain of the TGF-β type II receptor, leads to conformational changes in the intracellular domain of the receptors that promotes the phosphorylation and subsequent activation of the type I receptor (Shi and Massague, 2003; Wrana et al., 1994). There is also evidence that TGF-β type II receptors are constitutively active on the cell membrane, irrespective of its association with ligands or a type I receptor. However, the active type II receptor is not sufficient for the recruitment of the type I receptor (Derynck and Feng, 1997; Wrana et al., 1994). The TGF-β type II receptor first forms a complex with its ligand, which can then recruit the type I receptor initiating the downstream signal cascade (Fig 4).
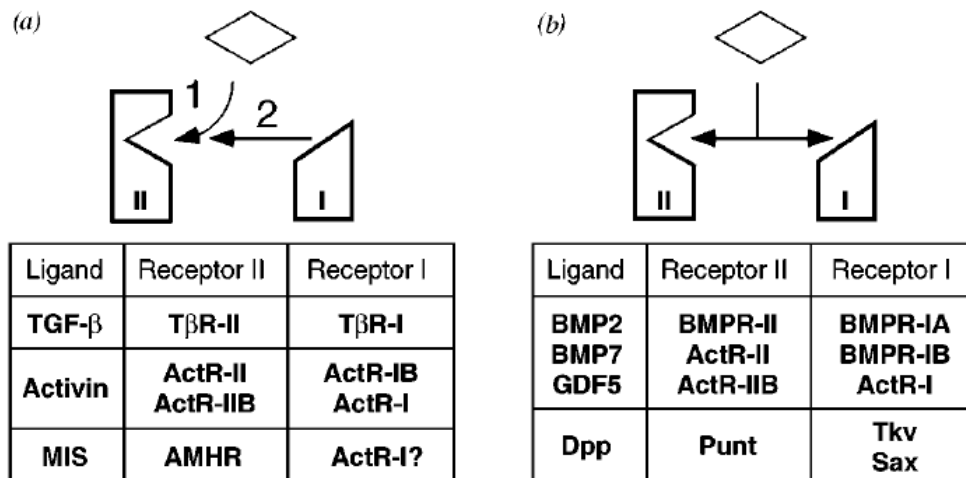
Figure 4. Two modules of TGF-β ligand binding to its receptors: (a) sequential binding (b) cooperative binding (Massague, 1998).

In TGF-β signaling pathways, the ligand/receptor complexes activate the downstream signal transduction and also trigger the internalization of both ligand and receptors (Ehrlich et al., 2001). Once the cargo is inside the cell, there is a set of sorting processes following which the cargo is sent to different destinations such as the lysosome or trans-Golgi while some return back to the cell surface for additional signaling events (Shapira et al., 2012). This sorting and recycle part will be discussed in details in section "the Endocytosis of TGF-β Receptors".

**The formation of ligand-receptors complex allows a close interaction**

It is found that the type II receptor is constitutively activated through auto-phosphorylation at multiple amino acid sites such as serine, threonine and tyrosine in the kinase domain with/without ligand occupancy. However, the type II receptor can only be recruited with type I receptor with the ligand binding

(Derynck and Feng, 1997; Wrana et al., 1994). In contrast to type II receptor, a type I receptor can only be activated by ligand-induced phosphorylation (Derynck and Feng, 1997). Following the formation of the ligand-receptors complex is a rapid trans-phosphorylation catalyzed by type II receptor kinase on threonine and serine residues in TTSGSGSGLP of the GS domain (Massague, 1998; Wrana et al., 1994). Since the GS domain plays a critical role in type I receptor activation as a switch, it is an indispensible regulatory factor in TGF-β signaling. There are also several negative regulators of TGF-β signaling which target this GS domain, including immunophilin FKBP12, which serves as the inhibitor to the TGF-β signaling pathway by blocking the phosphorylation site after binding to the unphosphorylated GS domain (Shi and Massague, 2003). However, phosphorylation of the GS domain in type I receptor can attenuate the binding ability of FKBP12 (Huse et al., 2001).

Ligand binding to the extracellular domain of the TGF-β type II receptor leads to a conformational changes in the intracellular domain of the receptors which allows the phosphorylation and subsequent activation of the type I receptor (Shi and Massague, 2003). Formation of a ligand-receptor complex allows a close interaction between type I and type II receptors and facilitates the subsequent activation of kinase domains in intracellular regions. The receptor activation triggers the downstream TGF-β signaling flow, and the further signal flow depends on TGF-β signal transducer SMAD proteins. SMAD proteins are the direct downstream substrate for the activated type I receptor kinase (Massague,

1998). In the canonical TGF-β signaling pathway, SMADs are activated through

phosphorylation by an activated type I receptor, where they subsequently

translocate into the nucleus to regulate specific gene expression with the

assistance of some SMAD (Fig 5).
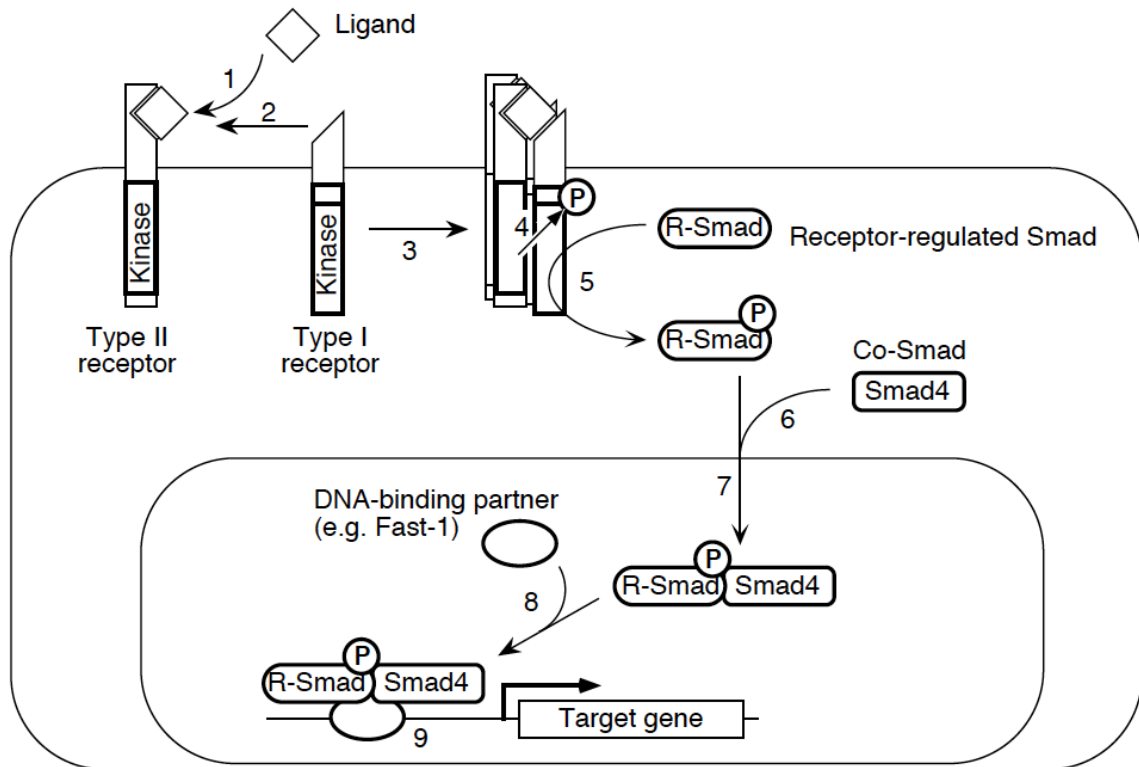


Figure 5. The overview of the TGF-β signaling pathway. This figure shows sequential binding of ligand to receptors. The ligand first binds to type II receptor, which then recruits type I receptor to form a heterotetramer receptor complex. Inside this complex the type I receptor is activated by the phosphorylation by type II receptor. Then the receptor complex activates the downstream R-SMAD through phosphorylation. The phosphorylated SMAD (pSMAD) enters the nucleus to regulate specific gene expression. The Co-SMAD functions as auxiliary protein for the signaling pathway (Massague, 1998).

## TGF-β signaling mediators: SMAD proteins

Receptor activation triggers the downstream TGF-β signaling pathway. SMAD

proteins are the direct downstream substrates for the activated type I receptor

kinase (Massague, 1998) and they are structurally related signal effectors (Derynck and Zhang, 2003). The nomenclature of SMAD proteins comes from the *Drosophila Mad* gene and the *C. elegans sma* gene (Derynck et al., 1996). SMADs were first identified from a genetic screen in *Drosophila* as the product of *Mothers against dpp (Mad)* (Sekelsky et al., 1995). Later *Mad* homologues were found in *C. elegans* and the Padgett lab identified *sma-2, sma-3* and *sma-4* as the nematode version of Mad. Mutations in these three genes result in a small worm body size (Savage et al., 1996). SMAD proteins can be divided into 3 subfamilies based on their roles in TGF-β signaling: 1) Receptor-regulated SMAD (R-SMAD). R-SMAD is the substrate for the activated type I receptor. The activated phosphorylated R-SMAD targets genes in the nucleus and later is in charge of specific gene expression (Massague, 1998). R-SMAD can be further subcategorized into two divisions, one includes the BMP-activated SMADs (SMAD 1, SMAD 5 and SMAD 9), the other one includes the TGFβ-activated SMADS, which includes SMAD 2 and SMAD 3 (Weiss *et al*., 2013). 2) Common-mediated SMAD (Co-SMAD). Co-SMAD participates in the signaling pathway to assist signal transduction by interacting with the activated R-SMAD (Massague, 1998). SMAD 4 is the only Co-SMAD known today. 3) Inhibitor-SMAD (I-SMAD). This type of SMAD, which includes SMAD 6 and SMAD 7, antagonizes signal transduction that is mediated by R-SMAD and the Co-SMAD (Massague, 1998). In the canonical TGF-β signaling pathway, R-SMAD is activated through phosphorylation by the activated type I receptor and is subsequently translocated

into the nucleus where it regulates specific genes with the assistance of the Co-SMAD (Fig 4).

SMAD proteins are usually about 400-500 amino acids in length. R-SMAD and Co-SMAD share a similar structural architecture (Weiss, 2013). Two conserved domains, Mad Homolog 1 (MH1) domain in the N-terminal tail and Mad Homolog 2 (MH2) domain in the C-terminal tail constitute SMAD, and are connected via a linker sequence. The MH1 domain binds specific DNA sequences and negatively regulates the MH2 domain. The MH2 domain is involved in the assembly of SMAD 4 and the R-SMAD (Ehrlich et al., 2001). R-SMADs contain an SSXS motif at their C-terminal end. Two serine residues in this motif are phosphorylated by the active type I receptor (Weiss, 2013).

I-SMAD has a different structure from R-SMAD and Co-SMAD. While I-SMAD contains a conserved MH2 domain like R-SMAD and Co-SMAD, the MH1 domain is variable (Derynck and Zhang, 2003). I-SMAD does not contain the SSXS motif at the C-terminal end, causing I-SMAD to act as antagonist in the signaling pathway (Weiss, 2013) (Fig 6).
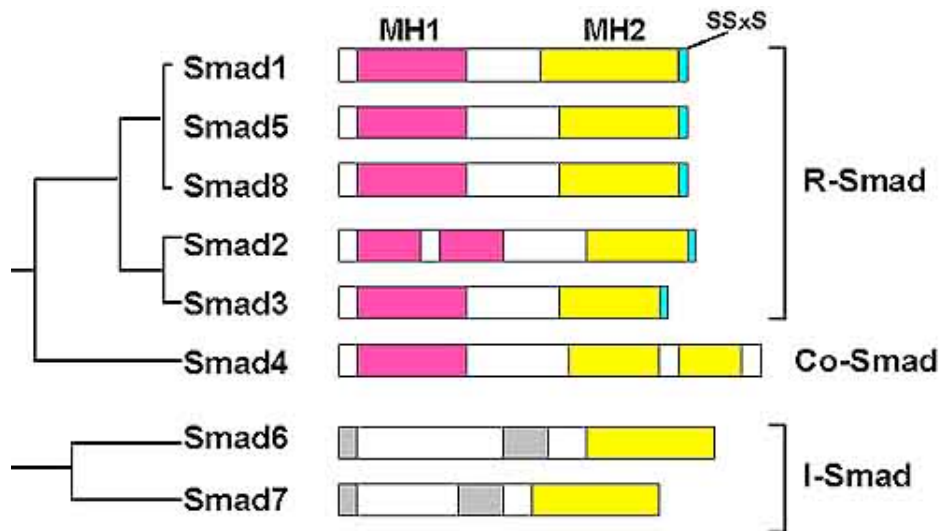
Figure 6. The domain organization and subcategories of SMAD family (Nishimura et al., 2003).

Similar to different members of the TGF-β receptor family that bind different TGF-β ligands, SMAD proteins are activated by different upstream activated TGF-β ligand-receptor complexes, and thus enabling diverse signal responses (Fig 7).

| Type II | Type I | R-Smad |
|---|---|---|
| BMPRII | ALK-2 (ActRI) | Smad1, Smad5, Smad8 |
| | ALK-3 (BMP-RIA) | |
| | ALK-6 (BMP-RIB) | |
| ActRII, ActRIIB | ALK-4 (ActRIB) | Smad2 |
| ActRIIB | ALK-7 | Smad2 |
| TβRII | ALK-5 (TβRI) | Smad2, Smad3 |
| | ALK-1 | Smad1, Smad5 |
| | ALK-2 | |
| AMHR | ALK-3 | Smad1, Smad5 |
| | ALK-2 | |
| | ALK-6 | |
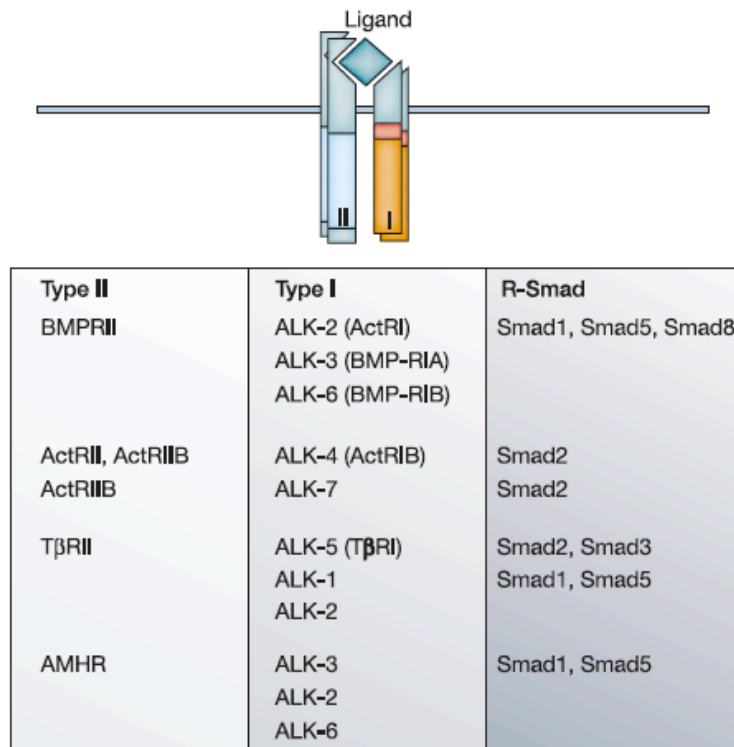
Figure 7. The different combinations of TGF-β ligand and receptors and sequential different signal transducers (Derynck and Zhang, 2003).

**Signaling transduction: SMAD-dependent pathway and SMAD-independent pathway**

As discussed above, SMAD proteins are extensively studied as signal transducers in the TGF-β downstream signal cascade, and are members of the canonical signaling pathway, or SMAD (dependent) pathway.

Ligand-induced phosphorylation of TGF-β receptors results in the activation of type I receptor. Following phosphorylation, the activated type I receptor binds to and phosphorylates the two serine residues in the C-terminal SSXS motif of R-SMADs (Shi et al., 1998). A nine-amino acid loop can directly interact with the L3

in the MH2 domain in R-SMADs, thereby playing a key role in receptor signaling and SMAD binding-specificity (Shi et al., 1998; Weiss, 2013).

In addition to amino acid loops in type I receptor and R-SMADs, a special amino acid sequence downstream of the L3 loop determines binding specificity between type I receptor and R-SMADs (Shi et al., 1998; Weiss, 2013). The GS domain in the type I receptor can be phosphorylated by type II receptor. Following phosphorylation, this domain can provide a stable docking site for the downstream sequence from the L3 loop. This contributes to the stabilization of protein-protein interaction and type I receptor-SMAD binding specificity (Shi et al., 1998).

After a series of protein-protein interaction and internal conformational changes, the activated SMADs accumulate inside the nucleus and eventually regulate the transcription of the target gene. This is the classic pathway for the SMAD-dependent signaling responses to TGF-β receptor activation, a well-characterized pathway in TGF-β signaling. SMAD-dependent TGF-β signaling is ubiquitous intracellularly and it is responsible for facilitating biological signal responses universally. However, many studies have found that there are also non-SMAD signal pathways that are activated either by TGF-β receptors via protein-protein interaction or transducer-triggered phosphorylation. In most cases, the TGF-β non-SMAD signaling pathway is closely associated with the MAPK/Erk signaling pathway or the PI3K/Akt signaling pathway.

The mitogen-activated protein kinase (MAPK) family consists of serine/threonine protein kinases.  MAPKs are involved in a variety of fundamental developmental events such as cell proliferation, cell differentiation, apoptosis and stress response. The most important function of MAPKs is the regulation of cell cycle entry. Canonical MAPKs include extracellular signal-regulated kinase 1 and 2 (Erk1/2 or p44/42), Erk5, C-Jun N-terminal kinases 1-3 (JNK1-3) and p38 (Darling and Cook, 2014).

There is wide spectrum of extracellular factors such as mitogens, cytokines, growth factors, and environmental stressors that act as active stimuli for MAPKK kinases (MAPKKKs). These stimuli can activate MAPKKKs through either a receptor-dependent or independent way. The activated MAPKKKs then activate the downstream MAPK kinase (MAPKK) via protein phosphorylation, and the activated MAPKK in turn activates downstream MAPKs by phosphorylation. These activated MAPKs can activate the MAPK-activated protein kinases (MAPKAPKs). MAPKAPKs include RSK, MNK, MK2/3/5. Most biological functions caused by MAPKs are transduced and regulated by these activated MAPKAPKs (Darling and Cook, 2014). Deregulation of MAPK/Erk pathway results in uncontrollable cell growth, a step shared in the development of cancers (Koul et al., 2013).

The serine/threonine protein kinase Akt (also known as protein kinase B or PKB), which was originally discovered as one of the proto-oncogenes, is important as a signaling kinase due to its functions in the regulation of cell proliferation, cell growth, cell survival and metabolism (Hers et al., 2011). The stimulation of Akt signaling begins with extracellular stimuli binding to and interacting with the extracellular domain of receptor tyrosine kinase (RTK). Stimuli primarily include phosphatidylinositol (3,4,5) trisphosphates (PIP3, the product of phosphoinositide 3-kinase (PI3K)) (Hers et al., 2011). PIP3 is a type of lipid that can provide a special docking site on the cell membrane facing to specific proteins with pleckstrin-homology (PH) domains. Both PDK1 and its downstream transducer Akt have PH domains. Once the cell is stimulated, Akt in the cytosol can translocate to the plasma membrane with PH domains, where PDK1 can partially activate Akt by phosphorylating a threonine residue at position 308. Activation of Akt comes from mTORC2 phosphorylating the amino acid serine at position 473 on Akt (Bhaskar and Hay, 2007).

Akt can interact with a variety of signal transducers in different signaling pathways. Abnormal function of PI3K/Akt signaling pathway is associated with cancer (Hers et al., 2011). Akt is a major factor that mediates cell survival via inhibiting certain pro-apoptotic proteins. Akt can also contribute to the inhibition of cell death by antagonizing pro-apoptotic signals (Bozulic and Hemmings, 2009). The PI3K/Akt pathway regulates cellular development via the interaction with the

TSC1/TSC2 complex. Akt can also facilitate cell proliferation by phosphorylating CDK inhibitor p21 and p27 (Bhaskar and Hay, 2007).

The TGF-β non-SMAD pathway also interacts with the Erk pathway. It is reported that TGF-β type I receptor can phosphorylate Erk mediator while the type II receptor can mediate phosphorylation of the amino acid tyrosine (Zhang et al., 2009). This can lead to TGF-β receptor associated-activation of Scr kinases, triggering a cascade of phosphorylation and recruitment of different mediators, eventually to activate Erk pathways (Weiss, 2013; Zhang et al., 2009) (Fig 8). The MAPK/Erk signaling pathway is reported to play roles in cell development and cell migration, and Erk is involved in the epithelial to mesenchymal transition (EMT), which is also a major process regulated by TGF-β. EMT is required during embryonic development, but a pathological sign in tumor development (Brabletz et al., 2018). The Erk non-SMAD pathway is a classic example of a TGF-β-induced EMT signal response.
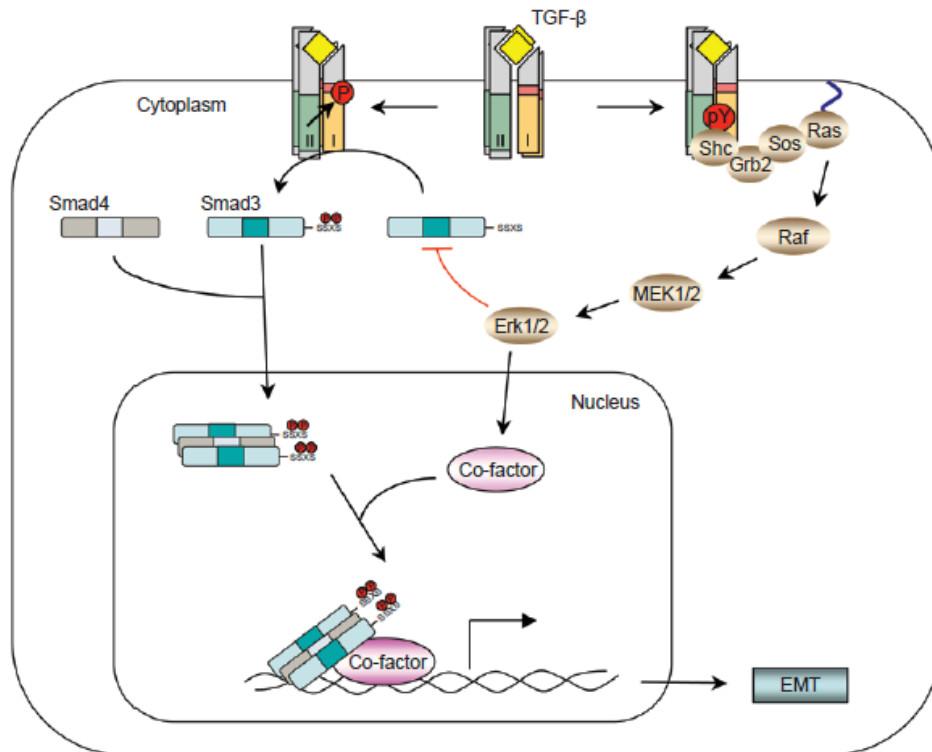
Figure 8. The Erk non-SMAD pathway. The ligand and its receptors form a hetero-tetramer receptor complex, and inside this complex, the type I receptor is activated by type II receptor through phosphorylation. Then the receptor complex can either activate downstream R-SMAD via phosphorylation or activate Scr kinases. In former model further activated SMAD enters the nucleus to regulate specific gene expression like EMT; in the latter model recruited Grb2 and Shc and are recruited to go through a series of signaling cascade to activate Erk pathways, and finally regulate EMT. Co-SMAD functions as auxiliary protein (Massague, 1998).

Another well-studied TGF-β non-SMAD pathway is the C-Jun N-terminal kinase (JNK) and p38 MAPK signaling cascade. Usually in a conventional mitogen-activated protein kinase (MAPK) signaling pathway, stress will activate MAP3Ks, and this activation initiates the signaling pathway. MAP3Ks can activate downstream mediator MAP kinase kinases (MKKs), and the activated MKKs, especially MKK4 and MKK3/6, further activates downstream targets such as JNK and p38 (Plotnikov et al., 2011; Zhang et al., 2009).

TGF-β can rapidly activate JNK via MKK4. Furthermore, upstream of the MAPK

signal cascade, an activating MAP3K, termed MAP3K7 in the case of MKK3/6

and MKK4, is a TGF-β-activated kinase 1 (TAK1) (Zhang et al., 2009). Therefore,

TGF-β can also activate the JNK/p38 MAPK pathway by the activation of TAK1

(MAP3K7) through the interaction with TRAF, the member of TNF receptor

associated protein family (Weiss, 2013) (Fig 9). The consequence of the

JNK/p38 non-SMAD pathway is EMT or cell apoptosis.
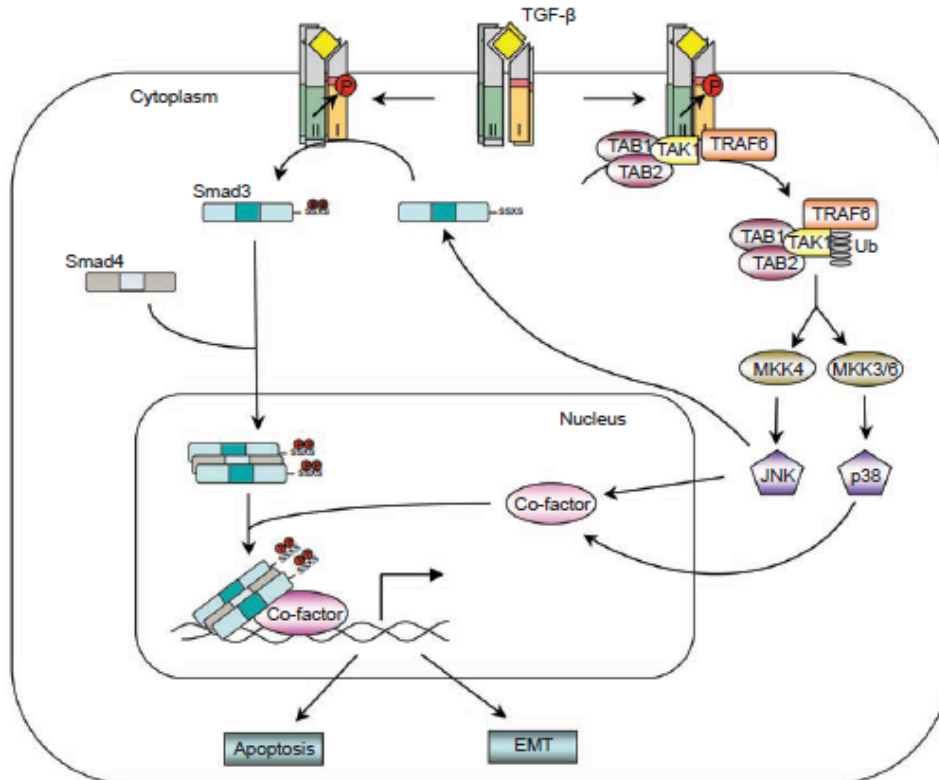


Figure 9. The JNK/p38 non-SMAD pathway. The ligand and its receptors form a hetero-tetramer receptor complex, and inside this complex, type I receptor is activated by type II receptor through phosphorylation. Then the receptor complex can either activate downstream R-SMAD via phosphorylation or activate JNK/p38 pathways. In the former model activated SMAD enters the nucleus to regulate specific gene expression like apoptosis or EMT; the latter model recruited several factors via the activation of MKK4/MKK3/6, to finally regulate apoptosis or EMT. Co-SMAD functions as auxiliary protein (Massague, 1998).

Rho-like GTPase non-SMAD pathways have also been revealed. TGF-β ligand-receptor complex directly or indirectly activates Rho-like GTPase, such as RhoA, Rac or Cdc42 (Zhang et al., 2009). The activation of Cdc42/Rac regulates cell adhesion or tight junction between cells. Activation of RhoA, via the activation of Smurf/PKC, directly or indirectly regulates cell adhesion or actin polymerization and eventually induces EMT response (Weiss, 2013; Zhang et al., 2009). Rho-like GTPases are functional in cell motility and gene expression. However, this TGF-β-Rho-GTPase non-SMAD pathway, mostly regulates EMT (Weiss, 2013; Zhang et al., 2009) (Fig 10).
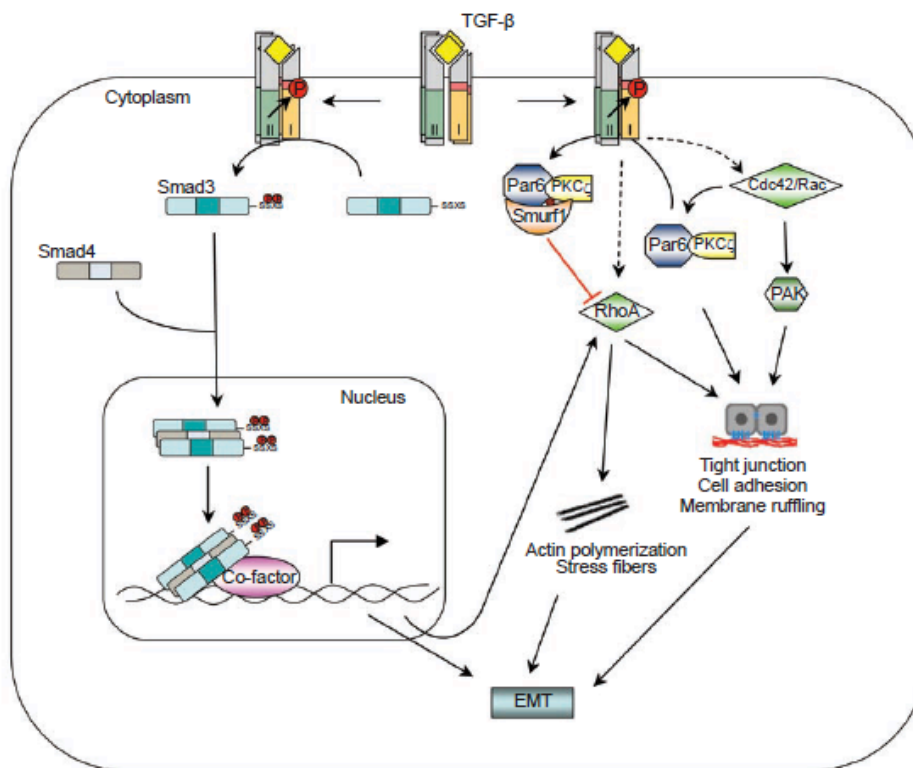


Figure 10. The Rho-like GTPase non-SMAD pathway. The ligand and its receptors form a hetero-tetramer receptor complex, and inside this complex, type I receptor is activated by type II receptor through phosphorylation. Then the receptor complex can either activate downstream R-SMAD via phosphorylation or activate JNK/p38 pathways. In the former model SMAD enters the nucleus to regulate specific gene expression like apoptosis or EMT; in the latter model factors are recruited to activate JNK/p38 pathways by the activation of MKK4/MKK3/6, and finally regulate apoptosis or EMT. Co-SMAD functions as an auxiliary protein (Massague, 1998).

PI3K may play a role in the TGF-β non-SMAD signaling pathway. TGF-β can activate PI3K through the activation of Akt, which is the downstream effector to PI3K (Weiss, 2013). This PI3K/Akt pathway induced by TGF-β is involved in TGF-β-regulated EMT (Zhang et al., 2009). The TGF-β induced PI3K/Akt non-SMAD pathway accounts for fibroblast proliferation and morphological transformation mediated by TGF-β signaling in addition to playing a role in EMT. C-Abl, a tyrosine kinase, can serve as the downstream target of activated PI3K and thus contribute partially to the TGF-β-mediated fibroblast response (Zhang et al., 2009). The PI3K/Akt non-SMAD pathway antagonizes the responses induced by SMAD-mediated signaling (Shin et al., 2001). This counteraction usually is considered as a form of protection.
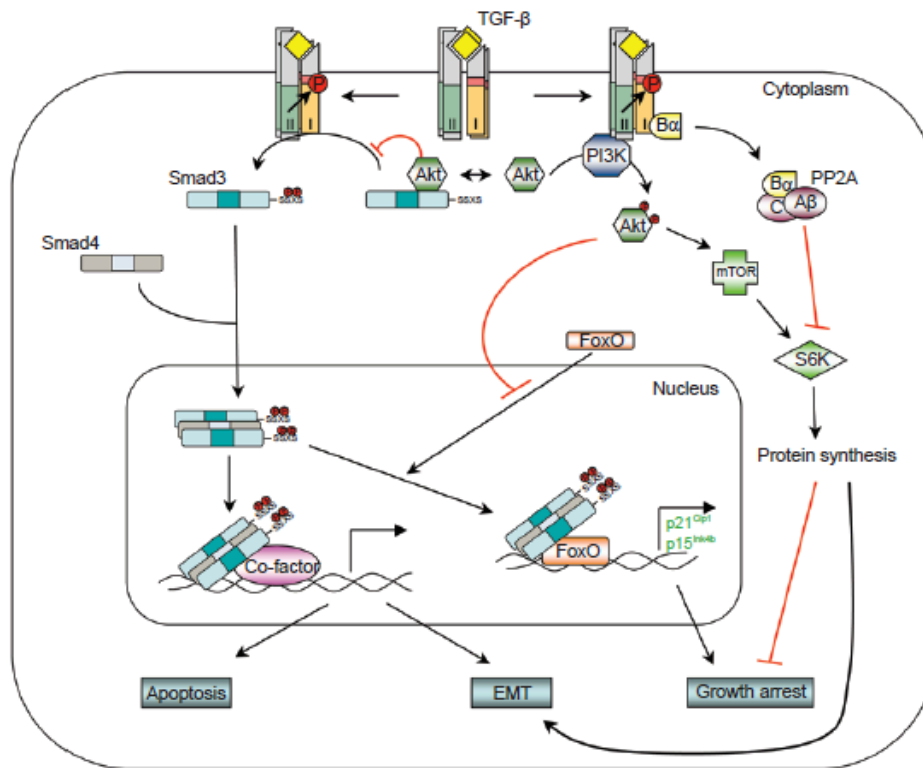
Figure 11. The PI3K/Akt non-SMAD pathway. The ligand first binds to type II receptor then type II receptor recruits type I receptor to form hetero-tetramer receptor complex. The downstream R-SMAD is activated by this receptor complex via phosphorylation. The phosphorylated SMAD enters the nucleus to regulate specific gene expression. The Co-SMAD functions as an auxiliary protein for SMAD (Massague, 1998).

**TGF-β signaling pathway in *C. elegans***

Years of investigation have characterized the TGF-β signaling pathway in *C. elegans*. These studies contribute to a better understanding of the components and functions of TGF-β signaling pathways, including transcriptional targets and the regulators. Unlike the pathway, a plethora of homologous TGF-β members in mammals, there are only five TGF-β signaling ligands found in *C. elegans,* and these TGF-β ligands are neither redundant nor essential to the worms (Patterson and Padgett, 2000). These characteristics provide a clearer background for researchers to study TGF-β signaling in the worm, and contribute to the study of

TGF-β signaling in other backgrounds. The chart below shows different components of TGF-β factors in *C. elegans,* including ligands, receptors and downstream signal transducers, and their similar orthologs in *Drosophila* and mammals (Table 1).

| Component | *C. elegans* gene name | *Drosophila* gene name | Human gene name | Molecule or family |
|---|---|---|---|---|
| | *dbl-1* | *dpp* | BMP5 | |
| | *daf-7* | *dawdle* | GDF11 | |
| | *unc-129* | - | - | |
| | *tig-2* | *gbb* | BMP8 | Transforming Growth Factor-β |
| Ligand | *tig-3* | dActivin | BMP2 | |
| | *sma-6* | *tkv* | BMPRIB | |
| Type I receptor | *daf-1* | *babo* | TGF-βRI | |
| Type II receptor | *daf-4* | *put* | ACTRIIB | ser/thr kinase receptor |
| | *sma-2* | *Mad* | Smad1 | |
| | *sma-3* | *Mad* | Smad5 | |
| | *daf-8* | *Smox* | Smad8 | |
| R-Smad | *daf-14* | *Smox* | Smad2 | |
| | *sma-4* | *Medea* | Smad4 | |
| Co-Smad | *daf-3* | *Medea* | Smad4 | |
| I-Smad | *tag-68* | *Dad* | Smad6 | Smad |

Table 1. The TGF-β superfamily signaling is conserved in eukaryotes (wormbook.org).

In *C. elegans,* TGF-β signaling is involved in two major processes, dauer formation (dauerlarva, an arrested developmental form of worms in response to harsh environment) pathway, and the Sma/Mab pathway (small body size) (Fielenbach and Antebi, 2008). In the dauer pathway, DAF-7 is the specific TGF-β ligand and activates the dauer-TGF-β signaling pathway through DAF-1 and DAF-4 receptors, respectively (Ren et al., 1996a). When nematodes encounter a harsh environment, DAF-7 expression is suppressed, shutting down the dauer-TGF-β pathway. As a result, worms undergo sharp shifts in metabolism, which

results in great changes on molecular level as well as on morphological level, and then they prefer to enter a non-growing and non-producing state as a type of self-protection. This type of protection is termed as dauer stage (Fielenbach and Antebi, 2008; Ren et al., 1996b; Suzuki et al., 1999). *C. elegans* in this stage contains a special type of cuticle, the oral orifices are shut down, and the pharynxes are constricted (Vowels and Thomas, 1992).

In this work, I have focused on the Sma/Mab TGF-β signaling pathway in *C. elegans*. This pathway regulates worm body size, the copulatory spicule and the development of male-specific sensory rays. DBL-1 is the ligand for this pathway and it regulates worm body size in a dose-dependent manner. Higher concentration of DBL-1 results in longer worm and vice-versa (Suzuki et al., 1999). Type I and type II receptors for DBL-1 are encoded by *sma-6* and *daf-4* genes respectively, and SMA-2, SMA-3 and SMA-4 are the signal transducers in the Sma/Mab pathway (Fielenbach and Antebi, 2008; Savage et al., 1996). SMA-2 and SMA-3 are R-SMAD homologs and cooperate with each other in signal transduction while SMA-4 is a Co-SMAD in the signaling pathway. From Table 1, it is clear that Sma/Mab TGF-β signaling pathway is similar to BMP signaling pathway in mammals. The schematic representation of whole Sma/Mab signaling pathway is shown in Fig 12.
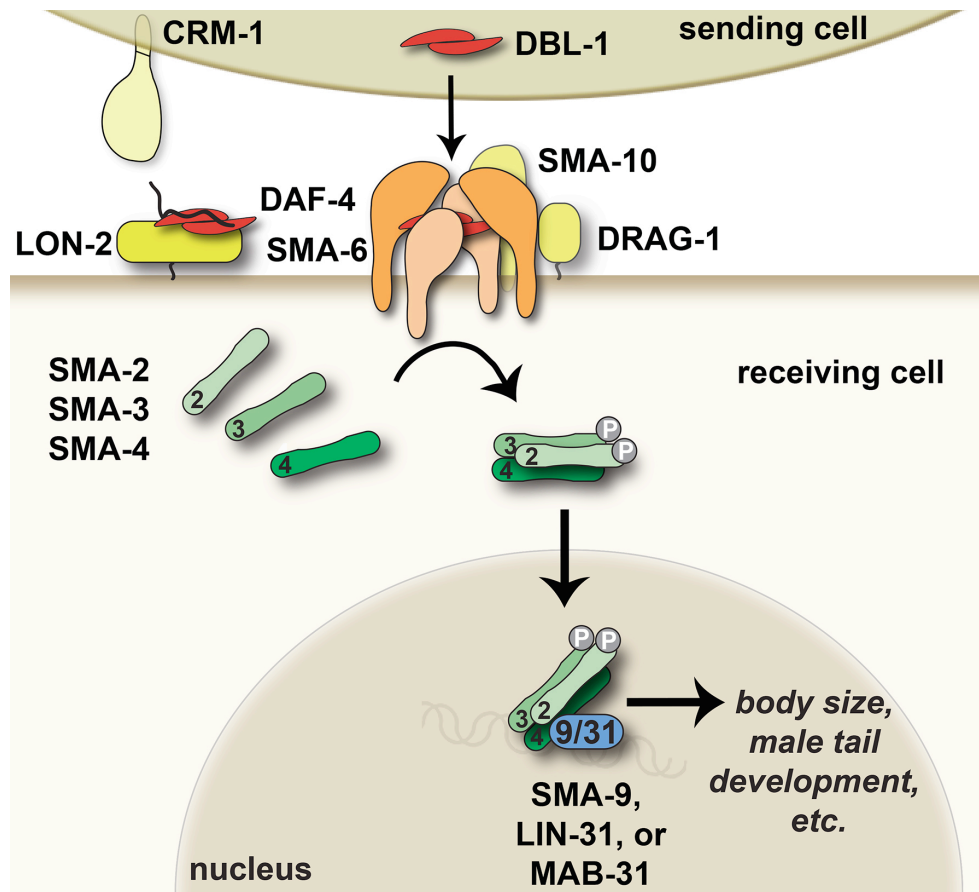
Figure 12. The Sma/Mad TGF-β signaling pathway in *C. elegans*. DBL-1 is the ligand for sma/Mad signaling pathway. DBL-1 interacts with TGF-β type I and type II receptor SMA-6 and DAF-4. There are various regulators involved in this signaling pathway. Extracellular regulators like CRM-1 act in the extracellular environment to promote DBL-1 signaling. SMA-10 and DRAG-1 facilitate DBL-1 signaling at cell membrane. LON-2/glypican is the inhibitor to DBL-1 on the cell membrane. Signal transducers in Sma/Mab are SMA-2, SMA-3 and SMA-4. Transcription factors that cooperate with SMADs include SMA-9/Schnurri, LIN-31/forkhead, and MAB-31 in the cell (Girard et al., 2007).

## The endocytosis of proteins on plasma membrane

Cells are not static; there are vigorous communications between extracellular environment and contents inside the cell. These communications enable cells to uptake nutrition, to exfoliate wastes or signal. There are numerous dynamic cargo deliveries inside the cell; these contribute to protein renewal and degradation.

**Endocytosis and trafficking**

Eukaryotic cells use different mechanisms to uptake molecules from the extracellular environment. One general way is to transport molecules via protein channels or carriers embedded within the phospholipid bilayer (Cooper et al., 2007). Although this mechanism of transport is suited for small molecules, endocytosis is a more efficient mechanism when it comes to macromolecules in a foreign medium. In 1963 Christian de Duve coined the term "endocytosis" to describe both the ingestion of macromolecules and the intake of particles in vesicles. In a well-defined endocytosis process, the particle will be internalized into cell after being surrounded by the cell membrane bilayer followed by a pinching off of the bilayer bud from the cell membrane to become a vehicle in which the ingested particle resides. The former one is referred to as phagocytosis and the latter one is termed as pinocytosis (Cooper et al., 2007).

Phagocytosis is the process in which relatively large particles (usually >0.5 μm) are internalized by mononuclear organism, neutrophils and macrophages (Cooper et al., 2007). There are specific ligands on different ingestible particles, and these ligands have specific cell-surface receptors on the phagocyte. The interaction between the ligand and its receptor initiates the internalization of the particle, and this interaction activates the polymerization of actin filaments into longer filaments to form the network at the site where the particle was internalized. After internalization, actin is stripped away from the vesicle (Allen

and Aderem, 1996). This step is followed by the fusion of vesicle with lysosomes

and degradation of the target (Richards and Endres, 2014) (Fig 13).
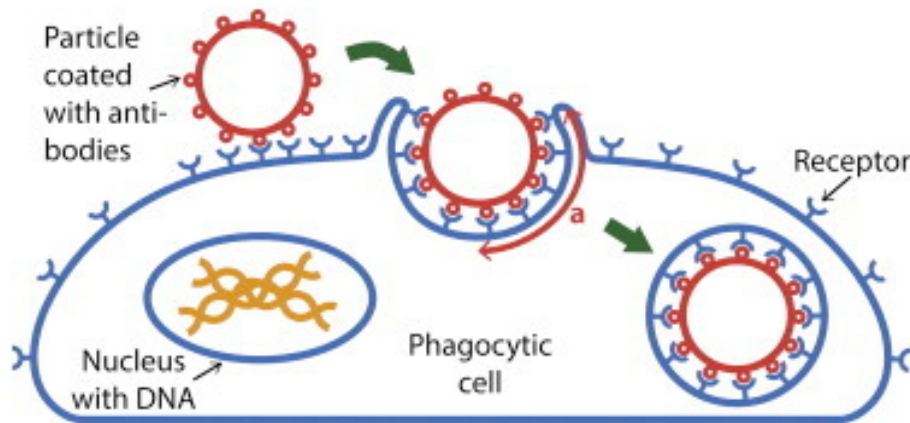


Figure 13. Schematic representation of Phagocytosis. Receptors on the cell surface can recognize and bind the particle with specific antibodies, including pathogen, dead cell, or other molecules. When receptors bind more and more particles, the cell would engulf all ligand molecules. Once upon the engulfment, a phagosome is formed. Phagosome can fuse with lysosomes, which results in the digestion of ligand particles (Richards and Endres, 2014).

Pinocytosis is a sub-category of endocytosis and includes receptor-mediated

endocytosis, which is the most well studied category of pinocytosis. The

macromolecule outside the cell (referred as ligand) first needs to bind to the

receptor localized on the plasma membrane. Then the small membrane area

around the receptor, together with either clathrin or lipid raft, form a vesicle,

which contains the receptor-ligand complex. Then the vesicle buds off from the

plasma membrane in a form of a small carrier vesicle that fuses with the early

endosome, late endosome, lysosome or recycles back to membrane, and

becomes a small carrier vesicle that fuses with early endosome, then late

endosome (Cooper et al., 2007). A series of dynamic membrane-enclosed

vesicles contribute to this endocytic pathway, and these vesicular structures are termed as endosomes (Hu et al., 2015).

Endosomes are the carriers of ingested cargoes, and they are divided into three different categories according to their functions in endocytosis: early endosome (sorting endosome), recycling endosome and late endosome (Hu et al., 2015). Early endosomes (EE) are defined as vesicles primarily located in the peripheral region in the cell. Numerous endocytosis routes of cargoes exist in a cell; however, EE serves as a key transportation point in cellular cargo trafficking. Usually EE is thought to be the initiation of cargo sorting (Jovic et al., 2010). The destination of cargo (back to the membrane, go to the lysosome for degradation or go to the trans-Golgi network for modification) is determined in this vesicle compartment. EEs can transfer cargo proteins between different regions in the cell membranes, similar to transfer proteins between the apical and basolateral membrane domains (Cooper et al., 2007).

Early endosomes maintain an acidic pH (usually it is about 6.0) internally. In most cases, EE is formed around the peripheral cytoplasm to uptake the extracellular receptor-ligand complex. The acidic microenvironment of the EE contributes to the facile separation of receptor and ligand (Cooper et al., 2007). Following the dissociation, receptor and ligand are delivered to different compartments for cargo sorting, and then move for next destinations. Due to this sorting function in the determination of separated ligand and its receptor, EE is also termed as

sorting endosome (SE) when it comes to its operational role (Maxfield and

McGraw, 2004). This sorting function in SE is very important in the intracellular

endocytic pathway, especially for cargo recycling (back to the plasma

membrane). The study of endocytosis in mammalian cells revealed two pathways

inside the cell, and both of them are loop pathways. Based on the time cargo

travels in each loop, they are termed as the short loop and the long loop (Li and

DiFiglia, 2012). In the short loop pathway, the cargoes internalized from

membrane are recycled and rapidly (in 2-3 minutes) transported through early

endosomes. However, in a long loop, internalized cargoes initially go to the early

endosomes and then travel to recycling endosomes and then back to the cell

membrane. This process takes about 10 minutes (Li and DiFiglia, 2012). The

factors that direct the delivery and fusion in internalization pits, early endosomes

and later endosomes are numerous and not fully understood yet, but there are

two key elements that regulate this dynamic membrane delivery process: the

Rab family of small guanosine triphophatases (GTPases) and SNARE proteins.

Rab proteins regulate the transport of endocytic vesicles. There are quick

switches of different members of Rab GTPases during the transitions of ingested

cargo (Cooper et al., 2007; Hu et al., 2015). For example, in the short loop

mentioned above, Rab5 is in charge of the transfer between internalized vesicles

to early endosomes, while Rab4 takes care of cargoes recycling back to the cell

membrane. Mechanisms of early/recycling endosomes transition in the long loop

route remain unclear; so far we only know that the process of cargo sent back to

the plasma membrane from recycling endosomes is accompanied by Rab 11 (Hu

et al., 2015) (Fig 14).
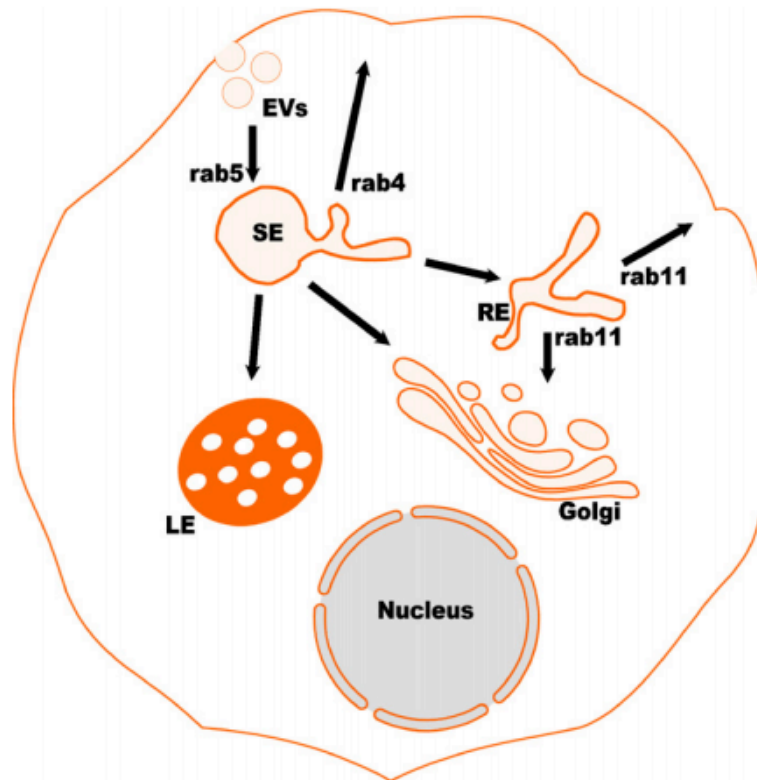


Figure 14. Schematic representation of different endosomal vesicles, the corresponding factors, and the recycling route for internalized cargoes. Receptors and their ligands on the cell surface are internalized into a bud and are pinched off from the cell membrane into cells to form endocytic vesicles (EVs). Rab5 facilitates the fusion of EVs with each other or with sorting endosomes (SEs). Some internalized molecules in SEs are retained inside the vesicles, which then become late endosomes (LEs); some other internalized molecules are recycled back to cell surface. The recycling of cargoes back to cell surface is divided into two categories: short loop and long loop. In the short loop Rab4 will keep more molecules in the SE back to cell surface directly. In long loop, the internalized cargoes are segregated into recycle endosomes (REs) by tubule-like structure, and are then sent back to cell surface by the regulation of Rab11. Sometimes Rab11 directs the cargoes in REs to first go to the Golgi system before going back to cell surface **(Li and DiFiglia, 2012)**.

SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors)

are located on vesicles and receptor endosomes and are required for vesicle

recognition, docking and fusion (Hong, 2005). On a functional level, SNAREs can

be divided into v-SNAREs and t-SNAREs. The former is usually associated with vesicles containing proteins or lipids from membranes and the latter is located in target compartment (Cooper et al., 2007). The specificity of fusions between vesicles and endosomes comes from the specific interaction between v-SNARE on an upstream endocytic vesicle with its cognate t-SNARE on the downstream receiving vesicle compartment (Hong, 2005). Various small Rab GTPase proteins and SNAREs are involved in vesicle-mediated transport events (Cooper et al., 2007; Hong, 2005).

Recycling endosomes (RE) are defined as the sub-compartment of early endosomes that receive cargoes from early endosomes/sorting endosomes (SE). REs function as transition stations for engulfed cargoes, from which cargoes either go back to the plasma membrane or go to the downstream sub-compartments (Li and DiFiglia, 2012).

In some cases, mis-folded or down-regulating proteins inside the cell or endocytosed cargoes from the plasma membrane that need to be degraded are sent to lysosomes. The endosomal-lysosomal system plays a major role in post-Golgi proteins degradation. During the formation of the inward buds containing cargoes from the plasma membrane, cargoes that destined for lysosomal degradation are incorporated into intraluminal vesicles (ILVs). These vesicles bud from limiting membranes (Piper and Katzmann, 2007). As these vesicles accumulate, a special intermediate subset of endosomes that are defined as

multivesicular bodies (MVBs) are created (Hu et al., 2015; Piper and Katzmann, 2007).

Multivesicular bodies have different roles depending on their potential fates. When contents are destined for lysosomes or recycling, MVBs will fuse with late endosomes; when contents targeting intraluminal vesicles for releasing to the extracellular enviornment, MVBs fuse with plasma membrane (Edgar, 2016). This functional differentiation also defines different roles of intraluminal vesicles (ILVs). ILVs interconnect with former MVBs destined for protein degradation. ILVs directed by MVBs become exosomes (Edgar, 2016). This work focuses on intracellular endocytosis exclusively.

For both early and late endosomes, cargoes move near the nucleus. Accompanying this process is Rab conversion, where the association of early endosomes with Rab 5 is replaced by an association of late endosomes with Rab 7 (Hu et al., 2015). Despite MVBs being fused with lysosome for protein degradation, late endosomes are another major vesicular compartment delivering cargoes to the lysosome for degradation. Late endosomes are also in charge of the generation of intraluminal vesicles (ILVs) (Hu et al., 2015).
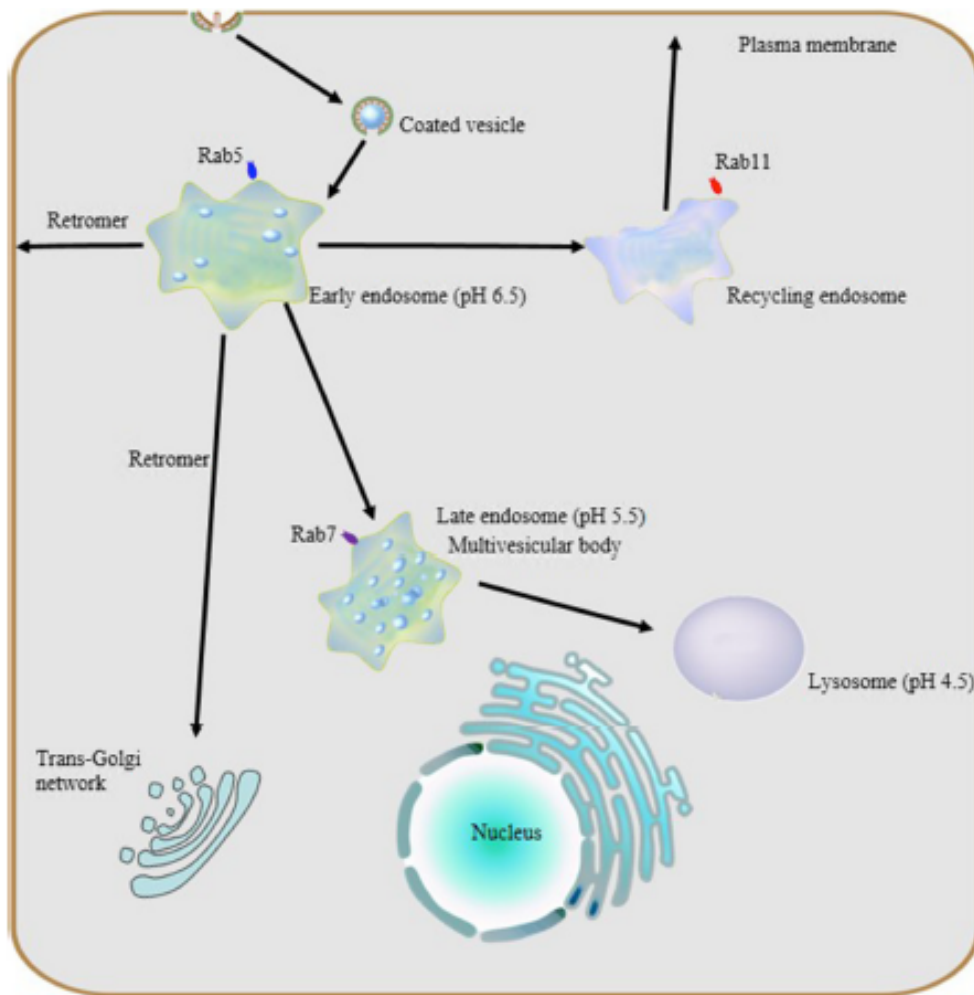
Figure 15. The overview of cargo trafficking in a cell. Cargoes on the cell surface are internalized into the cell via coated vesicles, which then fuse with early endosome. The early endosome serves as the major sorting compartment in endocytosis pathway. Some cargoes are delivered to recycling endosomes and then go back to cell surface for reuse. Some are sent to late endosome, from where some cargoes are sent to lysosome for degradation. Along this endosomal-lysosome route, the pH value becomes more and more acidic. Some cargoes in early endosomes go to retromer and from there go to trans-Golgi networking for modification, and are then delivered back to the cell membrane (Hu et al., 2015).

As mentioned earlier, endocytosed proteins are either degraded or are reused. These two distinct destinations for proteins are determined by two cargo sorting systems: ESCRT and retromer. Proteins to be degraded are labeled with ubiquitination marker. Ubiquitinated proteins can be recognized by a cargo-sorting receptor known as ESCRT. ESCRT that is located on the membrane of

endosomal vesicles directs the degradation of ubiquitinated protein (Hu et al.,

2015). Retromer complexes are found in eukaryotic cells, and are conserved in

all eukaryotes from yeast to humans (Hu et al., 2015). It is a protein complex

that is composed of sorting nexin dimers SNX and vacuolar protein sorting trimer

Vps (Hu et al., 2015). The major functions being mediation of retrograde retrieval

of cargo from endosomes to the trans-Golgi network (TGN) (Hu et al., 2015).

Retromer complexes have capabilities for specific-cargo recognition and are

conserved through all eukaryotic cells (Hu et al., 2015). Retromer complexes are

encoded by vacuolar protein sorting (VPS) genes. VPS-35, VPS-29 and VPS-26

compose a trimeric core while VPS-5 and VPS-17 compose sorting nexin (SNX)

proteins (Hu et al., 2015). The overview of cargo trafficking in a cell is shown in

Fig 15.

**The endocytosis of TGF-β receptors**

In TGF-β signaling, the interaction between ligand and its receptor both activates

the downstream signal transduction, and causes the internalization of both ligand

and receptors (Chen, 2009). In most cases TGF-β receptors go through classic

clathrin-dependent internalization, but some receptors get into the cell via lipid-

raft/caveolae-mediated internalization (Conner and Schmid, 2003). Once the

cargo is inside the cell, through a set of sorting processes the cargo is sent to

different cellular locations such as to the lysosome or trans-Golgi, or returns to

the cell surface for additional signaling events (Chen, 2009). The effect of

endocytosis on TGF-β receptors is complicated. In some cases the endocytosis

of the receptor-ligand complex increases the probability of the interaction

between the activated receptors and intracellular substrates, which results in

signaling augment afterwards (Conner and Schmid, 2003). In this case,

endocytosis plays a positive role for TGF-β signaling. Sometimes endocytosis

causes negative results. For example, the receptor-ligand complex can be

delivered to lysosome for degradation, and thus the signaling is shut down

(Conner and Schmid, 2003; Gonzalez-Gaitan, 2003). The above two pathways

and their effects on signaling are shown in Fig 16.
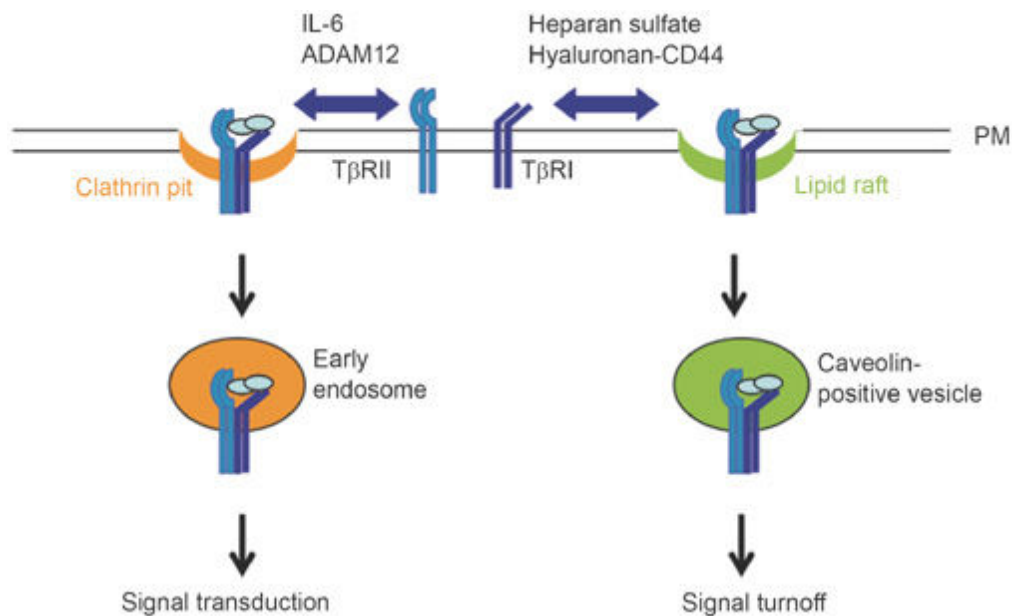


Figure 16. The different endocytic pathways of TGF-β receptors and their outcomes. TGF-β receptors can be internalized by either a clathrin-dependent pathway or nonclathrin-dependent pathway. Internalization of TGF-β receptors via the clathrin-dependent pathway enhances TGF-β signaling, while internalization by lipid rafts promotes receptor degradation (Chen, 2009).

Besides receptor internalization and endocytosis, there are several ways to

regulate endocytosis of receptors in cells. For example, it is found that the

cooperation between type I and type II receptors is a prerequisite for optimal

internalization, and di-leucine motifs in the kinase domain in both type I and type II receptors are required for ligand-receptor internalization in clathrin-mediated endocytosis (Ehrlich et al., 2001; Zwaagstra et al., 2001).

Although endocytosis of TGF-β does not play a key role in TGF-β signaling, this process can contribute to TGF-β-induced SMAD activation and the downstream transcriptional level responses in certain cases (Chen, 2009). There are also several signals in TGF-β receptors that are required for their endocytosis.

**The internalization pathways of TGF-β receptors**

In a cargo delivery process, endocytosis serves as the vehicle to transfer the internalized receptors from plasma membrane into different compartments in the cell. There are two endocytic pathways, clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) (Chen, 2009). TGF-β receptors can be internalized by either pathway, however clathrin-dependent pathway is the major endocytosis way for TGF-β receptors (Chen, 2009). Leof and colleagues demonstrated that both TGF-β type I and type II receptors can be endocytosed rapidly. Only the kinase activity of TβRII is required for the internalization of type I and type II receptor complex as shown by an artificial chimeric TGF-β type I and type II receptor complex system (Anders et al., 1997; Anders et al., 1996). It is also reported that the deletion of kinase domain in TGF-β type I receptor does not change the down-regulation of TGF-β receptor complexes, which is consistent with the above results (Garamszegi et al., 2001b).

**Clathrin-dependent internalization pathway**

The clathrin-mediated pathway is extensively studied and is the best characterized internalization pathway for various membrane protein receptors (Chen, 2009); clathrin serves as the scaffold for the vesicle buds in cargo delivery (Takei and Haucke, 2001). A clathrin-coated pit is set up through the recruitment of adaptor complex protein AP2, clathrin and other accessary proteins; then cargoes on the cell surface are concentrated into this pit gradually. Eventually, the mature clathrin-coated pits are pinched off from the membrane (Takei and Haucke, 2001). At this point, the clathrin coat is uncoated from the cargo vesicle, and this uncoated vesicle moves to and fuses with the early endosome, and as a result, the inside cargo receptors are sent to early endosome (Hinshaw, 2000). The detailed mechanism of the clathrin dependent pathway is shown in Fig 16.

The associated of TGF-β receptors with the clathrin-dependent pathway is evidenced by: 1. Endocytosis of TGF-β receptors is hijacked due to potassium depletion in mouse fibroblast cell line AKR-2B (potassium ion is required for clathrin lattice formation, potassium depletion can result in the blockade of clathrin-mediated internalization of cargoes on the cell surface) (Anders et al., 1997). 2. The pulse-chase of membrane TGF-β type I receptor showed that its internalization is paralyzed by potassium depletion. 3. Both TGF-β type I and

type II receptors are associated with adaptor complex protein AP2 subunit, a key

factor in the clathrin dependent pathway (Lu et al., 2002).
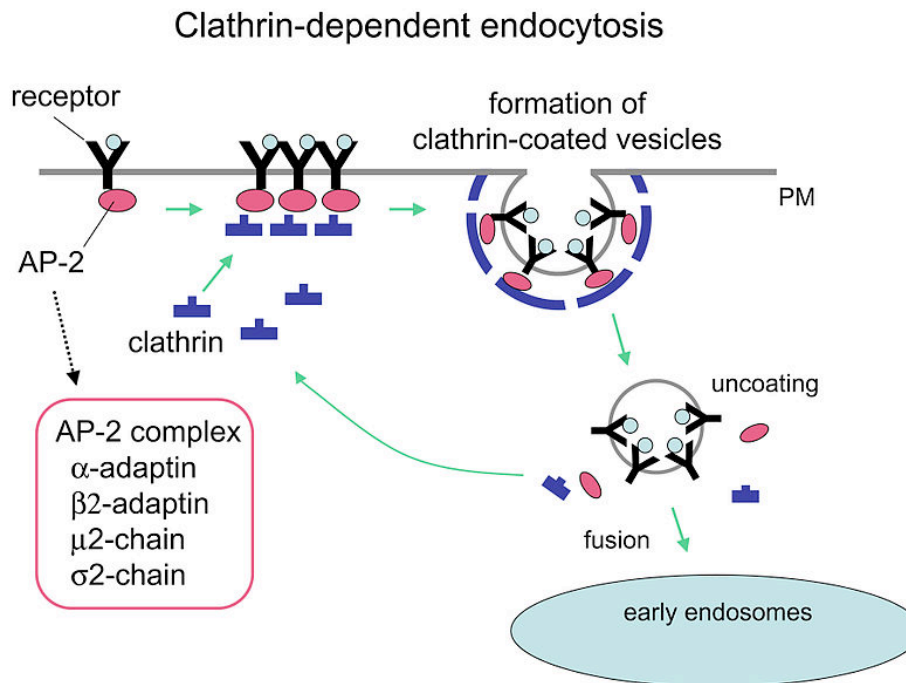


Figure 17. Schematic representation of the mechanism of clathrin-dependent endocytosis and relevant factors involved in the pathway. AP-2 complex at the cytoplasmic domain of the receptor works as a link between the ligand-receptor complex and clathrin inside the cell. When there is enough ligand-receptor, a clathrin-coated pit will form on the cell membrane facing inside. Later this clathrin-coated pit is pinched off from the membrane to form an independent vesicle in the cell. After a while, both clathrin and AP-2 complex will separate from the vesicle, which is defined as uncoating, and this uncoated vesicle will be transferred to fuse with early endosomes (Grant and Donaldson, 2009).

TGF-β receptors can be constitutively internalized in clathrin-dependent pathway

and are usually recycled (Chen, 2009). Different types of endosomes transfer the

internalized cargoes to the sorting machinery (Takei and Haucke, 2001).

Recycling of the receptors occurs either through the recycling endosomes or

through the retromer. As mentioned earlier, retromer delivers the cell-surface

receptors from endosomes to the trans-Golgi apparatus/network (TGN) (Seaman,

2012). Although the function of retromer is primarily to retrieve proteins from endosomes to TGN, sometimes the un-liganded cargo proteins may be sent to lysosomes for mis-degradation (Hu et al., 2015). In the TGF-β-induced SMAD signaling pathway, the signal activity of ligand-bound receptors can be augmented in early endosomes, facilitated by signal promoting factors like SARA and Hrs, which is thought to enhance TGF-β signaling activity (Chen, 2009; Seaman, 2012). The schematic diagram below shows the overall pathway of TGF-β receptors inside the cell (Fig 18).
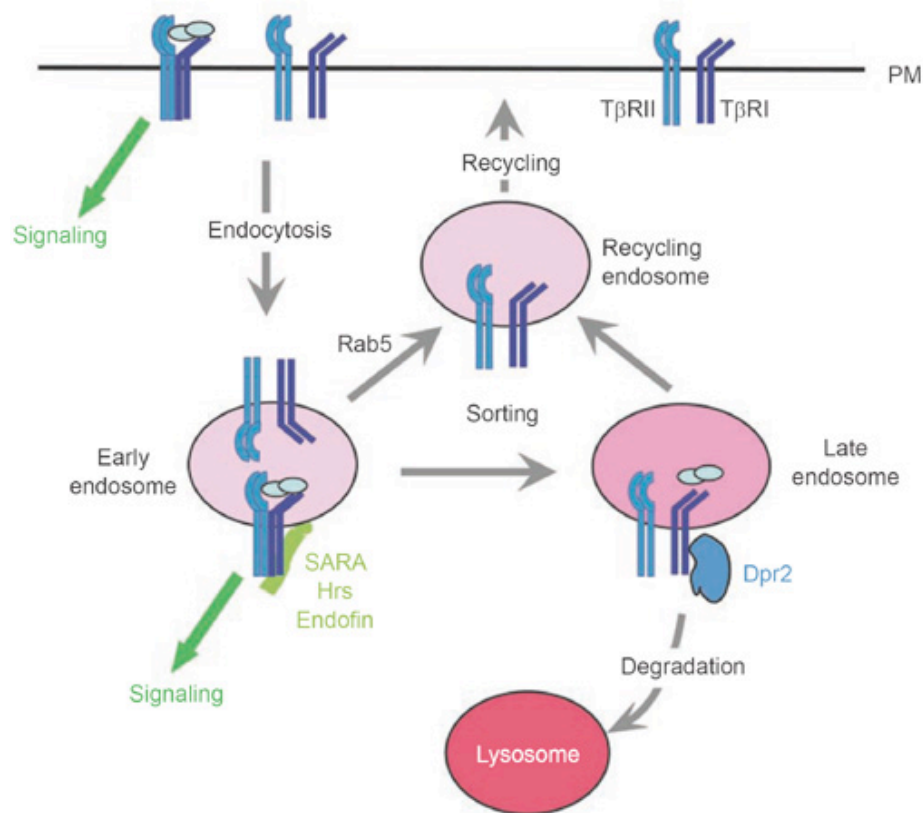


Figure 18. Schematic representation shows several endocytosis pathways and outcomes of TGF-β receptor interaction inside the mammalian cells. Some receptors with ligand will go directly for signaling without endocytosis; some empty receptors will be sent to the early endosome, from there some of them bind their ligand and interact with endocytosis factors SARA, Hrs, and Endofin then go downstream for signaling. Some of them are sorted to the recycling endosome

then sent back to cell surface. Some are delivered to the late endosome for modification and recycling, or go to the lysosome from late endosome for protein degradation (Chen, 2009).

**Clathrin-independent internalization pathway**

Unlike the clathrin-dependent pathway, cholesterol- and sphingolipids-enriched lipid rafts characterize the cargo pits in clathrin-independent endocytosis (Chen, 2009). This pathway is termed the clathrin-independent internalization pathway, or caveolae-/lipid raft-mediated pathway. Caveolae are defined as caveolin associated, low density, specialized detergent-resistant micro-domains. "Lipid raft" is a common and general name for the assembly of specific lipids into an ordered small fragmented space inside the membrane bilayer (Pelkmans, 2005). There are several sub-categories of cargo delivery in lipid raft-mediated pathways, and here I have focused on caveolae-mediated pathway.

Caveolae is not as well studied as clathrin-coated pits and there are many gaps in the mechanistic understanding of caveolae function. However, studies have shown that caveolae/lipid rafts mediate several signaling processes. Caveolae/lipid rafts serve as a signaling center for G-protein-coupled receptors, are calcium and protein tyrosine kinases. Sometimes caveolae/lipid rafts play as the entrance for virus to get into cell (Pelkmans, 2005). In addition to signaling functions, lipid rafts also contribute to the internalization of several types of proteins, including glycosylphosphatidylinositol (GPI)-anchored proteins, toxins, growth hormone receptors and other endothelin receptors. After internalization through the caveolae-/lipid raft mechanism, cargoes are transported to caveosomes; eventually the cargoes are delivered to the lysosome for

degradation. Factors regulating these caveosomes remain unknown (Chen, 2009).

**The endocytosis motif defined in TGF-β receptors**

In this cargo delivery, endocytosis serves as the vehicle to transfer the internalized receptors from plasma membrane into different departments in cell. A sequence in the C-terminus tail on type I receptor, termed as NANDOR box, is essential for the trans-phosphorylation of type I receptor and downstream signaling activity but it has no influence on the intracellular delivery (Garamszegi et al., 2001b). The name NANDOR comes from the definition non-activating and non-down-regulating (Garamszegi et al., 2001b). Interestingly, it has also been found that the truncation of the C-terminus tail of type II receptor eliminated its endocytosis. This region is referred to as the LTA motif (Murphy et al., 2007). A tryptophan residue located a few amino acids upstream of the NANDOR box is necessary for the basolateral delivery of type I receptor (Zhou et al., 2004).

Screening of the NANDOR sequence in the C-terminus tail of type I receptor has demonstrated the importance (Garamszegi et al., 2001b). In human TGF-β type I receptors, the NANDOR box is a small region located in the C-terminal tail between residues 482-491. Most amino acids that reside in this region are highly conserved through all human TGF-β homologs (ACVL1, ACVL2, ACV1, BMPR1 and TGFRI). It has been reported that a tryptophan residue located a few amino acids upstream of the NANDOR box in ACVL1 is necessary for the baso-lateral

delivery of type I receptor. The mutation induced substitution of this W at position 521 to alanine will eliminate internalization of type I receptor. However, the downstream TGF-β signaling can still be detected by the phosphorylation of signal transducer pSMAD (Zhou et al., 2004), which indicates that either the internalization of type I receptor is not necessary for downstream TGF-β signaling activation, or there is an unknown signaling pathway for TGF-β downstream signaling cascade.

Similar to the NANDOR box in type I receptor, Murphy et al. 2007 found that the truncation of the C-terminus tail of type II receptor eliminated its endocytosis (Murphy et al., 2007). The defined region referred to as the LTA motif starts with three residues: leucine, threonine and alanine. The LTA motif is a small region screened in the C-terminal tail of type II receptor that consists of residues 529-538. This motif is conserved in all human TGF-β homologs (TGFR2, BMPR2 and AVR2). The assay to track the location of TGF-β type II receptor in MDCK cell culture (which is a classic cell model for polarization study) showed that under normal circumstances, the WT type II receptor located at the basolateral surface and cannot be seen on apical surface.  If all the non-alanine amino acids are substitute by alanines in this LTA motif, the type II receptor loses the ability to remain on the basolateral membrane surface but distributes all around the plasma membranes (Murphy et al., 2007). If only two or three adjacent amino acids located inside LTA motif are mutated to alanines, the distribution of mutant receptors all over plasma membrane still occurs, which indicates that every

amino acid in LTA motif is important for spatial distribution control of TGF-β type II receptor (Murphy et al., 2007).

To get a better understanding of the endocytic motifs mentioned above more clearly in TGF-β receptors, I aligned a part of the C-terminal tail for both type I and type II receptors from nematodes to mammals respectively and highlight the special amino acids and endocytic regions as below (Fig 19). From the alignment, it is easy to note that most of the amino acids either in the NANDOR box or in the LTA motif are highly conserved across *C. elegans* to human homologs, and the endocytic regions in both type I and type II receptors have a significant overlap with each other, indicating that they share sequence similarities. Type I and type II receptor could interact with each other in receptor trafficking. The W residue required for type I receptor internalization is conserved through all type I receptor homologs, and there is also a highly conserved W at the same location in type II receptor. Although there is no convincing evidence yet, the W residue might be involved in type II receptor endocytosis similar to type I receptors.

```
        Type I Receptors
                                      NANDOR box
         ALK-1         MRECWYPNPSARLTALRIKKTLQKI 25
         ALK-2(act)    MKECWYQNPSARLTALRIKKTLTKI 25
Human    ALK-3(BMP)    MSECWAHNPASRLTALRIKKTLAKM 25
         ALK-4         MRECWYANGAARLTALRIKKTLSQL 25
         ALK-5(TbR)    MRECWYANGAARLTALRIKKTLSQL 25
Drosophila TKV         MQECWHPNPTVRLTALRVKKTLGRL 25
           BABOON      MKECWYPNPVARLTALRIKKTLASI 25
C. elegans ─ SMA-6     MEDSWHSIPHFRHSALKLKKEMAEL 25
                       :  .*       * .:    :* :


        Type II receptors
                                      LTA motif
         TBRII         LTECWDHDPLARLTAQCVAERFSE 24
Human    ACTIVIN       IEECWDHDAEARLSAGCVEERVSL 24
         BMP           IEDCWDQDAEARLTAQCAEERMAE 24
Drosophila PUNT        MEECWDHDAEARLSSSCVMERFAQ 24
           WIT         CEDCWDHDADARLTSLCAEERMQE 24
C. elegans ─ DAF-4     TEEMWDPEACARITAGCAFARVWN 24
                       : ** :. **::: *.  *.
```

Figure 19. The overlap of endocytosis motifs in both TGF-β type I and type II receptors from vertebrates to nematode. Both of the motifs are highly conserved.


**The trafficking of receptors in *C. elegans***

The clathrin-dependent endocytosis of TGF-β receptors can follow three paths in

*C. elegans*. 1. Receptors can be sent to the early endosomes from the vesicle

buds formed from plasma membrane. Then the internalized receptors are

recycled back to plasma membrane directly from early endosomes. 2. The

internalized receptors are delivered to retromer from early endosomes and sorted

to trans-Golgi network (TGN). 3. Receptors in the retromer are sent to late

endosomes and finally to the lysosome for degradation, or receptors are destined

to lysosomes from early endosomes, which eventually mature into late

endosomes (Chen, 2009; Seaman, 2004). Our lab has found that type I and type

II receptors, SMA-6 and DAF-4, go to distinct pathways in *C. elegans*. The study

of internalization levels of both type I and type II receptors with the depletion of

AP-2 (adaptor protein 2 which is required for clathrin-dependent cargo

internalization) showed that SMA-6 accumulates on the cell surface while DAF-4 remained unchanged. This demonstration indicates that the internalization of type I receptor SMA-6 and type II receptor DAF-4 are clathrin-dependent and clathrin-independent, respectively (Gleason et al., 2014). SMA-6 is is found in the lysosome when the retromer-dependent pathway is blocked. SMA-6 binds directly to retromer by binding the core protein required for retromer function, referred to VPS-35 in *C. elegans* (Gleason et al., 2014). However, it does not appear that DAF-4 binds to the retromer directly and the retromer has little effect on the endocytosis of DAF-4 (Gleason et al., 2014). This suggests that type II receptors are recycled in a different manner from type I receptor; DAF-4 likely does not go through the retromer for further cargo sorting. Since DAF-4 is the only type II receptor in *C. elegans* and type II receptors are key to the activation of type I receptors, it is important to study endocytosis of DAF-4 and SMA-6.
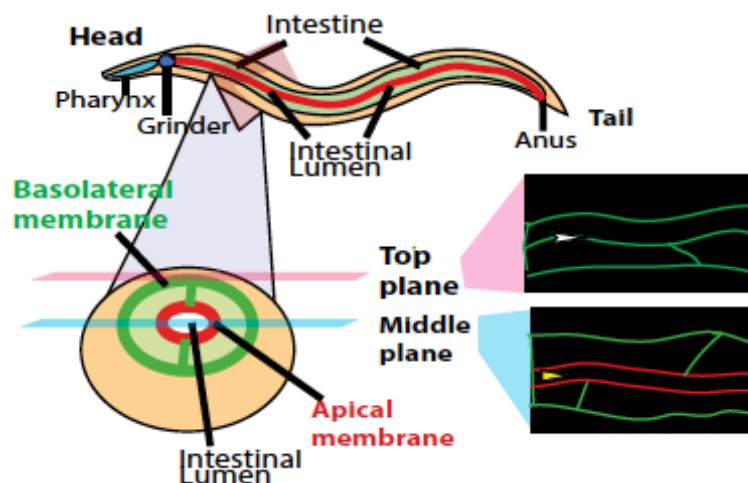


Figure 20. Schematic representation of the *C. elegans* intestine system. Green lines represent the basolateral cell surface while red lines define the apical cell surface (Gleason et al., 2014).

The *C. elegans* intestine system is used to study the receptor trafficking in this project. The nematode intestine system is well characterized for the study of endocytosis. The intestine is composed of large layer epithelial cells that are positioned in almost symmetric pairs around lumen. This specific positioning allows the trafficking of molecules from cell membranes into the cytoplasm. Intestine cells in *C. elegans* are polarized cells, and therefore provide a good model to study the spatial distribution of TGF-β receptors. In addition, *C. elegans* TGF-β receptors SMA-6 and DAF-4 are both expressed in worm intestine and are needed for function in the intestine (Fielenbach and Antebi, 2008). Further, we and others have used the intestine for receptor studies and have developed many cell biological tools for these studies. These facts make the worm intestine an ideal system to study the changes in TGF-β receptors with cancer/MFS-associated mutations. The schematic representation of worm intestine system is shown in Fig 20.

## Diseases associated with malfunctioning TGF-β signaling

Abnormal function of TGF-β signaling pathways can result in many diseases including cancer, immune response, Marfan syndrome (MFS) and MFS-like syndromes, cardiovascular diseases, and bone malformation.

TGF-β signaling is important in various cancer and tumors. The Cdk inhibitor can regulate cell cycle responses to TGF-β signaling to result in cell cycle arrest in the late G1 stage (Massague, 1998). TGF-β signaling is also thought to play

important roles in the regulation of epithelial-mesenchymal transition (EMT)
(Massague, 1998). Although EMT is critical in embryonic development, EMT is
usually regarded as a symbol of initiation in tumorigenesis. A majority of
missense mutations in TGF-β receptors have been identified in patients with
colon, pulmonary, kidney and many other cancers (Massague, 1998). The role of
these mutations in cancer promotions remains unknown and requires
investigation.

TGF-β signaling activity does not only mediate the cell proliferation and
differentiation and death associated tightly with tumorigenesis, but is also
involved in the regulation of an important mediator cytokine that is required in the
regulation of immune responses. TGF-β signaling is important for T lymphocytes
(Li et al., 2006).
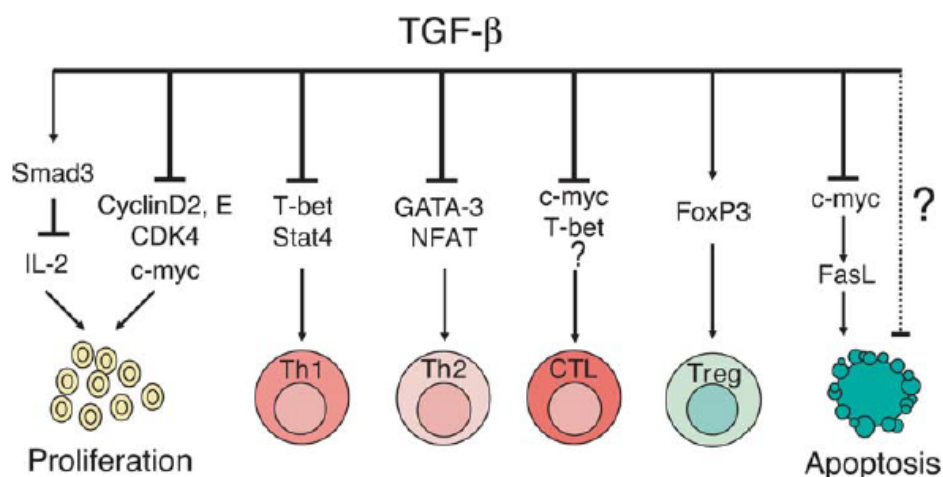


Figure 21. Schematic representation of the regulation of TGF-β signaling in the T cell responses
(Li et al., 2006).

In addition to its relationship with tumorigenesis and regulation of the immune responses, abnormal activity of TGF-β signaling can lead to Marfan syndromes/MFS-like syndromes, and these disorders are often associated with clinic manifestations like bone malformation and cardiovascular problems. These symptoms overlap with Marfan syndromes/MFS-like syndromes, so bone malformation and cardiovascular problems listed above related with abnormal TGF-β signaling will be discussed in this thesis.

**Marfan syndrome and Marfan-like syndromes**

TGF-β signaling plays a role in various diseases including Marfan syndrome (MFS). Diagnosis of MFS is based on clinical signs and family history analysis. MFS patients are usually screened in varied ages with the following clinical symptoms: abnormally taller gesture, long digits (including arms and legs), curved spine, heart failure, aortic enlargement, lung collapse, eye lens dislocation, and abnormal formation of bones (Benke et al., 2013a). President Lincoln was a suspected MFS patient due to his abnormally tall figure, though this has not been confirmed since DNA test is not available in the interest of privacy (Reilly, 2000).

An important criterion of MFS diagnosis is disproportionate and slender stature as mentioned above (De Paepe et al., 1996). However, a study reported that MFS patients are not necessarily taller than the population average; however they are generally taller than the family average (Erkula et al., 2002). Patients

with MFS can suffer from heart failure, aorta and blood vessel problem,

respiratory disorder and abnormal bone formation (Benke et al., 2013b). The

dislocation of lens in eyes and detachment of retina can be observed in some

MFS patients, which may lead to vision loss (Benke et al., 2013a; Dietz, 1993).

Symptoms that are more difficult to detect directly are also lethal to some severe

patients.

Several disorders related with MFS, share similar clinical signs as MFS. These

disorders are referred to as MFS-like diseases. Some well-known examples of

MFS-like disorders include Ehlers-Danlos syndrome (EDS), Loeys-Dietz

syndrome (LDS) and familial Thoracic Aortic Aneurysm and Dissection (familial

TAAD).  These disorders, like MFS, are genetically inherited connective tissue

diseases and may cause death. EDS is generally characterized by fragile joints,

skin hyperextensibility and joint hypermobility (the Ehlers Danlos society,

https://www.ehlers-danlos.com/what-is-eds/). LDS is regarded as a sub-type of

EDS and well characterized with aortic enlargement problem, abnormally twisted

blood vessels or artery disconnections (Van Laer et al., 2014). Familial TAAD is

similar to LDS in that both are disorders that primarily affect the aortic system.

But familial TAAD can affect the upper part of the aorta (thoracic aorta) close to

the heart (Jondeau and Boileau, 2012).

MFS and MFS-like diseases are caused by mutations in either *FBN1* gene or

TGFBR1/TGFBR2 gene. Mutations in *FBN1*-associated with MFS have been

found for a while, and recent studies demonstrated that specific mutations in

TGFBR1/TGFBR2 are related to MFS-like diseases. Usually MFS/MFS-like

patients with mutant *FBN1* are diagnosed as Marfan syndrome type I (MFS1)

while patients with mutations screened in TGFBR1/TGFBR2 are generally

categorized as Marfan syndrome type II (MFS2) (Zangwill et al., 2006). Since the

very first MFS2 was screened in LDS patients, LDS was generally used

interchangeably with MFS2. Gradually researchers and clinicians realized that

there were many sub-categories in MFS2 depending on syndrome

manifestations. In fact LDS patients show more general clinical signs than MFS2

patients (Horbelt et al., 2010a). The relationship between LDS, MFS2 and familial

TAAD is shown in Fig 21.
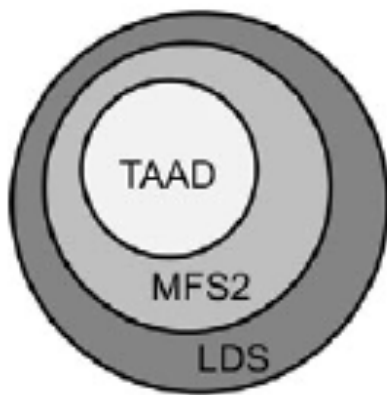


Figure 22. The relationship of Familial Thoracic Aortic Aneurysm and Dissections (TAAD), Marfan syndrome type 2 (MFS2) and Loeys–Dietz syndrome (LDS) in clinical manifestations (Horbelt et al., 2010a).

**Marfan syndrome type I (MFS1): mutations in FBN1 gene**

Marfan syndrome is primarily caused by mutations in *FBN1*. *FBN1* gene (15q21)

encodes protein fibrillin-1, which is an essential factor for the generation of

connective tissue. Although relevant mutations are screened in FBN1, Marfan syndrome caused by mutant fibrillin-1 features a disregulation of TGF-β ligands (Kaartinen and Warburton, 2003a). The fibrillin-1 protein encoded by the *FBN1* gene is incorporated into the microfibril network in the extracellular matrix, providing structural support in connective tissues (Kaartinen and Warburton, 2003a).

As mentioned, TGF-β ligands are important factors for various biological processes. The negative regulation of TGF-βs exists through soluble proteins that can prevent ligands from interacting with TGF-β receptors located on the membrane (Massague, 1998). TGF-βs are synthesized as pre-propolypeptides in a precursor form that goes through proteolytic digestion in the Golgi apparatus. Proteolysis leaves two products assembled in dimers; one is the dimer protein cleaved from the N-terminal tail referred to as latency-associated peptide (LAP), while the other one is cleaved from the C-terminal end defined as mature TGF-βs. Importantly, the N-terminal portion of LAP is non-covalently bound to TGF-βs after secretion. This latent form cannot bind to the cell surface receptors, preventing continuation of a TGF-β signal (Kaartinen and Warburton, 2003a; Khalil, 1999; Massague, 1998). The complex is composed of dimer TGF-βs as well as LAP and is termed as small latent complex (SLC). In this complex, TGF-βs are rendered in a mature but biologically inactive form (Kaartinen and Warburton, 2003a).
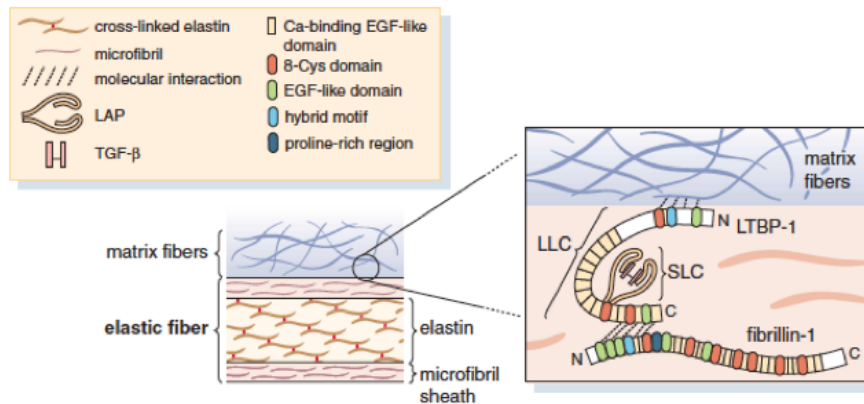
Figure 23. The mature TGF-β ligands are rendered by elastic fibers in matrix fibers. The TGF-β ligand binds to small latency complex (SLC), and SLC interacts with the C-terminus of latency TGF-β binding protein (LTBP) to become a larger latency complex (LLC). The N-terminus of LTBP binds to matrix fibers, which fix the LLC in the elastic fiber layer (Kaartinen and Warburton, 2003).

Another key factor playing a role in this process is a large secretory protein refered as latent TGF-β-binding protein (LTBP) (Kaartinen and Warburton, 2003a; Khalil, 1999; Massague, 1998). Although there is a "latent" in its name, LTBP does not infer latency to TGF-β ligands, but it mediates the secretion and association of latent TGF-β complex (SLC) to the extracellular matrix (ECM) (Saharinen et al., 1996). The LTBPs bound to SLC can form a large latent complex called LLC. The LLC complex targets TGF-β complex (SLC) to fibrillin microfibrils through its C-terminal end (Kaartinen and Warburton, 2003a). The fibrillins encoded by FBN1 possess structural motifs that include calcium-binding EGF-precursor–like units that can be separated by either 8-cysteine or TGF-β– binding domains. The 8-cysteine motifs and latent TGF-β–binding proteins (LTBPs) belong to the same protein family (Kaartinen and Warburton, 2003a). The schematic representation of localization and maturation of TGF-β ligands is shown in Fig 22 above.

Mutations in *FBN1* gene lead to impaired fibrillin-1 synthesis and subsequently

defective generation of connective tissue, which ultimately leads to the release of

TGF-β ligands. Mutations in fibrillin-1 not only alter intercellular communication,

but also change the extracellular concentration of TGF-β ligands (Chaudhry et

al., 2007). The elevated level of TGF-β ligands causes the excessive TGF-β

signaling (Benke et al., 2013b; Kaartinen and Warburton, 2003b). Neptune et al.,

2003 demonstrated a correlation between abnormally high levels of TGF-β

ligands and signaling in lung tissue in fibrillin-1 -/- mice (Neptune et al., 2003)

(Fig 23).
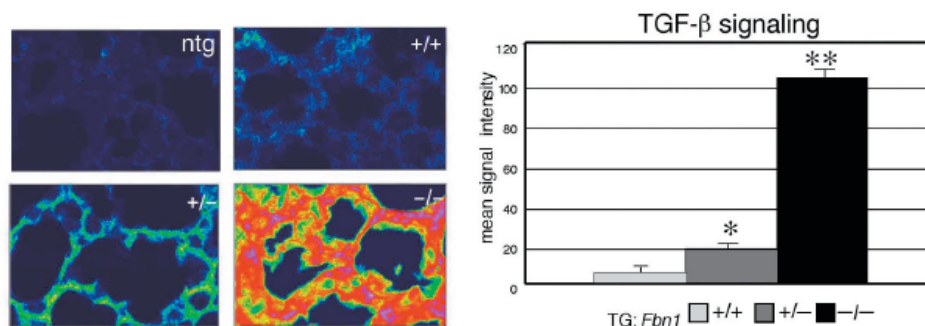


Figure 24. Active TGF-β expression and signaling in lung tissue of mice with deficiency of fibrillin-1. Left panel showed fluorescence microscopy of GFP-tagged TGF-β ligand in lung tissue from PD7 wild type (+/+), fbn1 deficient heterozygous (+/–) and fbn1 deficient homozygous (–/–) transgenic mice. Right panel is the measurement of fluorescence intensity of left panel. Compared with wild-type mice (+/+), average intensity was 4 and 25 times greater fbn1 deficient heterozygous (+/–) and fbn1 deficient homozygous (–/–) mice respectively (Neptune et al., 2003).

Besides the above MFS/MFS-like disorders caused by mutations in *FBN1*, there

are some less common MFS-like diseases resulting from mutant FBN1 such as

Weill-Marchesani syndrome (WMS). Weill-Marchesani syndrome is also a

dominantly heritable connective tissue disorder with a frequency of about 1 in

100,000. Patients show opposite clinical manifestations compared to regular

MFS/MFS-like patients do: shorter stature, stiff joints and eye abnormalities.

Although WMS impairs connective tissue function, the most significant clinical

sign is the ocular problem. Most patients will develop glaucoma (an eye disease

with abnormally high eye pressure) that can lead to blindness (Hubmacher and

Apte, 2011).

Since the impaired network of microfibril and elastin is a major cause for

significant increased TGF-β level, it is crucial to get a better understanding of

microfibril and elastin homeostasis. A series of secreted enzymes with multi-

domain and matrix-associated zinc metalloendopeptidases known as ADAMTS

have a variety of roles in tissue development. ADAMTS is short for A Disintegrin

and Metalloproteinase with Thrombospondin motifs (Kelwick et al., 2015). There

are 19 defined sub-members in the human ADAMTS family (Kelwick et al.,

2015).

ADAMTS10 can bind to fibrillin-1 with high affinity and is crucial for the

biosynthesis of microfibril. ADAMTSL6β, a member of this family, is required for

connective tissue structure and regeneration (Benke et al., 2013b). Certain

mutations screened in ADAMTS10, in addition to mutations in FBN1, lead to

WMS-like disease, manifesting eye lens dislocation (Benke et al., 2013b).

Intriguingly, ADAMTSL6β is found to negatively regulate the increased level of

TGF-β ligands in the extracellular environment. The administration of

ADAMTSL6β to a mouse model with MFS disorder rescued microfibril formation

(Saito et al., 2011). These data suggested a novel cure for mutant *FBN1*

associated connective tissue disorder.

**Marfan syndrome type II (MFS2): mutations in TGFBR1/TGFBR2 gene**

The majority of specific disorders like MFS/MFS-like and some cancers can be

attributed to heterozygous germ line mutations of either the type II (TGFBR2) or

type I (TGFBR1) TGF-β receptor genes. Less commonly are mutations in other

signaling components of TGFβ, such as SMAD3 (Proost et al., 2015;

Wooderchak-Donahue et al., 2015), TGFβ2 (Lindsay et al., 2012), TGFβ3

(Bertoli-Avella et al., 2015b) and the repressor SKI (Schepers et al., 2015).

Interestingly, in almost every case described, the mutation is a missense

mutation. Thus the mutant gene encodes a protein product; null mutations are

not found in these diseases.

Mutations in TGF-β receptors associated with Marfan syndromes have been

recently found in clinical research and showed more interesting features than

MFS-associated mutations screened in FBN1 gene, and MFS caused by mutant

TGFBR2 is defined as MFS2.  Some patients with TGFBR2 carrying mutations

show MFS clinical symptoms; however the severity is different from real MFS.

This type of MFS is defined as Marfan-like syndromes (MFS-like) (Matyas et al.,

2006). Screening of these patients showed there are different mutations in TGF-β

receptors, especially in the cytoplasmic region, most of them are point mutations

and a few are deletions. The pathologic mechanism for MFS2 is still a mystery,

since the majority of mutations are located in the kinase domain in TGF-β

receptors. It was initially thought that mutations in the kinase domain would result

in loss of kinase activity, which would subsequently paralyze downstream signal

transduction (Horbelt et al., 2010a). In summary, MFS2 is caused by

misregulation of TGF-β signaling. This is in contrast to MFS1, which results from

a higher TGF-β activity due to the excess release of TGF-β ligands.

Recent studies have shown that some mutant TGF-β receptors maintain kinase

ability. Western blot analysis from fibroblast cell samples of MFS-like patients

that used antibodies directed against the phosphorylated SMAD of mutant type II

receptor R528H in response to ligands showed that although the acute

responsiveness of the mutant receptor is reduced, the long-term signal

transduction is not disrupted (Loeys et al., 2005). MFS-like patients carrying the

R528H mutation showed disrupted connective tissue in aortic blood vessel walls,

which is consistent with the classic clinical symptoms in MFS. These results

imply that there should be other factors that sabotage the function of TGF-β

signaling (data and figures are shown in Fig 24).
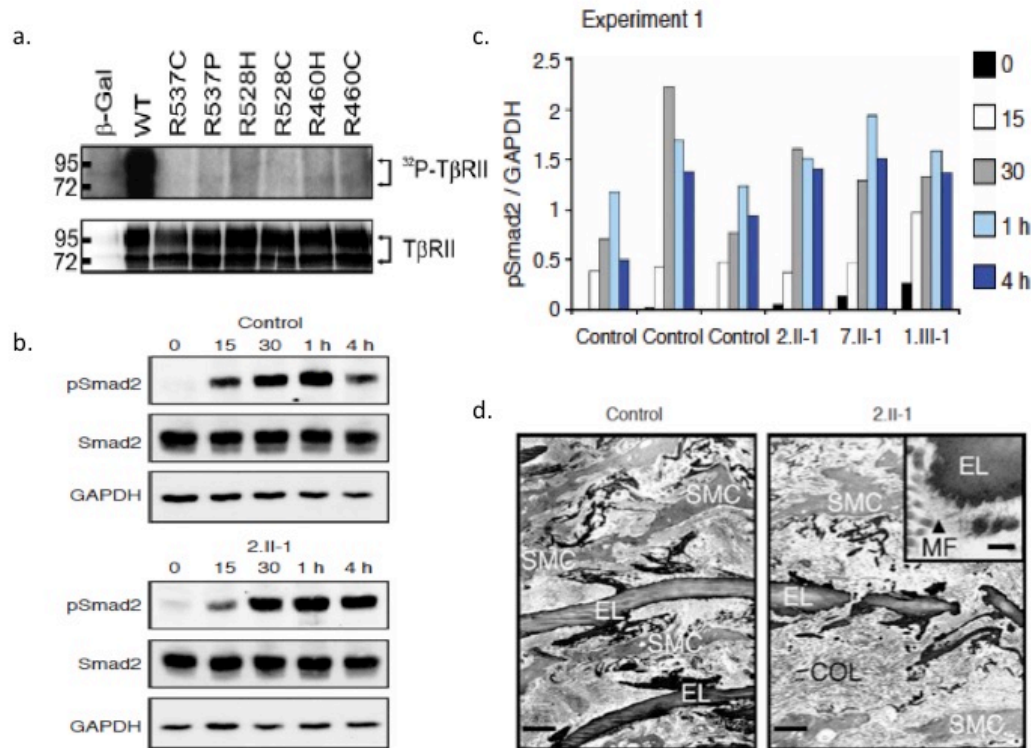
Figure 25. Contradictory results on kinase activity of TGF-β type II receptor with MFS-like associated mutation and histology of aortic walls. (a) *In vitro* kinase activity of mutated TGF-β type II receptor. FLAG-tagged TGF-β type II receptors were immunoprecipitated and employed in *in vitro* kinase assay with [r-$^{32}$P] ATP. The upper panel presents autoradiogram that shows $^{32}$P-phosphorylated TGF-β type II receptors, and the lower panel is the western blot of the amount of total TGF-β type II receptors in the immunoprecipitates (Horbelt et al., 2010a). (b) The immunoblots showed the rate of phosphorylation of SMAD2 (pSMAD2) in fibroblasts samples of control and MFS patient carrying specific point mutation R528H at 0 min, 15 min, 30 min, 1 h and 4 h after addition of exogenous TGF-β ligands (Loeys et al., 2005). (c) The quantification corresponds to (b). Three sets of samples taken from MFS patients with different point mutations, 2.II-1 is the patient with R528H mutation (Loeys et al., 2005). (d). Low- and high-power (inset) ultrastructural images showed the loss of elastic fiber architecture in the aortic walls in MFS patient 2.II-1 (R528H) compared with WT control. SMC, smooth muscle cell; COL, collagen; EL, elastin deposit; MF, microfibrils. Low-power scale bars, 2.5 mm; inset scale bar, 0.25 mm (Loeys et al., 2005).

As indicated in Fig 24, many results in the field contradict each other. In vitro kinase assays did not detect any kinase activity in cell culture while patients' samples showed delayed but active TGF-β signaling. These results imply that there should be other factors that sabotage the function of TGF-β signaling.

Considering the location of the missense mutations, could it be possible that some disease-associated mutations disrupt receptor endocytosis?

**Disease-associated mutations screened in patients**

In this work we will focus on the disease-associated mutations screened in TGF-β receptors. Screening of MFS and MFS-like patients showed there are hundreds of different mutations in TGF-β receptors, especially in the cytoplasmic region. In most cases, only one missense mutation on either type I receptor or type II receptor is sufficient to cause the disease (Loeys et al., 2005; Matyas et al., 2006). How do these mutations in TGF-β receptors cause MFS or MFS-like syndromes? Since most mutations are located in the C-terminal kinase domain of TGF-β receptors, it was initially thought that mutations in the receptor kinase domain merely result in loss of kinase activity, which subsequently paralyzed downstream signal transduction (Horbelt et al., 2010b) (Fig 24). However, recent studies show that mutant TGF-β receptors maintain kinase function and the phosphorylated R-SMADs can be detected in downstream. One missense mutation R486W of BMP type I receptor is found in a brachydactyly (BD) type A2 patient, which is a dominant heritable bone disorder. Interestingly, R486 is the first amino acid of endocytic motif NANDOR box in the type I receptor, and a kinase assay demonstrated that mutant BMP type I receptor with R486W retained kinase activity (Lehmann et al., 2003b) (Fig 25). In MFS-like patients, western blot analysis that monitored the phosphorylated SMAD of mutant type II receptor R528H in response to ligands showed that although the acute

responsiveness of mutant receptor is reduced, long-term signal transduction is

not disrupted (Loeys et al., 2005) (Fig 24).
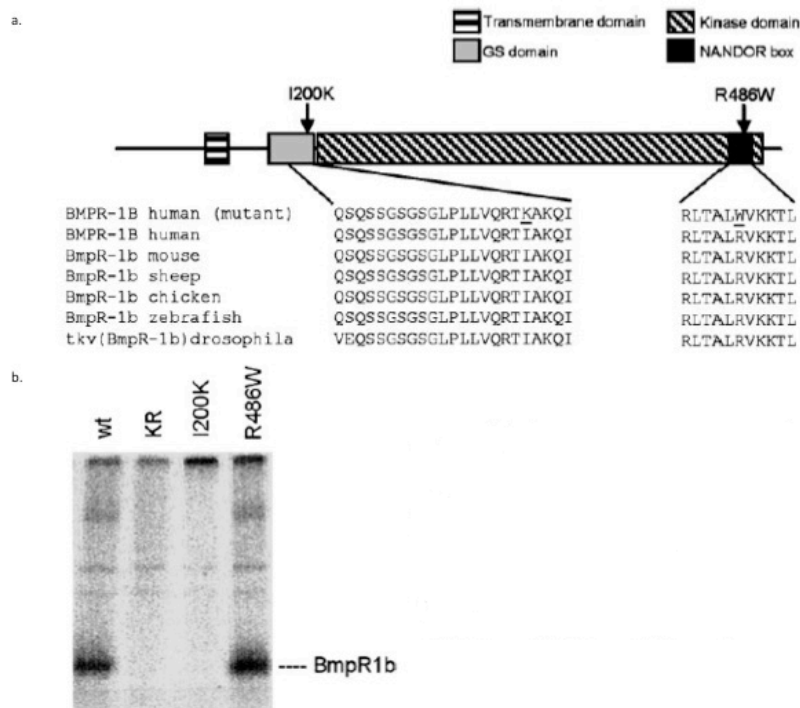


Figure 26. Schematic representation of point mutation R486W in BMP receptor and relevant kinase assay. The upper panel indicates the amino acid sequence alignment for BMP homologs, and the location of R486W is underlined. The domain organization of BMP receptor is also represented. The lower panel shows the in vitro kinase assay. Compared with WT control, kinase deficient control and I200K point mutation, R486W still showed kinase activity (Lehmann et al., 2003b).

Not only are MFS/MFS-like mutations found in TGF-β receptors, but cancer-

related point mutations are also identified in the cytoplasmic domain of TGF-β

receptors. As discussed earlier, misregulation of TGF-β signaling pathway is

tightly associated with tumorigenesis.

Missense mutations identified in cancer/MFS-related patients screened so far,

are represented in a schematic in Fig 26. There is one missense mutation

highlighted in yellow color that is the mutation screened by our lab in *Drosophila*

*punt*. From this figure, it is clear that although the disease-associated mutations

in TGF-β receptors are dispersed through the whole cytoplasmic region, there

are two regions in C-terminal tail in which mutations are concentrated, and they

are defined as mutation cluster I and II respectively.



Figure 27. The overview of different types of disease-associated mutations in the C-terminal tail in human type I and type II receptors. There is a region inside where a lot of mutations reside called the mutations cluster. Different colors represent different disease categories. Red codes represent mutations screened from MFS/MFS-like patients, blue codes are cancer-related mutations, and green colors are mutations screened in both cancer as well as MFS/MFS-like patients. There is another mutation cluster located in the upstream of cytoplasmic domain, which is called mutation cluster I. The mutation cluster shown in this figure is mutation cluster II (modified from M. Reiss).

Interestingly, most of these mutations are the same ones also screened in many

cancer-related TGF-β receptor mutations. Over 40 different missense mutations

in TGF-β receptors have been identified in human cancer cell lines or tumor

specimens (Chen, 2009; Garrigue-Antar et al., 1995a; Grady et al., 1999; Lu et

al., 1998; Lu et al., 1999; Nagayama et al., 2002; Schiemann et al., 2004; Wang et al., 2000). These mutations include eight changes in the TGFBR2 gene and four in the TGFBR1 gene. Similar to MFS-like mutations, several cancer-associated mutations can be found in the TGFBR2 gene cluster in the same C-terminal domain (Bierie and Moses, 2006; Kim et al., 2000; Levy and Hill, 2006). In most cases, it was assumed that the identified mutations represented null mutations. However, these mutations confer some activity (as is also the case for MFS-like mutations) (Chen, 2009; Garrigue-Antar et al., 1995a; Grady et al., 1999; Lu et al., 1998; Lu et al., 1999; Nagayama et al., 2002; Schiemann et al., 2004; Wang et al., 2000).

**Some disease-associated mutations may alter TGF-β trafficking**

Since the two endocytosis motifs, the NANDOR box in the type I receptor and the LTA motif in the type II receptor, are inside mutation cluster II in the C-terminal tail of TGF-β receptors, it is important to learn if those cancer/MFS-associated mutations affect the trafficking of TGF-β receptors. Both endocytic motifs are highly conserved from mammals to *Drosophila*, and they are in same position in two receptors as observed from sequence alignments. Strikingly, almost all of the amino acids in this special region are involved with either MFS-like or cancer-associated missense mutations. Taken together, it is possible that several mutations screened in MFS-like/cancer patients can lead to improper endocytosis of TGF-β receptors.

It has shown that mutations in endocytosis LTA motif of the TGF-β type II receptor result in unusual apical delivery of the receptors inside the MDCK cell line (Murphy et al., 2007). Madin-Darby Canine Kidney (MDCK) cell is a mammalian cell line that serves as good model for the study of cell polarization. It is reported that wild type TGF-β type II receptors can be delivered to basolateral surface in a cell, but cannot be sent to the apical surface. However, mutations of specific amino acids to alanines in LTA motif allowed the mutant TGF-β type II receptor to go to the apical surface as well as basolateral surface. These amino acids are described as LTAxxVAxxF. These data indicated part of the amino acids in TGF-β type II receptor LTA motif could control the spatial distribution of TGF-β type II receptor (Fig 27).
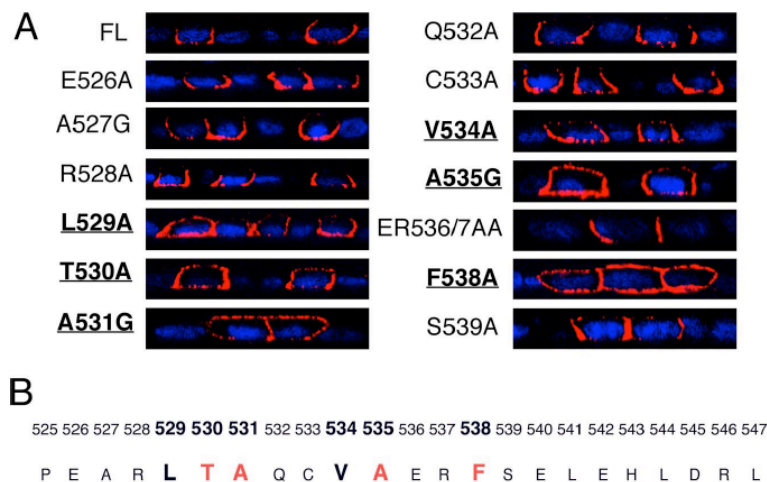


Figure 28. The LTA motif (529-538) regulates the distribution of TGF-β type II receptor on the basolateral surface of the cell. (A) Point mutation of amino acids of the LTA motif to either alanine or glycine in TGF-β type II receptor. The mutant receptor was transfected into polarized MDCK monolayers and then stained with GM-CFS antibody. Confocal imaging showed the receptor distribution for both WT control and mutants. Some mutants showed that point mutations result in loss of control of TGF-β type II receptor retention on basolateral surface. (B) The amino acid sequence of the LTA motif. The critical amino acids for apical basolateral delivery are highlighted in red (Murphy et al., 2007).

The control of the spatial distribution of TGF-β type II receptor by the LTA motif

may provide a way to understand the pathology of MFS2. MFS1 is due to a

higher signaling activity of TGF-β pathway; therefore MFS2 patients also gain

higher signaling activity in TGF-β pathway. However, whether those disease-

associated mutations destroy the kinase activity in TGF-β signaling remains

unclear. It is reported that TGF-β ligands tend to accumulate on the apical

surface of a cell, while WT TGF-β type II receptor should go to basolateral

surface (Murphy et al., 2007; Yakovich et al., 2010). In normal circumstances,

this spatial gap will separate TGF-β ligands and receptor naturally and control

TGF-β signaling to some extent. However, the mutations in the LTA motif will

drive TGF-β type II receptors to the apical surface, where TGF-β ligands reside.

Is it possible that the mutated TGF-β type II receptors interact with the ligand and

together with type I receptor to signal? If that is the case, could it explain why

there is still kinase activity detected in some in vivo assay using MFS/MFS-like

patient samples? I will focus on the above questions to study some specific

MFS/MFS-like associated missense mutations in *C. elegans*.


### *C. elegans* employed as a research model for MFS-like mutations in TGFBRII

The intestine system in *C. elegans* is employed for receptor trafficking in this

project. To learn how the disease-associated mutations affect TGF-β receptors, it

is necessary to design constructs carrying specific point mutations and introduce

them into *C. elegans* to generate transgenic worm strains. Not all amino acids in

endocytosis motifs are conserved from mammals to *C. elegans*, but the conserved amino acids in TGF-β receptors are involved in either cancer or MFS/MFS-like diseases, and some of them are even presented in both categories. As a result, I will focus on how the mutations affecting these conserved amino acids impact the trafficking of TGF-β receptors. Based on the screening of cancer/MFS mutations from patients and the studies on the endocytosis of TGF-β receptors, I selected missense mutations that would be investigated in the context of this work (Table 2).

| Type I receptor | | | NANDOR box | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TβRI | W475 | ..... | R482 | L | T | A | L | R487 | I | K | K | T | L |
| SMA-6 | W585 | ..... | R592 | H | S | A | L | K597 | L | K | K | E | M |
| Missense mutation | A | | | | | | | A Q(MFS) | | | | | |
| Type II receptor | | | LTA motif | | | | | | | | | | |
| TβRII | W521 | .... | R528 | L529 | T530 | A | Q | C533 | V | A | E | R537 | F |
| DAF-4 | W580 | .... | R587 | I588 | T589 | A | G | C592 | A | F | A | R596 | V |
| Missense mutation | A | | C(MFS) H(MFS) K(cancer) | F(MFS) | A(cancer) | | | F(?) T(?) Y(?) | | | | C(MFS) G(cancer) P(MFS) | |

Table 2. Cancer/MFS-like associated mutations located in endocytosis motifs in worm type I and type II receptors.

Type I receptor: A few mutations have been identified in the type I receptor, therefore I focused on the missense mutation in NANDOR box and the W475 residue that is required for receptor basolateral delivery. Triple alanine mutation of RIL486AAA in type I receptor TVK in *Drosophila* showed the reduced intensity level of TVK in embryo wing disc (Ying et al., unpublished data). Considering the missense mutation of arginine to glutamine at the same position in type I receptor

ALK-4 in humans is screened in MFS-like patients (Matyas et al., 2006), the substitution of the lysine to glutamine (K597Q) at the same position was made in *C. elegans* type I receptor SMA-6.

Type II receptor: type II receptor contains a greater number of missense mutations present in TGF-β associated diseases and several of those mutations are involved in more than one category. Since the LTA motif is critical for the endocytosis of type II receptors and also are present in the mutation cluster in C terminal tail, we selected the amino acids that are highly conserved in this region as well as the W residue outside LTA motif, since the W521R mutation is screened in MFS patients (Matyas et al., 2006).

In our work, we found that specific missense mutations located in endocytic motif change the trafficking route of DAF-4 receptor in transgenic nematodes. These mutant receptors also showed reduced protein levels. However, this reduction in receptor level does not attenuate the signaling activity, the body length of mutant worms indicates that these missense mutations are dominant-negative phenotypes, suggesting that these mutant receptors maintain kinase activity. Since SMA-6 and DAF-4 cooperate with each to deliver signal flow, the tracking of wild-type SMA-6 showed that the mutant DAF-4 could result in the apical surface distribution of some SMA-6, indicating that DAF-4 may play a role in the interaction between SMA-6 and retromer.

## Chapter II: Mimicking Human Marfan and Marfan-like Syndrome Mutations Leads to Altered Trafficking of the Type II TGFβ Receptor in *C. elegans*

For this chapter, I did all the experiments, including amino acid sequence analysis, mutation analysis, vector construction, generation of stable transgenic line by microparticle bombardment, confocal imaging and data collection. Our lab manager Nanci Kane did all worm microinjections and helped to modify this manuscript. Our previous postdoc, Dr. Mehul Vora, helped to create amino acid sequence alignment of figure 1A, helped to quantify the data with Graphpad Prism to generate all the column charts (figure 2A-D, figure 3A, 3B and 3D-F, figure 4A-C), and helped to write this manuscript. Our previous lab member, Dr. Ryan Gleason, gave me suggestions about the vector construct design and helped to modify this manuscript.

Peter Schweinsberg, from Dr. Barth Grant's lab, guided the operation of the microparticle bombardment equipment.

Dr. Asli Ertekin and Davide Tavella from the Francesca Massi lab analyzed the protein structure and possible conformational changes caused by specific mutations.

Now this paper is submitted to the journal *PlosOne* for publication.

**Abstract**

The transforming growth factor-β (TGF-β) family plays an important role in many developmental processes and when mutated often contributes to various diseases. Marfan syndrome is a genetic disease with an occurrence of approximately 1 in 5,000. The disease is caused by mutations in fibrillin, which lead to an increase in TGF-β ligand activity, resulting in abnormalities of connective tissues which can be life-threatening. Mutations in other components of TGF-β signaling (receptors, Smads, Schnurri) lead to similar diseases with attenuated phenotypes relative to Marfan syndrome. In particular, mutations in TGF-β receptors, most of which are clustered at the C-terminal end, result in Marfan-like (MFS-like) syndromes. Even though it was assumed that many of these receptor mutations would reduce or eliminate signaling, in many cases signaling is active. From our previous studies on receptor trafficking in *C. elegans*, we noticed that many of the receptor mutations that lead to Marfan-like syndromes overlap with mutations that cause mis-trafficking of the receptor, suggesting a link between Marfan-like syndromes and TGF-β receptor trafficking. To test this hypothesis, we introduced three of these key MFS and MFS-like mutations into the *C. elegans* TGF-β receptor and asked if receptor trafficking is altered. We find that in every case studied, mutated receptors mislocalize to the apical surface rather than basolateral surface of the polarized intestinal cells. Further, we find that these mutations result in longer animals, a phenotype due to over-stimulation of the nematode TGF-β pathway and, importantly, indicating that function of the receptor is not abrogated in these mutants. Our nematode models

of Marfan syndrome suggest that MFS and MFS-like mutations in the type II receptor lead to mis-trafficking of the receptor and possibly provide an explanation for the disease, a phenomenon that might also occur in some cancers that possess the same mutations within the type II receptor (e.g. colon cancer).

## Author summary

The transforming growth factor-β (TGF-β) family plays an important role in many basic biological processes and when mutated often contributes to various diseases. Marfan syndrome (MFS) is a genetic disease with an occurrence of approximately 1 in 5,000. The disease is caused by mutations in fibrillin, which lead to an increase in TGFβ ligand activity, resulting in abnormalities of connective tissues, which can be life threatening. However, some patients with normal fibrillin genes also show symptoms and pathologies associated with Marfan syndrome, known collectively as Marfan-like Syndromes (MFS-like). When these patients were assessed for mutations in other components of the TGF-β pathway, several mutations clustered in a small region of the receptors, primarily in the type II TGF-β receptor, were found.

We find that mimicking these mutations in the nematode type II TGF-β receptor causes the mutant receptor to traffic to regions of the cell where it is not normally found. Importantly, these mutations do not abrogate the function of the receptor, suggesting that mis-localization of the receptor might be previously unknown

cause of disease etiology. We hypothesize that receptor mutations present at the C-terminus lead to disruptions in interactions with trafficking regulators leading to symptoms of MFS/MFS-like syndromes – a novel disease mechanism of MFS/MFS-like syndromes that might also extend to cancers bearing similar mutations.

## Introduction

Marfan syndrome is an autosomal dominant genetic disorder of connective tissue that affects the ocular, skeletal, cardiovascular and pulmonary systems. Major cardiovascular manifestations, including aortic root dilatation, dissection and rupture, pulmonary artery dilatation, mitral and aortic valve insufficiency, often lead to death in early adult life. The pleiotropic manifestations of Marfan syndrome can be directly attributed to germline mutations in fibrillins, which are members of the family of latency-associated TGF-β binding proteins (LTBPs). Several studies have provided convincing evidence that fibrillin mutations are associated with ineffective sequestration of TGF-β ligand in the matrix, which is believed to lead to excessive levels of bioactive TGF-β in the tissue microenvironment (Dietz, 2015; Dietz et al., 2005).

Given the involvement of increased TGF-β ligand in Marfan syndrome, it is not surprising to find that mutations in other components of the TGF-β pathway can result in related disorders, collectively termed MFS-like syndromes (Horbelt et al., 2010a; Matyas et al., 2006; Robinson et al., 2006), including Marfan syndrome 2 (MFS2), Loeys-Dietz syndrome (LDS), Ehlers-Danlos syndrome (LDS-2), Thoracic Aortic Aneurysms and Dissections (TAAD) and Shprintzen-Goldberg syndrome (SGS). The common thread of each of these disorders is that they show milder manifestations of many of the phenotypes seen in Marfan syndrome.

The TGFβ family includes a large family of secreted, soluble proteins that act as growth factors; they are dimeric multifunctional regulators playing important roles in various embryonic and developmental processes from invertebrates to mammals (Massagué and Chen, 2000). Based on their roles in signal transduction, the TGF-β receptors are subdivided into three classes, type I, II and III (Massague and Wotton, 2000). The transmembrane type I and type II receptors are the major signaling receptors interacting with ligands (Massague and Wotton, 2000).

Both type I and type II receptors are related to each other and are heterodimeric transmembrane kinases most closely related to serine/threonine kinases (Shi and Massague, 2003). There are cysteine-rich regions for ligand binding in the extracellular domain of the N-terminus in both receptors (Shi and Massague, 2003). The kinase domain, an essential factor in TGF-β receptor function, occupies most of the cytoplasmic domain in the C-terminus (Shi and Massague, 2003). Binding of ligand to the extracellular domain of the TGF-β type II receptor leads to a set of conformational changes in the intracellular domain of the receptor, which allows the phosphorylation and subsequent activation of the type I receptor (Massague and Wotton, 2000). Receptor activation triggers downstream TGF-β signaling. SMAD proteins are the primary downstream substrate for the activated type I receptor kinase in the canonical pathway (Massagué and Chen, 2000). R-SMAD is activated through phosphorylation by

an activated type I receptor and translocates into the nucleus to regulate specific gene expression with the assistance of co-SMAD.

Most of these MFS-like disorders can be attributed to heterozygous germline mutations of either the type II (TGFBR2) or type I (TGFBR1) TGF-β receptor genes (Fig. 1). Less commonly found are mutations in other signaling components of TGF-β, such as SMAD3 (Proost et al., 2015; Wooderchak-Donahue et al., 2015), TGFB2 (Lindsay et al., 2012), TGFB3 (Bertoli-Avella et al., 2015a) and the repressor SKI (Schepers et al., 2018). Interestingly, in almost every case described to date, the mutation is a missense mutation suggesting production of a full-length protein.

Substantial experimental evidence suggests that constitutive activation of the TGFβ signaling pathway is at least partly responsible for the vascular abnormalities seen in classic Marfan syndrome (Dietz, 2015; Dietz et al., 2005). However, the story with MFS-like syndromes is much more complicated, with reports of increased or decreased TGF-β signaling, depending on what was assayed and which signaling component was examined (Benke et al., 2013a; Horbelt et al., 2010a). In samples from Loeys-Dietz patients (LDS), aortic tissue showed elevated pSmad2/3 levels, but the mutated receptors showed little intrinsic kinase activity (Mizuguchi et al., 2004), highlighting the paradox. In summary, most researchers agree that the aortic root wall (the major focus of defects in the MFS-like diseases) shows increased TGF-β signaling in human

patients and in mouse models of MFS-like syndromes (Dietz, 2015) with a reduction of kinase signaling potential from the mutated receptors. How the heterozygous loss-of-function of the receptors contributes to increased downstream signaling remains paradoxical (Dietz, 2015; Dietz et al., 2005; Lindsay et al., 2012).

Progress has been made in understanding how these mutations found in MFS-like syndromes affect the function of the TGF-β receptors, but a clear understanding is still missing (Dietz, 2015; Dietz et al., 2005; Holm et al., 2011; Horbelt et al., 2010a; Li et al., 2014; Lindsay et al., 2012; Loeys et al., 2005; Robinson et al., 2006). For most MFS-like receptor mutations that have been examined, no receptor kinase activity has been detected in vitro, and no pSMAD2 stimulation was observed when exposed to ligand (Horbelt et al., 2010a). In these MFS-like mutants, internalization of the receptors was mildly diminished in some mutants, but not significantly distinguishable from wild type (Horbelt et al., 2010a), indicating that synthesis and secretion of the receptors were normal.

We noticed that mutated receptor sequences in MFS-like syndromes overlap extensively with sequences at the C-terminal tails of the type I (NANDOR Box: non-activating and non-down-regulating) and type II (LTA motif comprising of leucine, threonine and alanine) that are needed for receptor trafficking (internalization as well as sub-cellular localization of both receptors) (Garamszegi

et al., 2001a; Murphy et al., 2007).  In addition, Zhou et al. (2004), found a nearby tryptophan located a few amino acids upstream of the NANDOR box that is necessary for the basolateral delivery of the type I receptor, further supporting a role for this region in intracellular receptor trafficking (Zhou et al., 2004). An alignment of the C-terminal tails for type I and II receptors from nematodes to mammals highlights the related NANDOR box and LTA motif, special amino acids and endocytic regions (Fig. 1A). Most of the amino acids in the NANDOR box or the LTA motif are highly conserved across species, and the endocytic regions in both the type I and type II receptors have a significant overlap with each other.

Based on our observation that trafficking motifs in the type I and type II receptors overlap with MFS, MFS-like and some cancer mutations, we hypothesized that some of these phenotypes could be due to mis-trafficking of the TGF-β receptors. Therefore, we sought to test this hypothesis using the model organism *C. elegans* where trafficking studies on TGF-β are well established.

## Material and methods

### Worm strains

All *C. elegans* strains are related to the wild-type Bristol strain N2.  Strains were grown at 20°C on standard nematode growth media plates seeded with OP50 *E. coli*. All nematode manipulations were done according to standard protocols (Brenner, 1974). RNAi experiments used the feeding method (Timmons and Fire,

1998). Constructs for feeding experiments were obtained from the Ahringer library (Kamath et al., 2003). Larval stage L4 animals were treated to RNAi constructs for 24 h and imaged as young adults. The *daf-4* (*e1364*) mutant strain CB1364 was obtained from the CGC, and maintained at 15°C. For a list of strains, please see Table 4.

**Generation of constructs and mutants**

Intestinal expression was achieved using the following construct: *vha-6p::daf-4::gfp::unc-54 3'UTR* obtained from pRG78 (Gleason et al., 2014). The SL2 trans-splicing sequence was obtained from the Hobert lab and the *SV40::tdTomato::NLS* backbone from pCFJ1208 ( pCFJ1208 was a gift from Erik Jorgensen - Addgene plasmid # 44490) . The backbone plasmid pCFJ150 (contains *unc-119* (+), a rescue gene) was digested with SpeI and AflIII. The four fragments were stitched together using the NEB Hi-Fi DNA Assembly Master mix (catalog # E2621S) to generate pJL22.

Hypodermal expression constructs: The hypodermal specific promoter, *elt-3* was amplified from pRG63 (Gleason et al., 2014), *daf-4::gfp::SL2::SV40::tdTomato::NLS::unc54 3'UTR* from pJL22 and the vector backbone was prepared by digestion of pCFJ150 with SpeI and AflIII. A three-fragment assembly was performed using the NEB Hi-Fi DNA Assembly Master mix (catalog # E2621S) to generate the final expression vector.

Selected MFS/MFS-like mutations were introduced into the *daf-4* receptor using the Q5 Site-directed mutagenesis kit from NEB (Catalog # E0554S) and were codon optimized for *C. elegans* based on the optimal codon usage (*C. elegans* II. 2nd edition, Table I).

All low-copy stably transformed strains were obtained through micro-particle bombardment. The protocol used was previously described in (Lee et al., 2005), well as the PDS-1000 / He™ and Hepta™ Systems from Bio-Rad. Macro particles carrier disk (catalog # 1652335), 2,000 psi Rupture Disks (catalog #1652333), 1.0 µm Gold Microcarriers (catalog #1652263) and Hepta Stopping Screens (catalog #1652226) are all from Bio-Rad. All microparticle bombardment was conducted in the *unc-119 (ed3)* mutant background.

For all *pvha-6* and *pelt-3* constructs, 20µg of DNA were used for microparticle bombardment. All the transformed worm strains were outcrossed to N2 at least four times. For a list of nucleotide change in mutagenesis, please see table 3.

**Western blotting and quantification**

Animals were synchronized by alkaline bleaching and arrested at the L1 stage on unseeded NGM plates overnight. Arrested L1s were transferred to seeded plates and grown on standard NGM plates until L4 stage at 20°C. Fifty synchronized L4 stage worms for each strain were placed in 15µl M9 buffer and 15µl NextGel protein sample loading buffer (4x) (VWR, catalog # M260-5.0ML) was added,

flash frozen in liquid nitrogen and stored at -80°C until used for western blotting.

Samples were boiled for 5 min, then centrifuged at 13,000 rpm for 1 min.

Samples were then loaded onto 10% polyacrylamide gels (NEXT GEL, Amresco,

catalog # M256-500MLSG). Samples were run at 100V for 60-90 min. Transfer

was performed onto nitrocellulose membranes using the Bio-Rad Trans-Blot®

Turbo™ Transfer System. Membranes were probed with anti-GFP from Roche

(catalog # 11814460001), polyclonal goat anti-tdTomato from SCIGEN (catalog #

AB8181-200), anti-actin and visualized using the Li-Cor Infrared imaging system.


**Confocal imaging and quantification**

Live worms were mounted on 2% (wt/vol) agarose pads with 0.1% tetramisole

(MP Biomedicals, catalog # 0215211910) in M9 buffer. All confocal imaging was

obtained using the Leica SP5 TCS confocal microscope. A minimum of six

animals were imaged for each condition with a minimum of three biological

replicates. Imaging quantification was performed using the open-source Fiji

Software (Schindelin et al., 2012) on the third or fourth anterior pair of cells for

the measurement of cytoplasmic GFP (cell membrane not included) and nuclear

tdTomato.


**Body length measurement and quantification**

Whole body length measurement was performed by imaging on a standard

epifluorescence microscope with the 5x objective. A minimum of thirty L4 worms

(or L4 + 24 hrs) per condition were imaged with three biological replicates. Whole

worm body lengths from head to tail along the axis were measured using the line tool in Fiji Software.

**Structure model**

The TGFβRII structural model was generated with the Swiss-model online program (https://swissmodel.expasy.org/) using template 5e8v.1.A for the TGFβ type II receptor). Representation of specific amino acids in the structural model is depicted by using Pymol software. Minor conformational conflicts by disease-associated point mutations are shown by Pymol software (Schrodinger, 2015).

| WT | TGGGATCCCGAAGCGTGTGCACGGATTACAGCTGGATGTGCGTTCGCGAGGGTA |
|---|---|
| W580R | CGTGATCCCGAAGCGTGTGCACGGATTACAGCTGGATGTGCGTTCGCGAGGGTA |
| LTA→As | TGGGATCCCGAAGCGTGTGCACGGGCTGCTGCTGCTGCTGCTGCTGCTGCT |
| R587H | TGGGATCCCGAAGCGTGTGCACACATTACAGCTGGATGTGCGTTCGCGAGGGTA |
| R596P | TGGGATCCCGAAGCGTGTGCACGGATTACAGCTGGATGTGCGTTCGCGCCAGTA |

Table 3. List of nucleotide change of mutagenesis in MFS-associated missense mutations.

| Strain Name | Genotype |
|---|---|
| LT1009 | *vha-6p*::*daf-4*(WT)-gfp::sl2::sv40-NLS::tdTomato::nls::*unc-54(3'utr)* |
| LT1010 | *vha6*::*daf4*(W580R)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1011 | *vha6*::*daf4*(LTA→As)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1012 | *vha6*::*daf4*(R587H)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1013 | *vha6*::*daf4*(R596P)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1014 | *vha6*::*daf4*(WT)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1015 | *vha6*::*daf4*(W580R)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1016 | *vha6*::*daf4*(LTA→As)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1017 | *vha6*::*daf4*(R587H)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1018 | *vha6*::*daf4*(R596P)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1041 | *vha6*::*sma6*(WT)::GFP; <br> *vha6*::*daf4*(WT)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1042 | *vha6*::*sma6*(WT)::GFP; <br> *vha6*::*daf4*(W580R)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1043 | *vha6*::*sma6*(WT)::GFP; <br> *vha6*::*daf4*(LTA→As)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1044 | *vha6*::*sma6*(WT)::GFP; <br> *vha6*::*daf4*(R587H)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1045 | *vha6*::*sma6*(WT)::GFP; <br> *vha6*::*daf4*(R596P)::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1019 | *elt3*::*daf4*(WT)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1020 | *elt3*::*daf4*(W580R)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1021 | *elt3*::*daf4*(LTA→As)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1022 | *elt3*::*daf4*(R587H)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1023 | *elt3*::*daf4*(R596P)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |
| LT1046 | *elt3*::*daf4*(K388R)::gfp::sl2::sv40::tdTomato::nls::*unc54(3'utr)* |

Table 4. List of nematode worm strains used in this work

## Results

**Receptor mutations define a critical domain for disease and trafficking**

Patients afflicted with MFS-like diseases generally contain mutations in genes that are components of, or regulate, the TGF-β signaling pathway (Lerner-Ellis et al., 2014; Schepers et al., 2015). A large proportion of these mutations are map to the receptor genes. Three heterozygous missense mutations in the TGF-β type II receptor gene of MFS-like patients were chosen for detailed examination (Fig. 1): W521R (Matyas et al., 2006), R528H (which was identified in 6 out of 10 families with MFS-like syndrome) (Loeys et al., 2005), and R537P. The corresponding positions in the *C. elegans* type II receptor, DAF-4, are W580R, R587H, and R596P respectively.

Additionally, different missense mutations in TGF-β receptors have been identified in human cancer cell lines or tumor specimens (Chen et al., 1998a; Chen et al., 2001; Garrigue-Antar et al., 1995b; Grady et al., 1999; Lu et al., 1998; Lu et al., 1999; Lücke et al., 2001; Nagayama et al., 2002; Nerlich et al., 2003; Schiemann et al., 2004; Wang et al., 2000). As in the case of the MFS-like mutations, many of the cancer-associated mutations in the TGFBR2 gene cluster in the same C-terminal domain (Bierie and Moses, 2006; Kim et al., 2000; Levy and Hill, 2006) (Fig. 1). These mutations occur in the TGFBR2 gene (> 40 mutations) but also in the TGFBR1 gene (4 mutations) (Fig. 1). The MFS-like R528H mutation is also present in type II receptors in colon carcinoma cell lines (Chen et al., 1998b). Likewise, the mutation R537P is mutated in both MFS-like

patients and some cancers (Chen et al., 1995; Horbelt et al., 2010a). The overlap between the mutations present in MFS-like and TGF-β-linked cancers suggests that some of these cancers might also be caused by mis-trafficking of the receptors.

Further mutational analysis of TGF-β receptors shows this C-terminus to be important for signaling. Our previous genetic screens of the *Drosophila* BMP pathways identified eight missense mutations in the type II receptor *Punt* (highlighted in yellow and green in Fig. 1, unpublished data) at positions that were either identical, or adjacent to, residues mutated in MFS-like syndromes and/or some cancers. Additionally, a human mutation associated with brachydactyly maps to the BMP type I receptor in this same C-terminal domain, highlighting that both TGFβ and BMP receptors are susceptible to mutation in this conserved domain (Fig. 1). The mutations described above reside primarily in a motif that overlaps with the LTA motif (residues 529-538 underlined) known to disrupt the normal trafficking of the receptors, often leading to an increase in apical localization (Fig. 1).

We first sought clues to how this C-terminal domain might be involved in trafficking by examining its 3D structure. Using the available structure of the type II TGFβ receptor (PDBID: 5e8V), we observe that the LTA motif of the kinase domain is exposed to the exterior where interactions with other proteins, particularly trafficking regulators, might be expected to occur (Fig 1B). To

investigate how the MFS-like substitutions might interfere with the structure and function of the type II TGF-β receptor, we modeled them in Pymol (Schrodinger, 2015) to better understand the relationship between various residues in the LTA motif and the larger structure of the protein. In the native state of the wild-type type II TGF-β receptor, the residue W521 contributes to a hydrophobic core that stabilizes the pairing of helices 8 and 12. This hydrophobic core is composed of residues W521, L452, P498, W455, I500 and L512. Mutation of W521 to arginine can cause a destabilization of the pairing between helices 8 and 12, with a rearrangement of the other side-chains in the core to compensate for the loss of the hydrophobic moiety of the tryptophan. R528 and R537, in helices 13 and 14, respectively, are involved in long-range electrostatic interactions with oppositely charged residues. R528 forms a salt bridge with E428, in helix 7, and R537 forms a salt bridge with E519, in helix 12. These interactions contribute to stability of the helical bundle in the C-terminal domain of the TGF-β receptor. Mutation of R528H may affect the pairing of helix 13 with helix 7, but due to the limited extent of helix 13 (4 residues), the substitution may have little importance for the stability of the secondary and tertiary structure of the domain or interactions with partner proteins, as the two residues are relatively poorly exposed to the solvent. The effect of mutation R537 to proline, instead, may be important both for the stability of helix 14, since proline is a helix-breaker, and for the pairing of the solvent-exposed helix 14 with helix 12. Moreover, R537 is on the protein surface, and may belong to a binding site for partner proteins.

In summary, the structural modeling suggests that the mutations found in the MFS-like patients would disrupt the structure of a surface domain of the type II TGF-β receptor, perhaps altering its interaction with cytoplasmic trafficking and/or regulatory proteins or the function of the receptor.

A.

B.



C.



| W521R | R528H | R537P |
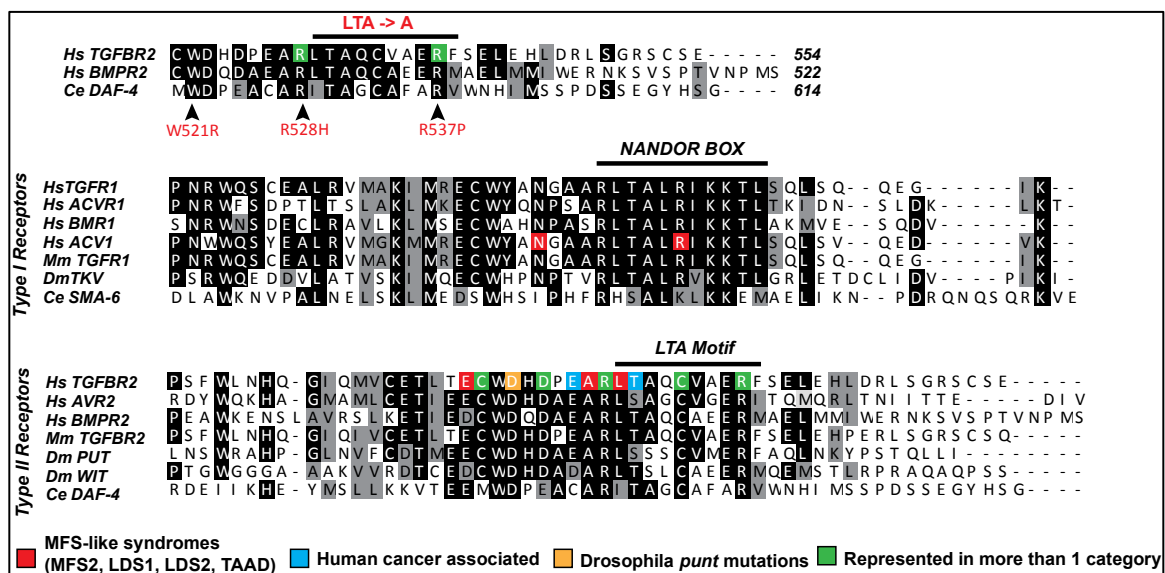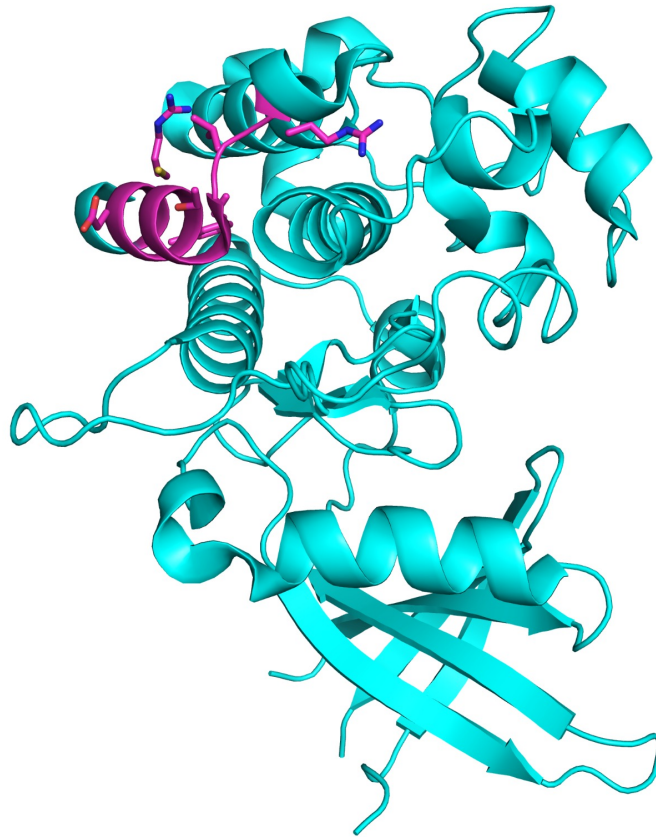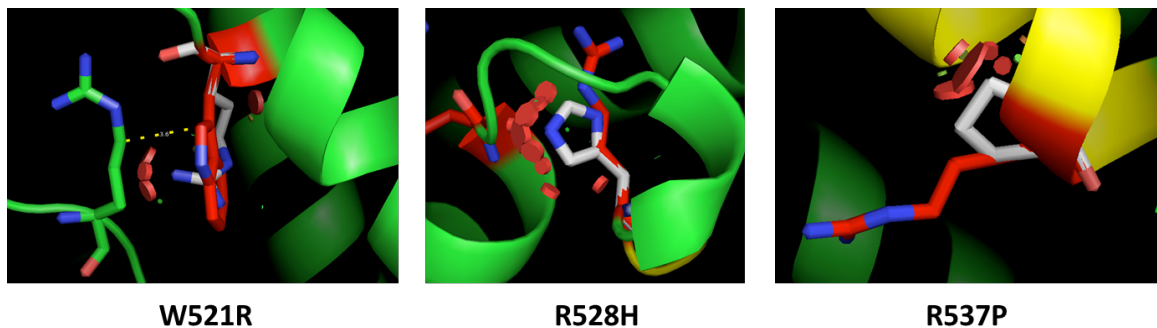
Figure 1. A. <u>Top Panel:</u> Amino acid alignment of the LTA motifs of the human type II TGF-β and BMP receptors with the type II receptor of *C. elegans*. Arrows indicate the three substitutions we have examined in detail in this study and the region where the LTA motif was substituted with alanines. <u>Middle and Lower Panels:</u> Amino acid alignments of the various type I and type II TGF-β receptors highlight the sequence conservation at the NANDOR box and LTA motif respectively. Black indicates identity, grey indicates conserved changes. For both panels, the various colored

boxes identify the residues found changed in the either MFS-like patients, cancers, a *Drosophila Punt* screen (unpublished data), or in multiple categories (red, blue, yellow and green respectively). As observed, all mutations exist as missense mutations in the type II TGF-β receptor. B. The LTA motif (highlighted in magenta onto the X-ray crystal structure of the kinase domain) is exposed to the environment, ripe for interaction with other proteins. Modeling of the MFS-like substitutions suggest changes that might not impact the function of the kinase domain, but rather alter the interactions with other proteins. C. MFS-like substitutions are expected to lead to stearic hindrances as modeled by Pymol.

**Type II TGF-β receptors bearing MFS-like substitutions are functional**

How each of the TGF-β receptor mutations affects receptor activity and downstream signaling has been controversial (Dietz, 2015). Most of these studies are *in vitro*, where the cellular context of TGFβ is removed, particularly those elements involved in receptor trafficking. Whether disease-associated mutations within the type II TGF-β receptors alter signaling strength and output is not clear; some studies have identified that mutations affecting the LTA motif disrupt the kinase activity while others have shown that SMAD phosphorylation is unaffected (Dietz, 2015; Dietz et al., 2005). Most of these residues do not alter SMAD2/3 *in vitro* phosphorylation (Murphy et al., 2007). Of note, two residue substitutions that resulted in a loss of SMAD phosphorylation were alanine substitutions rather than naturally occurring substitutions like those found in MFS-like, which may explain why these two substitutions behaved differently in these assays. Importantly, SMAD phosphorylation was comparable to wild-type levels for mutations at all other residues in the domain (Murphy et al., 2007). It is important to note that although SMAD2/3 phosphorylation levels have been studied for this region, there are no studies that assay signaling strength for alanine substitutions within this region or in the context of a disease-mutation. To identify whether the presence of MFS-like mutations affects function of the

receptors, we took advantage of the fact that TGF-β signaling governs body length in the worm (Estevez et al., 1993; Krishna et al., 1999; Savage-Dunn et al., 2000; Suzuki et al., 1999). Loss of TGF-β signaling (e.g. mutations in the type II receptor) leads to a smaller body length, and body length can be rescued (or enhanced) by transgenic overexpression of signaling components specifically in the hypodermis.

With the advent of CRISPR/Cas9 technology to seamlessly modify endogenous loci (Arribere et al., 2014), we sought to modify the endogenous *daf-4* locus. We received a C-terminal GFP-tagged *daf-4* strain from Dr. Jun Liu at Cornell University (a gift). Unfortunately the fluorescent signal from the GFP-tagged protein was undetectable or very weak so that it would not be useful for imaging purposes (data not shown), a problem that can arise when transcription levels of a gene are low (Roberts et al., 2017). Thus, in order to address the functionality and the localization patterns of the Type II TGF-β receptor, we chose to create transgenic animals that bear integrated low-copy transgenes (Schweinsberg and Grant, 2013) of wild-type or MFS-mutant receptors. The transgenic cassette consisted of an operon of the *daf-4* gene and an *NLS-tdTomato-NLS* separated by a SL-2 trans-splice leader sequence (Hastings, 2005) driven under the control of the hypodermal specific *elt-3* promoter (Figure 2A). The tdTomato acts as an internal control to facilitate normalization for transgene number across different strains. Transgenic animals were generated by microparticle bombardment of the constructs using established methods (Schweinsberg and Grant, 2013). After

selection of lines and outcrossing to our lab wild-type strain, we show that transgene expression levels are comparable across all transgenic lines as determined by tdTomato quantification (Figure 2B) where there was no statistical difference amongst the strains; thus any changes in body size amongst strains is an intrinsic function of the MFS-mutation.

We first show that overexpression of the wild type *daf-4* led to an increase in the body length of the wild type animal, re-iterating that enhanced signaling leads to a longer body length. Expression of a kinase-dead version of the receptor (Chen and Weinberg, 1995) led to a smaller animal (p < 0.0001, Figure 2C). Thus, the presence of a 'poison' receptor in a pool of wild type of *daf-4* receptors leads to a dominant negative phenotype of a small body size, perhaps through competition with the wild type receptor in the heterotetrameric signaling complex of type I and II receptors. Given these two results, we expected one of three different outcomes for the MFS-mutant receptors: 1) If we observe no change in the body length of the transgenic animal, the receptor may be non-functional, 2) If we observe a smaller body size, it could indicate that the mutant receptor behaves in a dominant negative manner with the wild-type receptor, and 3) If we observe a longer body size the receptor may be active and functional.

We first showed that mutating the entire LTA motif with alanines does not disrupt receptor function, as evidenced by the longer body length that requires active TGFβ signaling. Importantly, we have shown that the W580R and R587H

mutants result in functional receptors (Fig 2C) even though their expressed levels are lower than wild type receptors.

However, the presence of the wild type (endogenous) copy of the *daf-4* gene may influence the activity of the transgenic mutant receptors. Hence, we crossed each of our transgenic lines into the *daf-4* (*e1364*) mutant background (Gunther et al., 2000), a null mutant with a small body size phenotype. Hypodermal expression of wild type copies of the transgenic *daf-4* gene in this mutant completely rescues the small body of the *daf-4* (*e1364*) mutant (Fig 2D). We observe that all MFS-mutant transgenes rescue the small body size of the *daf-4* (*e1364*) mutant, albeit to different levels. This suggests that although all the MFS-mutant receptors are functional, there exists variation in their signaling strength. These data strongly suggest that MFS-mutations do not attenuate type II TGF-β receptor activity as assayed *in vivo* in a whole organism context.

A.
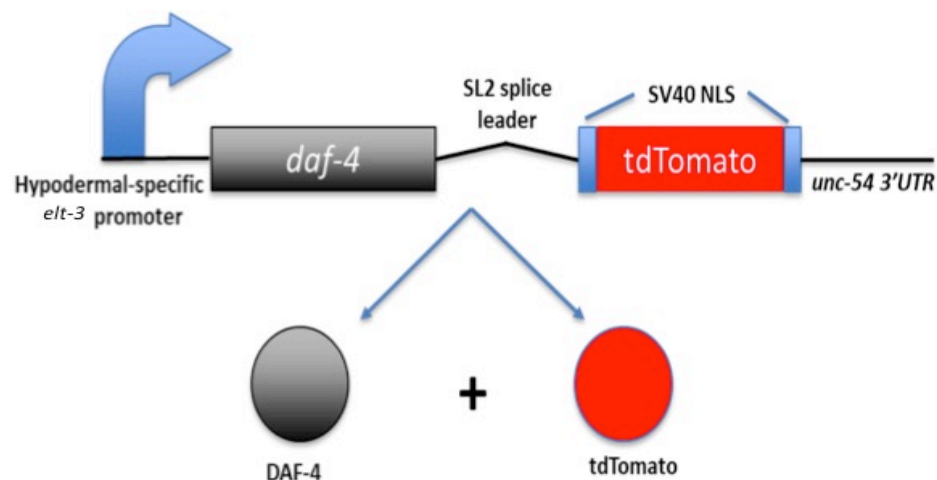
B.
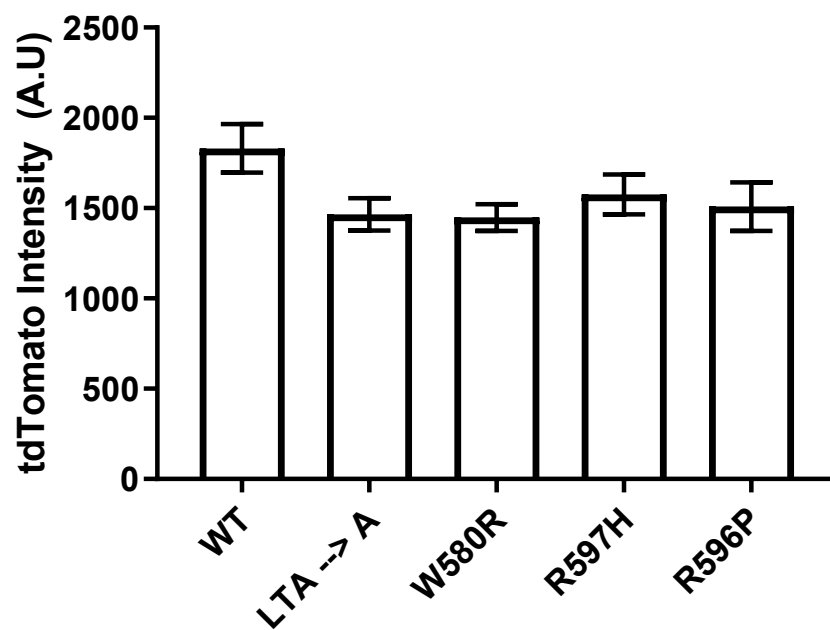
C.
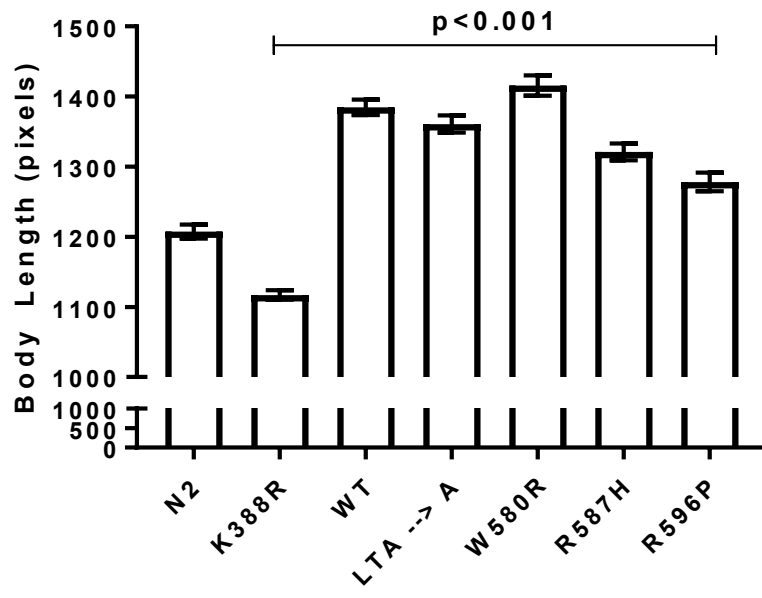
D.
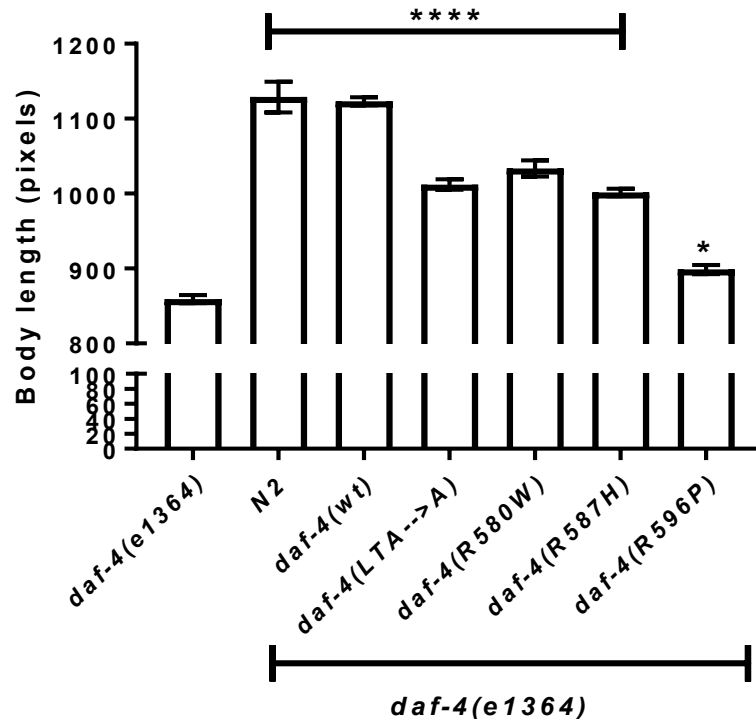


Figure 2. A. Schematic overview of the transgenic construct to determine the functionality of the type II TGF-β receptors bearing MFS-like substitutions. The SL2 splice leader makes it possible for the one-to-one expression of the DAF-4 and *NLS-tdTomato-NLS* cassette. This allows quantification of the levels of transcription from the hypodermal specific promoter *elt-3*. (B). Transgenic expression of the *daf-4*::GFP (wild-type or MFS-mutant) was normalized by the operonic expression of a tdTomato transgene. As observed, there is no statistical difference between the tdTomato intensity amongst the strains showing that the transgenes are expressed at similar levels between the strains. Thus, the variation in body size can be explained by the intrinsic property of the various MFS-mutations. C., D. Hypodermal specific transgenic expression of type II TGF-β receptors bearing either the LTA → A substitutions or the MFS-like mutations are capable of increasing body length in wild type animals (C), or fully rescuing the body size of *daf-4* (*e1364*) mutant animals (D). K338R is a substitution mutation that generates a kinase-dead receptor. Graph shows the mean body lengths values +/- S.E.M. Statistical comparisons were performed using a One-way ANOVA with Dunnett's correction for multiple comparisons against wild type or *daf-4* (*e1364*) strains. These data suggest that MFS-like mutations do not abrogate receptor function. A minimum of 30 animals were measured for each genotype.

## LTA motif and disease-associated mutations lead to reduced levels of type II TGF-β receptors

To visualize levels and localization of the mutant receptors *in vivo*, we expressed

them in the *C. elegans* intestine. The polarized epithelial cells of the intestine are

a well-characterized system for the study of receptor trafficking as well as a tissue in which TGF-β signaling is required and active (Gleason et al., 2014; Gunther et al., 2000; Sato et al., 2005). To this end, we developed a vector that contains an operon consisting of a C-terminal GFP-tagged *daf-4* receptor (wild-type and point mutants) and an *NLS-tdTomato-NLS* separated by a SL-2 trans-splice leader sequence (Hastings, 2005), expressed from the intestine specific *vha-6* promoter. Upon transcription and splicing, the result is a single GFP-tagged receptor and a single tdTomato molecule containing dual nuclear localization signals (Fig 3A). The GFP-tagged receptors allow for easy visualization of trafficking while the tdTomato acts as an internal control to detect subtle changes in the receptor expression levels as well as normalization for transgene number across different strains. This construct was integrated at a low-copy number into the worm using microparticle bombardment (Praitis, 2006; Praitis et al., 2001; Schweinsberg and Grant, 2013).

Confocal imaging (horizontal section of the worm intestine; white arrow represents basolateral surfaces while red arrow indicates apical or lumenal surfaces) and western blot analyses revealed that changes in the LTA motif, either substituting the motif with alanines or with MFS-like mutations, resulted in significantly decreased levels of receptors present in the cell (Fig 3B, C). To rule out the possibility that the reduced levels of mutant type II receptor DAF-4 were due to any aberrant synthesis and/or transport to the plasma membrane of the receptors, we examined the accumulation of DAF-4 at the plasma membrane.

This was done by knocking down *dpy-23*, which is the μ-2 adaptin member of the AP2 complex. *Dpy-23* is needed for clathrin-dependent endocytosis of various transmembrane receptors, including BMP/TGFβ (de Leon and Valdivieso, 2016; Gleason et al., 2014; Pan et al., 2008). Loss of *dpy-23* results in accumulation of the type I receptor SMA-6 at the plasma membrane (loss of internalization) and inhibition of BMP signaling (Timmons and Fire, 1998), and we used this particular assay as our control to determine efficacy of the RNAi knockdown of *dpy-23*. As previously observed, knockdown of *dpy-23* did not significantly impact the accumulation or levels of DAF-4 receptors, suggesting that biosynthesis and transport to the plasma membrane are likely not impaired by these mutations (Fig 3E).

Lysosomes are the degradative endpoints of most cell surface receptors (Bakker et al., 2017; Conner, 2016; Itoh and Itoh, 2018). Given that MFS-mutant receptors are present at lower levels within the cell, it is possible that the MFS-mutant receptors exhibit altered cellular kinetics and are degraded within the lysosome at increased rates. To examine this possibility, we performed a knockdown of *cup-5*, a gene necessary for lysosome function in *C. elegans* whose knockdown leads to dysfunctional lysosomes. Proteins that are normally degraded are expected to accumulate intracellularly in a *cup-5* mutant or knockdown background. We have previously shown that the loss of the retromer subunit, *vps-35,* leads to a premature degradation of the type I receptor SMA-6 within the lysosome and that this can be reversed by RNAi mediated knockdown

of *cup-5* (Gleason et al., 2014). We hypothesized that if the mutant DAF-4

receptors were being degraded in the lysosome, we would observe an

accumulation if the lysosome function was blocked. However, there was no

change in the receptor levels upon *cup-5* RNAi, suggesting that the MFS-like

mutations did not lead to an increased rate of degradation within the lysosome

(Fig 3F).

Taken together, the data from the *dpy-23* and *cup-5* RNAi experiments suggest

that the lowered levels of the MFS-mutant type II receptor are not a result of

defective synthesis, transport to the plasma membrane or degradation of the

receptor in the lysosomes.

A.

B.



| WT | W580R | LTA→As | R587H | R596P |
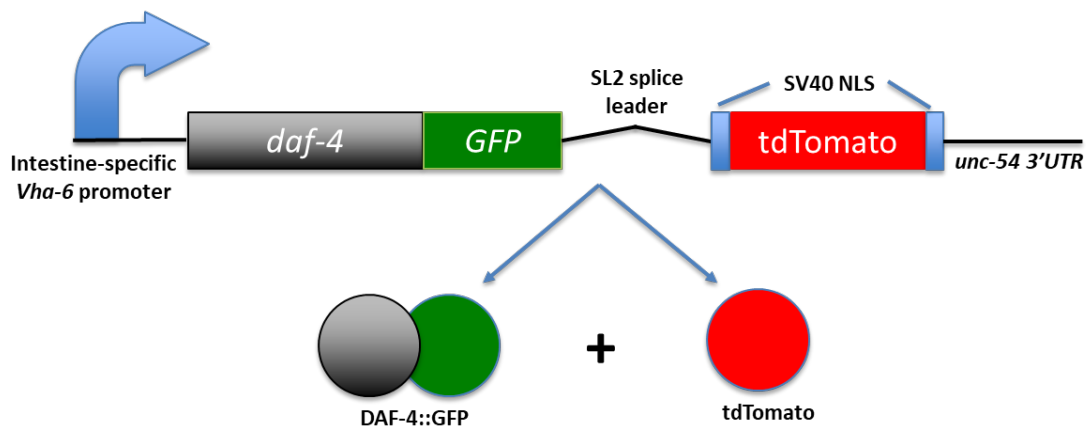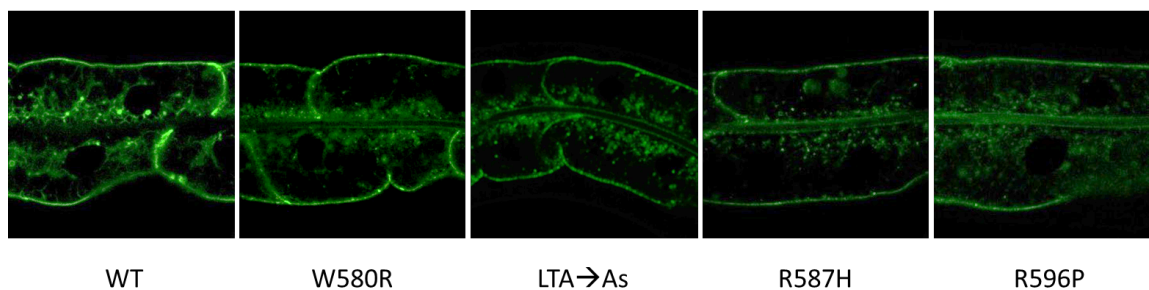
**Receptor levels ( Middle plane )**



** p < 0.05
*** p < 0.01
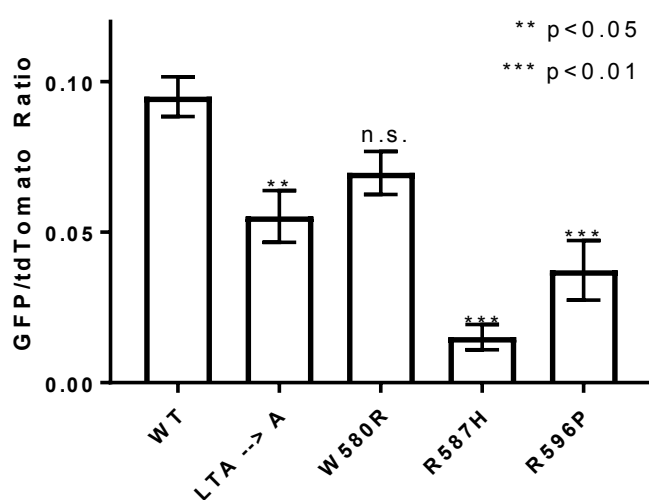
C.
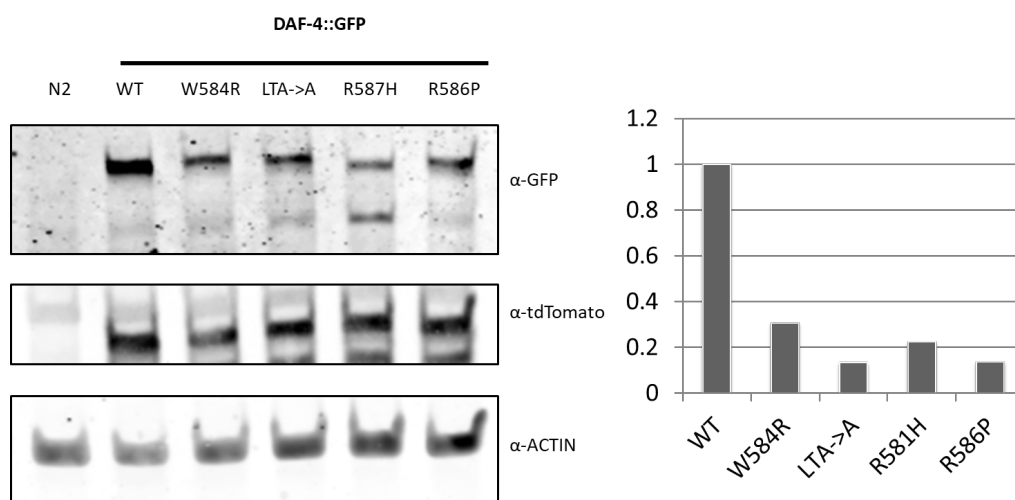
D.
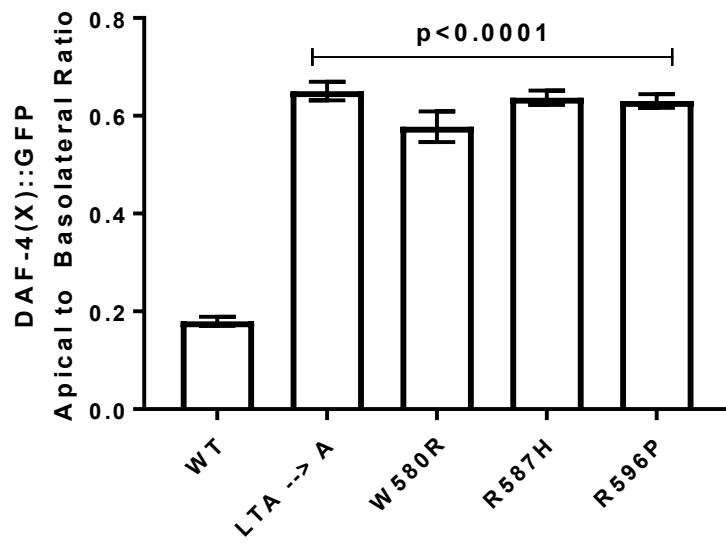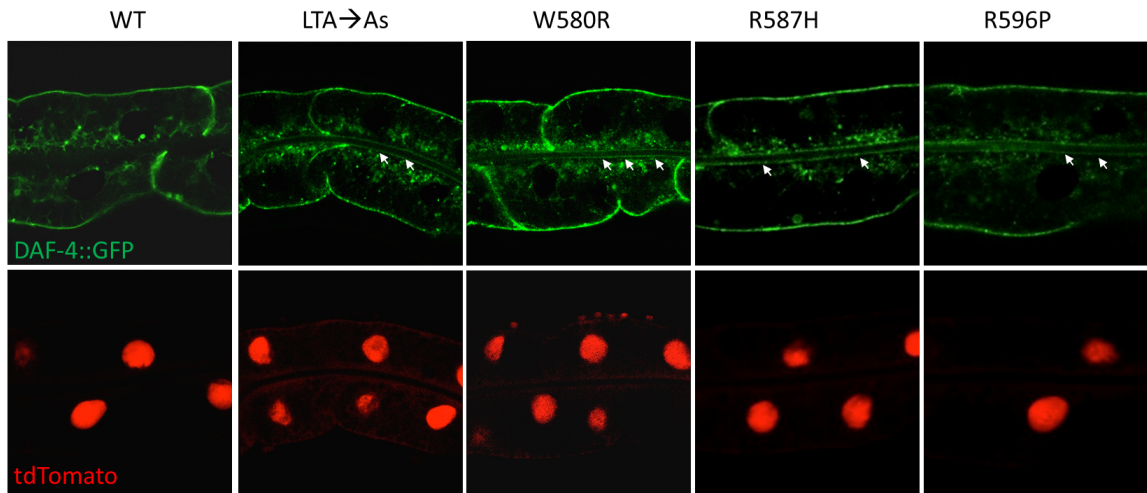
E.
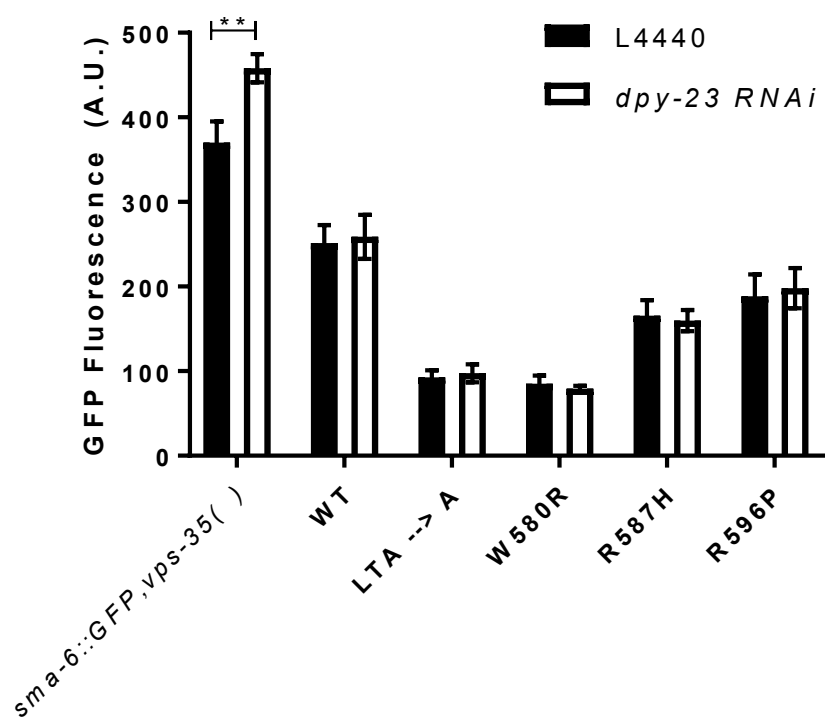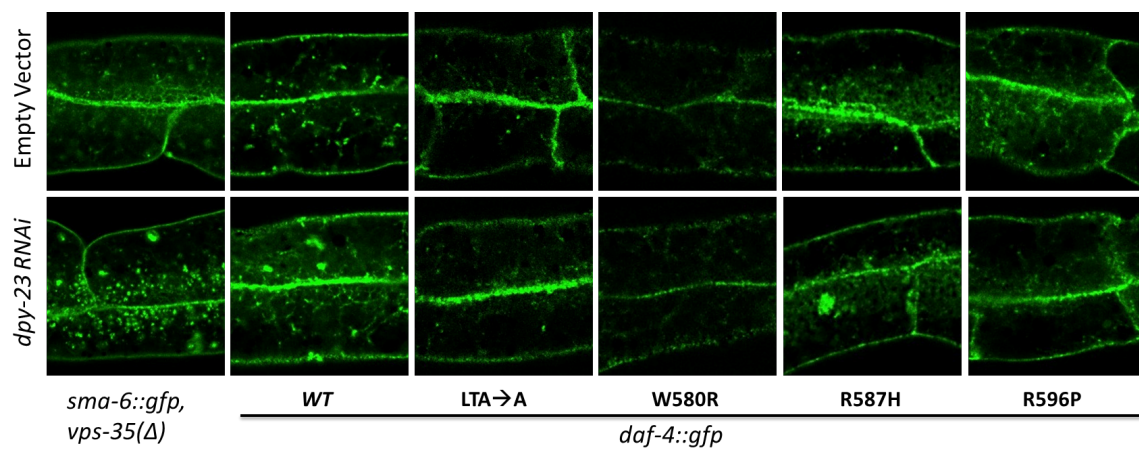
F.
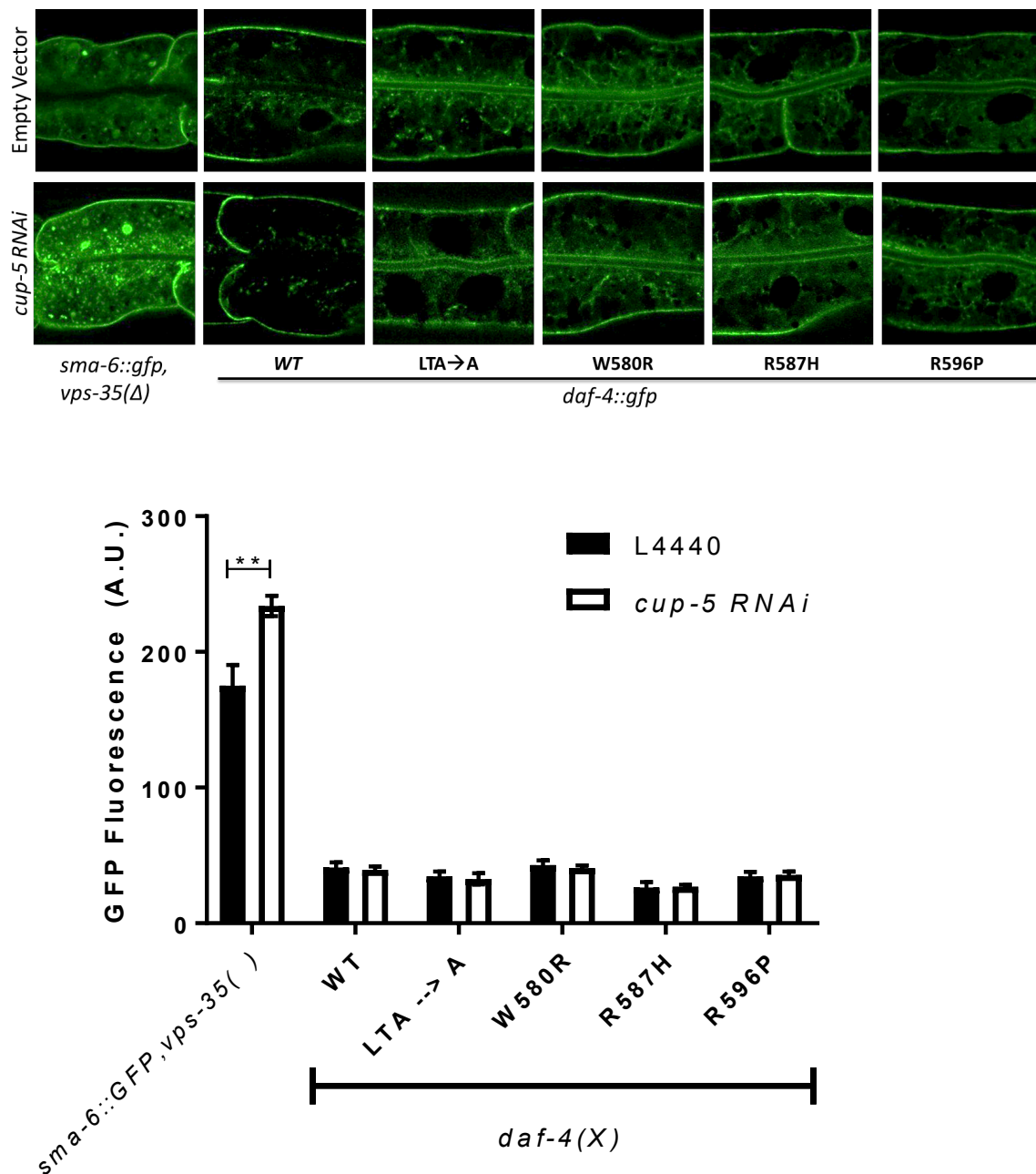


Figure 3. A. Schematic overview of the transgene construct to study cellular levels and localization patterns of type II TGF-β receptors bearing MFS-like mutations. The SL2 splice leader makes it possible for the one-to-one expression of the NLS-tdTomato-NLS cassette. This allows precise quantification of the levels of the GFP-tagged DAF-4 receptor. The coding sequences are expressed by the intestine specific *Vha-6* promoter. B., C. The LTA → A and MFS-like mutations lead to decreased DAF-4 receptors intracellularly as determined by confocal microscopy (B) and western blot (C). At least six animals were imaged for confocal imaging, and quantification of fluorescence intensity was carried out for intracellular abundance. Graphs indicate mean intensity +/- S.E.M. Statistical comparisons were performed using a One-way

ANOVA with Dunnett's correction for multiple comparisons against the wild-type strain. Western blot shown is a representative blot from at least three different biological replicates. D. Overexposure of the confocal images reveals that the low levels of mutant receptor present intracellularly are mis-trafficked to the apical surface of the intestine (white arrows) as compared to the basolateral surface. Quantification of the ratio of the apical to basolateral surface is shown in the attached graph. These data indicate that the LTA motif is required for the proper cellular distribution of the DAF-4 receptor and that the MFS-like mutations may disrupt interactions that could affect cellular distribution. Graphs indicate mean intensity +/- S.E.M. Statistical comparisons were performed using a One-way ANOVA with Dunnett's correction for multiple comparisons against the wild-type strain. E. RNAi mediated knockdown of *dpy-23* does not alter DAF-4 (mutX) levels. F. RNAi mediated knockdown of *cup-5* to reduce lysosomal function does not increase levels of DAF-4 (mutX) suggesting that the decreased levels of MFS-like mutant DAF-4 receptors are not as a result of degradation within the lysosome.

**Subcellular localization patterns are altered by MFS-mutations**

Although total receptor levels were reduced, we observed that the mutant receptors remaining within the cell displayed a drastically different localization pattern compared to the wild type receptors (Fig 3D). Images have been overexposed to easily identify localization changes; quantification is based on unmodified exposures. As we have previously observed (Gleason et al., 2014), the wild-type TGF-β type II receptors are located on basolateral membranes, and the ones recycled inside the cell present a net-like distribution (Fig 3D). Alteration of the LTA motif or introduction of the MFS-mutations leads to the loss of the net-like distribution and a significantly increased localization apical surface (Fig 3D). Structure-function of truncation mutants and alanine substitutions in the LTA motif have been shown to be important for localization to the apical and basolateral surfaces in polarized MDCK cell lines (Murphy et al., 2007). Our *C. elegans* model show that MFS-like substitutions found in patients, rather than alanine substitutions, result in altered trafficking of the type II TGF-β receptor from the basolateral surfaces to the apical surface of the cell.

**Mutant TGF-β type II receptors alter the level and trafficking of the type I receptor**

Signaling requires a heterotetrameric complex of type II and type I receptors; the

receptors must overlap spatially for the complexes to form upon ligand binding to

the type II receptor. Upon internalization, the two receptors separate at some

point and recycle back to the surface via distinct mechanisms, with the type I

receptor recycling via the retromer and the type II receptor utilizing an *arf-6*

mediated recycling pathway (Gleason et al., 2014).

We have shown that the presence of MFS-like mutations in the type II receptor

leads to the shift in localization from the basolateral surface to the apical surface

of the intestine (Fig 3D). The increase in body size in the wild-type background

and rescue of the small body phenotype of the *daf-4* (*e1364*) mutant with the

MFS-like mutant receptors, strongly suggests that activity is not diminished with

these mutations (Fig 2). Given the new localization pattern of the DAF-4 (MFS)

receptors and that SMA-6 would be required at the same locations, we asked

what the effect of the MFS-like mutations were on the cell biology of the type I

receptor, SMA-6. We tested the levels and localization of a functional *sma-6::gfp*

transgene within the intestine in the presence of either wild-type or MFS-like

mutant *daf-4* receptors (untagged). We observe a significant decrease in overall

levels of the type I receptor within the cell when co-expressed with the mutant

DAF-4 receptors (Fig 4A). The type I BMP receptor is known to recycle through a

retromer-mediated mechanism, and a loss of this retromer-receptor interaction

leads to degradation of the type I receptor in the lysosome. To identify whether

the type I receptor was indeed being degraded in the lysosome, we hypothesized

that perturbation of lysosome function would lead to a recovery of GFP-tagged

receptors that are normally degraded within the lysosome. An RNAi of *cup-5* led

to the restoration of SMA-6::GFP signal indicating that the presence of MFS-

mutant type II receptors leads to abnormal degradation of the type I receptor (Fig

4B).

In the presence of the wild-type DAF-4 receptor, SMA-6::GFP localizes primarily

to the basolateral surfaces of the intestinal cells (Fig 4C), as has previously been

shown (Gleason et al., 2014). In stark contrast, the presence of the mutant DAF-

4 receptors leads to a change in localization from the basolateral surface to the

apical surface, similar to what we observe for the mutant DAF-4 receptors

themselves i.e mislocalization proximal to the apical surfaces (Fig 3). The change

in localization of SMA-6 in the presence of the mutant DAF-4 receptors suggests

that the receptors are dependent on each other for trafficking. Whether this

dependency occurs after internalization and separation during recycling or soon

after biosynthesis and transport to the plasma membrane needs to be further

examined.

A.

B.



Effect of overexpression of MFS mutants on sma-6::GFP levels
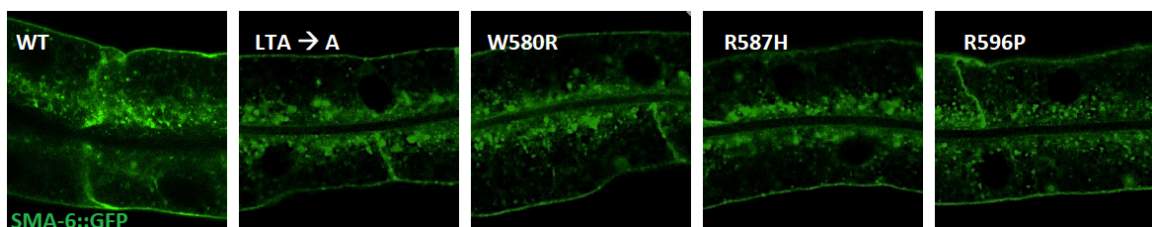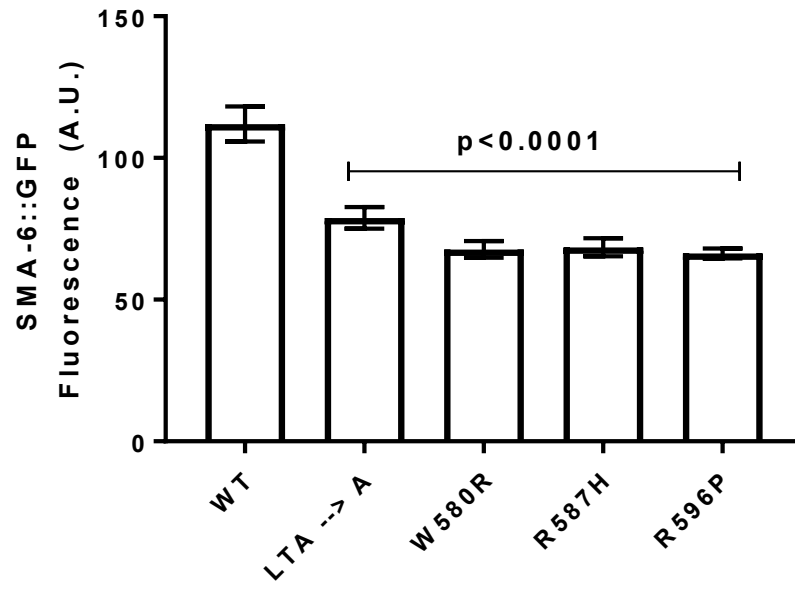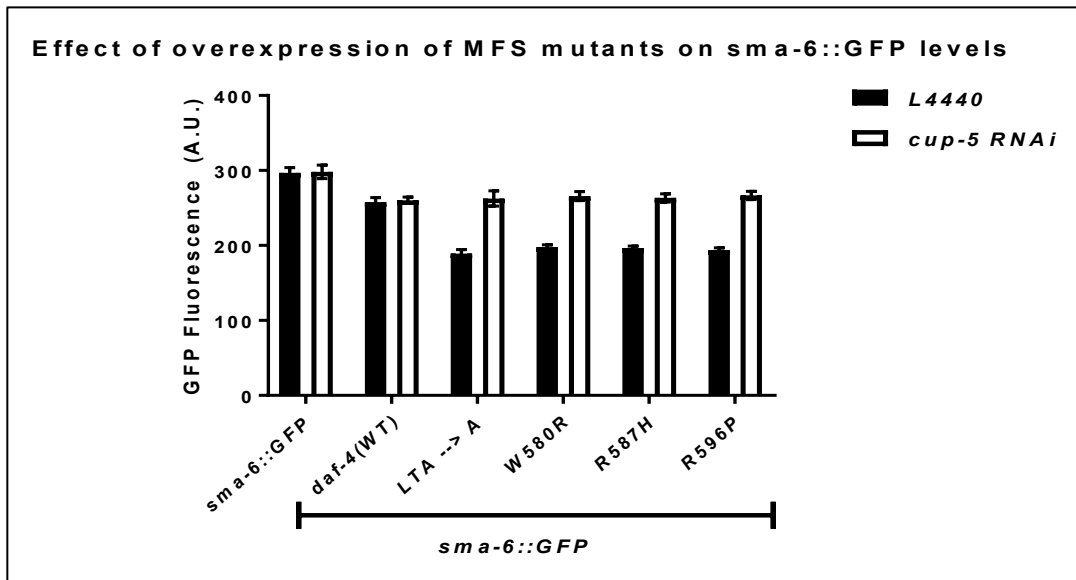
C.
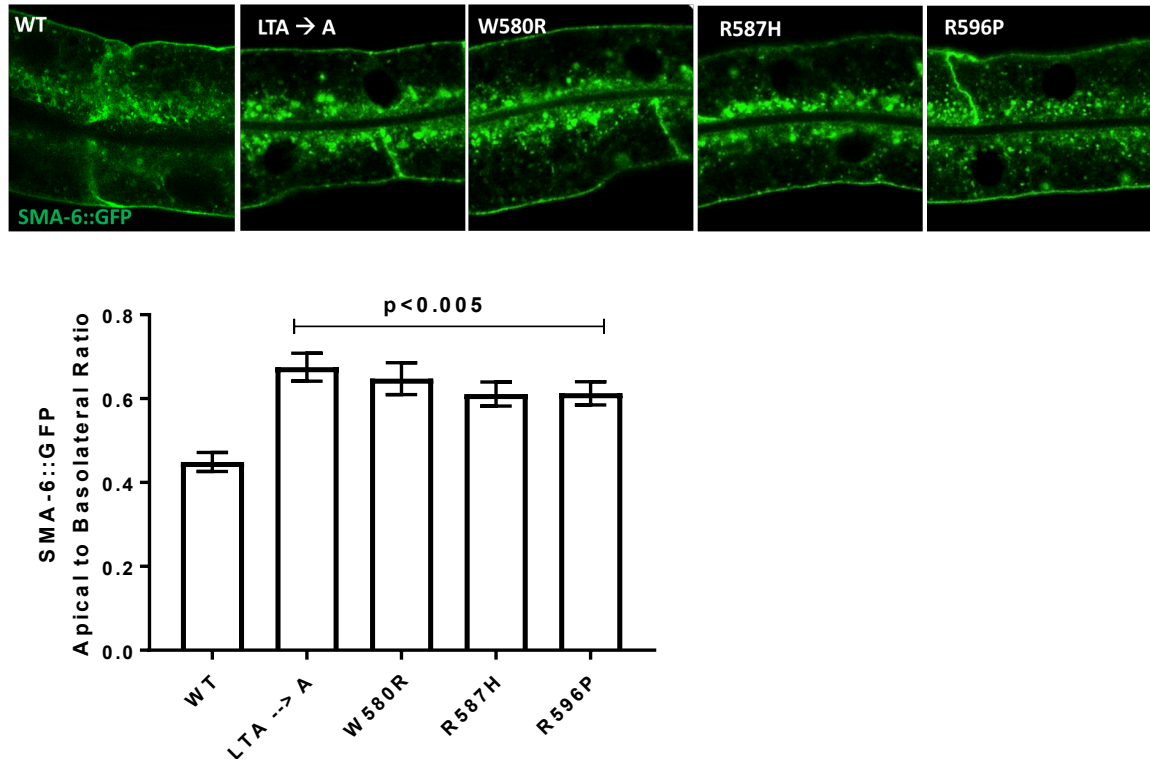




Figure 4. A. Type I TGFβ receptor levels are lowered in the presence of MFS-like mutant type II TGFβ receptors. The type I receptor SMA-6 was GFP tagged and expressed in the intestine along with a wild-type or MFS-like mutant type II receptor. Confocal imaging revealed that levels of the type I receptor are significantly reduced when mutant DAF-4 receptors are also present. B. SMA-6 degraded in the presence of the MFS-like type II receptor is restored by inhibition of the lysosome function by knockdown of *cup-5* RNAi. C. Overexposure of the confocal images reveals that the presence of mutant type II receptors also alters the intracellular distribution of the type I receptors from the basolateral surfaces to the apical surface of the intestine.

## Discussion

**MFS-like mutations have minimal effect on receptor function**

A hallmark of Marfan and MFS-like syndromes is an increase in TGF-β signaling. The prevalence of MFS-like mutations affecting the LTA motif of type II receptors, which lies within the kinase domain, can potentially lead to defective kinase activity. *In vitro* structure function assays determining signal output through levels of pSMAD or reporter genes are controversial – some studies show lowered pSMAD activation while others observed no change in pSMAD levels (Dietz, 2015; Dietz et al., 2005; Lindsay et al., 2012). In fibroblasts from a TGF-β kinase-deficient mouse, increased signaling was unexpectedly seen in assays of pSMAD2 (Denton et al., 2003). Another study showed evidence that mutant TGF-β receptors maintain kinase activity and that phosphorylated R-SMADs can be detected (Lehmann et al., 2003a). In MFS-like patients, it has been demonstrated that specific missense mutations screened within TGF-β receptors caused reduced acute responsiveness to ligand, but long-term signal transduction was not disrupted (Loeys et al., 2005). More recently, in another mouse model containing a MFS-like mutation engineered into the TGF-β type II receptor gene, impaired receptor signaling was observed in osteoblasts, but cultured osteoblasts had increased differentiation markers compared to wild type (Denton et al., 2003). The authors attribute these results to possible deficiencies in modeling the disease *in vitro*.

In our study, we created an *in vivo* model of MFS-like syndromes by introducing

human MFS-like mutations, and not alanine substitutions, into the *C. elegans*

type II TGF-β receptor and studying consequences in a whole animal context.

Our models clearly show that the type II receptors bearing MFS-like mutations

are functional and lead to excess signaling as determined by a body size assay.

The increase in body size in a wild-type background resembles the dominant

nature of these mutations in MFS-like patients.

## MFS-like mutations alter type II receptor trafficking and, indirectly, type I receptor trafficking

The LTA-domain is required for sorting of the type II TGF-β receptor to the

basolateral surface and acts in a dominant manner. When transferred to an

apically localized influenza haemagglutinin (HA) antigen, LTA motif directs a

dominant mis-localization to the basolateral surface (Murphy et al., 2007). While

several studies have examined important aspects of receptor function in MFS-

like diseases, the intracellular trafficking of the mutant receptors has not been

examined, particularly in the context of a whole animal. Given the coincidence of

these disease substitutions with an experimentally identified domain that is

required for receptor trafficking, examination of intracellular trafficking was

warranted, and likely contributes to the phenotype.

Our *in vivo* assays found that MFS-like associated mutations in or around the

defined LTA motif in the type II receptor affect the trafficking of *both* TGF-β

receptors, although the effect on the type I receptor would be indirect. Wild type

type II receptors tend to be distributed in a web-like manner throughout the cell, but do not have an appreciable apical localization (Fig 3B, D). However, this apical-to-basolateral distribution ratio is altered in the presence of mutant receptors, with a fraction of the mutant receptors now present at the apical surface. It is interesting to note that not all mutant receptors are apically localized. Since signaling complexes exist as heterotetrameric complexes, the composition of the complex might influence its trafficking route – a signaling complex made up of all mutant receptors might be apically localized while a complex containing a wild type and a mutant receptor might be basolateral or 'wild type' in its localization pattern.

**Altered trafficking of the MFS-like TGF-β type II receptor might localize it to areas with higher concentrations of ligand**

The TGF-β signaling complex is tetrameric in nature (Massague, 2008), and altered trafficking of a single receptor type might lead the entire complex to mis-localize to a location that affects signaling strength. This is one way to envision how trafficking might affect signaling, although other possibilities exist. Spatial regulation of ligands and receptors is common in the TGF-β family (Ramel and Hill, 2012). In humans, TGF-β receptors are directed to basolateral membranes while TGF-β ligands are secreted at the apical surface of polarized cells (Mitchell et al., 2004). Loss of the C-terminus LTA motif results in apically localized type II receptors, and apically targeted signaling complexes were fully functional as assayed by SMAD3 phosphorylation (Yin et al., 2017), suggesting that all requirements for signaling exist at the apical surface. Because the MFS-like

mutations alter trafficking patterns, it is possible that the misdirected receptor

complex is presented with a higher concentration of ligand at the apical surface.

Receptor trafficking dynamics might also be altered at the apical surface such

that the complex is retained for a time, allowing more signaling to occur before

internalization and separation prior to recycling. Because our images are

snapshots, it is not possible for us to identify whether receptor dynamics are

altered at the surface. Does the LTA motif first direct an apical transit before

basolateral sorting? If so, this would suggest that alterations to the motif might

affect 'transit time' through the apical boundary before final delivery to the

basolateral surface.

Although the LTA motif is known to direct trafficking, the molecules that interact

with it are currently unknown. We have previously shown that the *C. elegans* type

I receptor requires the retromer for recycling back to the surface while the type II

receptor appears to require the small GTPase *arf-6,* whose loss leads to intra-

endosomal accumulation of the type II receptor DAF-4 (Gleason et al., 2014).

However, loss of the retromer leads to redistribution from basolateral to a

combination of basolateral and apical localization of the type II receptor in MD-1

cells (Yin et al., 2013), suggesting that receptors that were basolaterally localized

have now been resorted to the apical membrane. Furthermore, loss of the

retromer also reduced recycling by 50-60% in MD-1 cells (Yin et al., 2013). The

type I receptor requires the retromer for recycling, and perhaps this interaction

can only take place in the context of the type II receptor LTA-motif-retromer

interaction (Yin et al., 2013). The presence of MFS-like mutations might interfere with this type I-type II-retromer complex and alter separation and recycling dynamics, thereby transiently increasing SMAD phosphorylation. The inability of the type I receptor to then interact with the retromer would lead to its sorting into the lysosome for degradation, as we have previously shown. This is consistent with our data showing that the levels of the wild type type I receptor are significantly reduced in the presence of the MFS-like mutant receptors (Fig. 4A). Until new data are collected, other molecular scenarios may also be envisioned - MFS-mutations may disrupt a type II receptor-retromer interaction and lead to a redistribution of signaling complexes to the apical surface. Interestingly, many of these same mutations are also present in many cancer cell lines and tumor specimens (Fig 1A). It seems likely that some of these mutations also affect trafficking of the receptors.

Another recent study showed that in MFS patients, the TGF-β regulator SARA (SMAD anchor for Receptor Activation) and SMAD2 is enriched within membrane fractions and leads to increased receptor interaction (Siegert et al., 2018). Importantly, the interaction of these mediators was significantly higher with the type II receptor. They further show that the TGF-β ligand has increased colocalization with SARA and EEA1 (an early endosome marker), which might lead to increased signaling. It would be very interesting to know whether the type II receptors in these patients contain mutations affecting their LTA domains.

Our *C. elegans* models of MFS-like syndromes offer a novel paradigm of MFS-like syndromes connecting receptor trafficking to disease. The mutations we have examined may provide possible therapeutic avenues; importantly some of these mutations are also found in cancers. If mutations are linked to trafficking defects in MFS-like syndromes then perhaps they share similar etiologies in other TGF-β receptor-induced cancers.

# Chapter III: General discussion and future plans

TGF-β signaling depends on its type I and type II receptors, which are both serine/threonine kinases, to transmit its signals. The kinase domain of the receptors is the most important domain of the TGF-β receptor. The binding of the TGF-β ligand to type I receptor activates the recruitment of type II receptor, which leads to the formation of ligand-receptor complex. Both type I and type II receptors are activated via trans- as well as auto-phosphorylation. This process is followed by the activation of downstream signal transducers to facilitate signal transduction.

Many TGF-β associated diseases are caused by mutations in TGF-β components. A majority of these mutations are located in TGF-β receptors, such as those found in Marfan syndrome (MFS) and the related MFS-like syndromes. In most cases, MFS and MFS-like are heritable heterozygous diseases and are caused by missense mutations in TGF-β receptors, especially those affecting the cytoplasmic domain. Sequence alignment showed that there is an overlap between disease-associated mutations and endocytosis motifs in TGF-β receptors. As a result, it is highly possible that these mutations may change the trafficking of receptors inside the cell. The location of these mutations have suggested that these mutations may inactivate the kinase activity of TGF-β receptors. It is reported that disease-associated point mutations could eliminate the kinase activity of TGF-β type II receptor in *in vitro* kinase assays (Horbelt et al., 2010a). However, our body length assay demonstrated that the mutant

receptors still retain kinase activity and suggest that mutations may confer some special characteristics on TGF-β receptors and signaling. In addition, the downstream activated signal transducer pSMAD is still detected in the fibroblast samples taken from MFS patients (Loeys et al., 2005) suggesting receptor activity.

Both TGF-β type I and type II receptors are dimers, and hence the receptor complex formed by type I and type II receptors is a heterotetromer. MFS and MFS-like syndromes are mostly heterozygous in patients, which indicates that there are wild-type receptors functioning in the ligand-receptor complex of an MFS/MFS-like patient. In this way, even if the mutation paralyzes the kinase activity of the receptor, one copy of wild-type receptor still maintains the downstream signaling activity. So it is important to test the kinase activity of mutant TGF-β receptors in a living system, and our *C. elegans* system showed that not only TGF-β type II receptor is still functioning in MFS background, but also the trafficking of mutant receptor is changed. Further support for my work comes from a recent study (Zhang, 2018), which showed that basolateral localization of BMP receptors is necessary for BMP signaling gradients in the early mouse embryo. Mislocalization of receptors is found when the LTA motif is mutated, resulting in ectopic BMP signaling.

Considering that type I and type II receptors cooperate with each other to conduct signaling activity, it is highly possible that the trafficking route and protein

level of wild type I receptor can be affected by the mutant type II receptor. Again,

our *C. elegans* system showed that trafficking route of some type I receptor is

changed in mutant type II receptor background, and the protein level reduced

too. This indicated that there is some interconnection between TGF-β type I and

type II receptors in receptor endocytosis as well as protein degradation. Previous

work done by our lab showed that in *C. elegans* type I receptor would interact

with retromer during endocytosis while type II receptor didn't show evidence that

it interacts with retromer (Gleason et al., 2014). The data from my work

suggested that although the endocytosis of type II receptor is independent of

retromer, it is involved in the interaction between type I receptor and retromer

through some mechanism. Further studies are warranted to identify the

molecular mechanism by which mislocalization of the receptors results in altered

signaling strength, which may lead to therapeutic approaches for Marfan-like

syndromes and select sets of cancers.

# References

Ageta, H., Tsuchida, K., 2011. Multifunctional roles of activins in the brain. Vitam Horm 85, 185-206.

Allen, L.A., Aderem, A., 1996. Mechanisms of phagocytosis. Curr Opin Immunol 8, 36-40.

Anders, R.A., Arline, S.L., Doré, J.J., Leof, E.B., 1997. Distinct endocytic responses of heteromeric and homomeric transforming growth factor β receptors. Mol Biol Cell 8, 2133-2143.

Anders, R.A., Gustafson, M., Edens, M., Limper, A.H., Leof, E.B., 1996. Pneumocystis carinii modulates cyclin-dependant kinase activity in a lung epithelial cell line. J Eukaryot Microbiol 43, 13S.

Arribere, J.A., Bell, R.T., Fu, B.X., Artiles, K.L., Hartman, P.S., Fire, A.Z., 2014. Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. Genetics 198, 837-846.

Bakker, J., Spits, M., Neefjes, J., Berlin, I., 2017. The EGFR odyssey - from activation to destruction in space and time. J Cell Sci 130, 4087-4096.

Benke, K., Agg, B., Szilveszter, B., Tarr, F., Nagy, Z.B., Polos, M., Daroczi, L., Merkely, B., Szabolcs, Z., 2013a. The role of transforming growth factor-β in Marfan syndrome. Cardiol J 20, 227-234.

Benke, K., Agg, B., Szilveszter, B., Tarr, F., Nagy, Z.B., Polos, M., Daroczi, L., Merkely, B., Szabolcs, Z., 2013b. The role of transforming growth factor-β in Marfan syndrome. Cardiol J 20, 227-234.

Bertoli-Avella, A.M., Gillis, E., Morisaki, H., Verhagen, J.M.A., de Graaf, B.M., van de Beek, G., Gallo, E., Kruithof, B.P.T., Venselaar, H., Myers, L.A., Laga, S., Doyle, A.J., Oswald, G., van Cappellen, G.W.A., Yamanaka, I., van der Helm, R.M., Beverloo, B., de Klein, A., Pardo, L., Lammens, M., Evers, C., Devriendt, K., Dumoulein, M., Timmermans, J., Bruggenwirth, H.T., Verheijen, F., Rodrigus, I., Baynam, G., Kempers, M., Saenen, J., Van Craenenbroeck, E.M., Minatoya, K., Matsukawa, R., Tsukube, T., Kubo, N., Hofstra, R., Goumans, M.-J., Bekkers, J.A., Roos-Hesselink, J.W., van de Laar, I.M.B.H., Dietz, H.C., Van Laer, L., Morisaki, T., Wessels, M.W., Loeys, B.L., 2015a. Mutations in a TGF-β ligand, TGFB3, cause syndromic aortic aneurysms and dissections. J. Am. Coll. Cardiol. 65, 1324-1336.

Bertoli-Avella, A.M., Gillis, E., Morisaki, H., Verhagen, J.M.A., de Graaf, B.M., van de Beek, G., Gallo, E., Kruithof, B.P.T., Venselaar, H., Myers, L.A., Laga, S., Doyle, A.J., Oswald, G., van Cappellen, G.W.A., Yamanaka, I., van der Helm, R.M., Beverloo, B., de Klein, A., Pardo, L., Lammens, M., Evers, C., Devriendt, K., Dumoulein, M., Timmermans, J., Bruggenwirth, H.T., Verheijen, F., Rodrigus, I., Baynam, G., Kempers, M., Saenen, J., Van Craenenbroeck, E.M., Minatoya, K., Matsukawa, R., Tsukube, T., Kubo, N., Hofstra, R., Goumans, M.J., Bekkers, J.A., Roos-Hesselink, J.W., van de Laar, I., Dietz, H.C., Van Laer, L., Morisaki, T., Wessels, M.W., Loeys, B.L., 2015b. Mutations in a TGF-β ligand, TGFB3, cause syndromic aortic aneurysms and dissections. J Am Coll Cardiol 65, 1324-1336.

Bhaskar, P.T., Hay, N., 2007. The two TORCs and Akt. Dev Cell 12, 487-502.
Bierie, B., Moses, H.L., 2006. TGF-β and cancer. Cytokine Growth Factor Rev 17, 29-40.

Bozulic, L., Hemmings, B.A., 2009. PIKKing on PKB: regulation of PKB activity by phosphorylation. Curr Opin Cell Biol 21, 256-261.

Brabletz, T., Kalluri, R., Nieto, M.A., Weinberg, R.A., 2018. EMT in cancer. Nat Rev Cancer 18, 128-134.

Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77, 71-94.
Chaudhry, S.S., Cain, S.A., Morgan, A., Dallas, S.L., Shuttleworth, C.A., Kielty, C.M., 2007. Fibrillin-1 regulates the bioavailability of TGFβ1. J Cell Biol 176, 355-367.

Chen, F., Weinberg, R.A., 1995. Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor β receptor kinases. Proc Natl Acad Sci U S A 92, 1565-1569.

Chen, R.H., Moses, H.L., Maruoka, E.M., Derynck, R., Kawabata, M., 1995. Phosphorylation-dependent interaction of the cytoplasmic domains of the type I and type II transforming growth factor β receptors. J. Biol. Chem. 270, 12235-12241.

Chen, T., Carter, D., Garrigue-Antar, L., Reiss, M., 1998a. Transforming growth factor β type I receptor kinase mutant associated with metastatic breast cancer. Cancer Res 58, 4805-4810.

Chen, T., Carter, D., Garrigue-Antar, L., Reiss, M., 1998b. Transforming growth factor β type I receptor kinase mutant associated with metastatic breast cancer. Cancer Res 58, 4805-4810.

Chen, T., Yan, W., Wells, R.G., Rimm, D.L., McNiff, J., Leffell, D., Reiss, M., 2001. Novel inactivating mutations of transforming growth factor-β type I receptor gene in head-and-neck cancer metastases. Int J Cancer 93, 653-661.

Chen, Y.G., 2009. Endocytic regulation of TGF-β signaling. Cell Res 19, 58-70. Conner, S.D., 2016. Regulation of Notch Signaling Through Intracellular Transport. Int Rev Cell Mol Biol 323, 107-127.

Conner, S.D., Schmid, S.L., 2003. Regulated portals of entry into the cell. Nature 422, 37-44.

Cooper, J.K., Sykes, G., King, S., Cottrill, K., Ivanova, N.V., Hanner, R., Ikonomi, P., 2007. Species identification in cell culture: a two-pronged molecular approach. In Vitro Cell Dev Biol Anim 43, 344-351.

Darling, N.J., Cook, S.J., 2014. The role of MAPK signalling pathways in the response to endoplasmic reticulum stress. Biochim Biophys Acta 1843, 2150-2163.

de Leon, N., Valdivieso, M.H., 2016. The long life of an endocytic patch that misses AP-2. Curr Genet 62, 765-770.

De Paepe, A., Devereux, R.B., Dietz, H.C., Hennekam, R.C., Pyeritz, R.E., 1996. Revised diagnostic criteria for the Marfan syndrome. Am J Med Genet 62, 417-426.

Denton, C.P., Zheng, B., Evans, L.A., Shi-wen, X., Ong, V.H., Fisher, I., Lazaridis, K., Abraham, D.J., Black, C.M., de Crombrugghe, B., 2003. Fibroblast-specific expression of a kinase-deficient type II transforming growth factor β (TGFβ) receptor leads to paradoxical activation of TGFβ signaling pathways with fibrosis in transgenic mice. J Biol Chem 278, 25109-25119.

Derynck, R., Feng, X.H., 1997. TGF-β receptor signaling. Biochim Biophys Acta 1333, F105-150.

Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C.H., Kern, S.E., Massague, J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B., Smith, J., Thomsen, G.H., Vogelstein, B., Wang, X.F., 1996. Nomenclature: vertebrate mediators of TGFβ family signals. Cell 87, 173.

Derynck, R., Zhang, Y.E., 2003. Smad-dependent and Smad-independent pathways in TGFβ family signalling. Nature 425, 577-584.

Dietz, H., 1993. Marfan Syndrome, in: Adam, M.P., Ardinger, H.H., Pagon, R.A., Wallace, S.E., Bean, L.J.H., Stephens, K., Amemiya, A. (Eds.), GeneReviews((R)), Seattle (WA).

Dietz, H.C., 2015. Potential Phenotype-Genotype Correlation in Marfan Syndrome: When Less is More? Circ Cardiovasc Genet 8, 256-260.

Dietz, H.C., Loeys, B., Carta, L., Ramirez, F., 2005. Recent progress towards a molecular understanding of Marfan syndrome. American journal of medical genetics Part C, Seminars in medical genetics 139C, 4-9.

Edgar, J.R., 2016. Q&A: What are exosomes, exactly? BMC Biol 14, 46.

Ehrlich, M., Shmuely, A., Henis, Y.I., 2001. A single internalization signal from the di-leucine family is critical for constitutive endocytosis of the type II TGF-β receptor. Journal of Cell Science 114, 1777-1786.

Erkula, G., Jones, K.B., Sponseller, P.D., Dietz, H.C., Pyeritz, R.E., 2002. Growth and maturation in Marfan syndrome. Am J Med Genet 109, 100-115.

Estevez, M., Attisano, L., Wrana, J., Albert, P., Massagué, J., Riddle, D., 1993. The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. Nature 365, 644-649.

Fielenbach, N., Antebi, A., 2008. *C. elegans* dauer formation and the molecular basis of plasticity. Genes Dev 22, 2149-2165.

Garamszegi, N., Doré, J.J., Penheiter, S.G., Edens, M., Yao, D., Leof, E.B., 2001a. Transforming growth factor β receptor signaling and endocytosis are linked through a COOH terminal activation motif in the type I receptor. Molecular Biology of the Cell 12, 2881-2893.

Garamszegi, N., Doré, J.J., Penheiter, S.G., Edens, M., Yao, D., Leof, E.B., 2001b. Transforming growth factor β receptor signaling and endocytosis are linked through a COOH terminal activation motif in the type I receptor. Mol Biol Cell 12, 2881-2893.

Garrigue-Antar, L., Barbieux, I., Lieubeau, B., Boisteau, O., Gregoire, M., 1995a. Optimisation of CCL64-based bioassay for TGF-β. J Immunol Methods 186, 267-274.

Garrigue-Antar, L., Munoz-Antonia, T., Antonia, S.J., Gesmonde, J., Vellucci, V.F., Reiss, M., 1995b. Missense mutations of the transforming growth factor β type II receptor in human head and neck squamous carcinoma cells. Cancer Res 55, 3982-3987.

Girard, L.R., Fiedler, T.J., Harris, T.W., Carvalho, F., Antoshechkin, I., Han, M., Sternberg, P.W., Stein, L.D., Chalfie, M., 2007. WormBook: the online review of *Caenorhabditis elegans* biology. Nucleic Acids Res 35, D472-475.

Gleason, R.J., Akintobi, A.M., Grant, B.D., Padgett, R.W., 2014. BMP signaling requires retromer-dependent recycling of the type I receptor. Proc Natl Acad Sci U S A.

Gonzalez-Gaitan, M., 2003. Signal dispersal and transduction through the endocytic pathway. Nat Rev Mol Cell Biol 4, 213-224.

Gordon, K.J., Blobe, G.C., 2008. Role of transforming growth factor-β superfamily signaling pathways in human disease. Biochim Biophys Acta 1782, 197-228.

Grady, W.M., Myeroff, L.L., Swinler, S.E., Rajput, A., Thiagalingam, S., Lutterbaugh, J.D., Neumann, A., Brattain, M.G., Chang, J., Kim, S.J., Kinzler, K.W., Vogelstein, B., Willson, J.K., Markowitz, S., 1999. Mutational inactivation of transforming growth factor β receptor type II in microsatellite stable colon cancers. Cancer Res 59, 320-324.

Grant, B.D., Donaldson, J.G., 2009. Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol 10, 597-608.

Gunther, C.V., Georgi, L.L., Riddle, D.L., 2000. A *Caenorhabditis elegans* type I TGFβ receptor can function in the absence of type II kinase to promote larval development. Development 127, 3337-3347.

Hastings, K.E., 2005. SL trans-splicing: easy come or easy go? Trends Genet 21, 240-247.

Hers, I., Vincent, E.E., Tavare, J.M., 2011. Akt signalling in health and disease. Cell Signal 23, 1515-1527.

Hinshaw, J.E., 2000. Dynamin and its role in membrane fission. Annu Rev Cell Dev Biol 16, 483-519.

Hogan, B.L., Blessing, M., Winnier, G.E., Suzuki, N., Jones, C.M., 1994. Growth factors in development: the role of TGF-β related polypeptide signalling molecules in embryogenesis. Dev Suppl, 53-60.

Holm, T.M., Habashi, J.P., Doyle, J.J., Bedja, D., Chen, Y., van Erp, C., Lindsay, M.E., Kim, D., Schoenhoff, F., Cohn, R.D., Loeys, B.L., Thomas, C.J., Patnaik, S., Marugan, J.J., Judge, D.P., Dietz, H.C., 2011. Noncanonical TGFβ signaling contributes to aortic aneurysm progression in Marfan syndrome mice. Science 332, 358-361.

Hong, W., 2005. SNAREs and traffic. Biochim Biophys Acta 1744, 493-517.

Horbelt, D., Guo, G., Robinson, P.N., Knaus, P., 2010a. Quantitative analysis of *TGFBR2* mutations in Marfan-syndrome-related disorders suggests a correlation

between phenotypic severity and Smad signaling activity. J Cell Sci 123, 4340-4350.

Horbelt, D., Guo, G., Robinson, P.N., Knaus, P., 2010b. Quantitative analysis of *TGFBR2* mutations in Marfan-syndrome-related disorders suggests a correlation between phenotypic severity and Smad signaling activity. J Cell Sci 123, 4340-4350.

Hu, Y.B., Dammer, E.B., Ren, R.J., Wang, G., 2015. The endosomal-lysosomal system: from acidification and cargo sorting to neurodegeneration. Transl Neurodegener 4, 18.

Hubmacher, D., Apte, S.S., 2011. Genetic and functional linkage between ADAMTS superfamily proteins and fibrillin-1: a novel mechanism influencing microfibril assembly and function. Cell Mol Life Sci 68, 3137-3148.

Huse, M., Muir, T.W., Xu, L., Chen, Y.G., Kuriyan, J., Massague, J., 2001. The TGF-β receptor activation process: an inhibitor- to substrate-binding switch. Mol Cell 8, 671-682.

Itoh, S., Itoh, F., 2018. TMEPAI family: involvement in regulation of multiple signalling pathways. Journal of biochemistry 164, 195-204.

Jondeau, G., Boileau, C., 2012. Genetics of thoracic aortic aneurysms. Curr Atheroscler Rep 14, 219-226.

Jovic, M., Sharma, M., Rahajeng, J., Caplan, S., 2010. The early endosome: a busy sorting station for proteins at the crossroads. Histol Histopathol 25, 99-112. Kaartinen, V., Warburton, D., 2003a. Fibrillin controls TGF-β activation. Nat Genet 33, 331-332.

Kaartinen, V., Warburton, D., 2003b. Fibrillin controls TGF-β activation. Nat Genet 33, 331-332.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P., Ahringer, J., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421, 231-237.

Kelwick, R., Desanlis, I., Wheeler, G.N., Edwards, D.R., 2015. The ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) family. Genome Biol 16, 113.

Khalil, N., 1999. TGF-β: from latent to active. Microbes Infect 1, 1255-1263.

Kim, S.J., Im, Y.H., Markowitz, S.D., Bang, Y.J., 2000. Molecular mechanisms of inactivation of TGF-β receptors during carcinogenesis. Cytokine Growth Factor Rev 11, 159-168.

Koul, H.K., Pal, M., Koul, S., 2013. Role of p38 MAP Kinase Signal Transduction in Solid Tumors. Genes Cancer 4, 342-359.

Krishna, S., Maduzia, L., Padgett, R., 1999. Specificity of TGFβ signaling is conferred by distinct type I receptors and their associated SMAD proteins in *Caenorhabditis elegans*. Development 126, 251-260.

Lee, D.W., Zhao, X., Scarselletta, S., Schweinsberg, P.J., Eisenberg, E., Grant, B.D., Greene, L.E., 2005. ATP binding regulates oligomerization and endosome association of RME-1 family proteins. J Biol Chem 280, 17213-17220.

Lehmann, K., Seemann, P., Stricker, S., Sammar, M., Meyer, B., Süring, K., Majewski, F., Tinschert, S., Grzeschik, K.-H., Müller, D., Knaus, P., Nürnberg, P., Mundlos, S., 2003a. Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. Proc Natl Acad Sci USA 100, 12277-12282.

Lehmann, K., Seemann, P., Stricker, S., Sammar, M., Meyer, B., Suring, K., Majewski, F., Tinschert, S., Grzeschik, K.H., Muller, D., Knaus, P., Nurnberg, P., Mundlos, S., 2003b. Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. Proc Natl Acad Sci U S A 100, 12277-12282.

Lerner-Ellis, J.P., Aldubayan, S.H., Hernandez, A.L., Kelly, M.A., Stuenkel, A.J., Walsh, J., Joshi, V.A., Schepers, D., Tortora, G., Morisaki, H., MacCarrick, G., Lindsay, M., Liang, D., Mehta, S.G., Hague, J., Verhagen, J., van de Laar, I., Wessels, M., Detisch, Y., van Haelst, M., Baas, A., Lichtenbelt, K., Braun, K., van der Linde, D., Roos-Hesselink, J., McGillivray, G., Meester, J., Maystadt, I., Coucke, P., El-Khoury, E., Parkash, S., Diness, B., Risom, L., Scurr, I., Hilhorst-Hofstee, Y., Morisaki, T., Richer, J., Desir, J., Kempers, M., Rideout, A.L., Horne, G., Bennett, C., Rahikkala, E., Vandeweyer, G., Alaerts, M., Verstraeten, A., Dietz, H., Van Laer, L., Loeys, B., 2014. The spectrum of *FBN1*, *TGFβR1*, *TGFβR2* and *ACTA2* variants in 594 individuals with suspected Marfan Syndrome, Loeys-Dietz Syndrome or Thoracic Aortic Aneurysms and Dissections (TAAD)

A mutation update on the LDS-associated genes *TGFB2/3* and *SMAD2/3*. Mol Genet Metab 112, 171-176.

Levy, L., Hill, C.S., 2006. Alterations in components of the TGF-β superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev 17, 41-58.
Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K., Flavell, R.A., 2006. Transforming growth factor-β regulation of immune responses. Annu Rev Immunol 24, 99-146.

Li, Q., Li, S., Mana-Capelli, S., Roth Flach, R.J., Danai, L.V., Amcheslavsky, A., Nie, Y., Kaneko, S., Yao, X., Chen, X., Cotton, J.L., Mao, J., McCollum, D., Jiang, J., Czech, M.P., Xu, L., Ip, Y.T., 2014. The conserved misshapen-warts-Yorkie pathway acts in enteroblasts to regulate intestinal stem cells in *Drosophila*. Dev Cell 31, 291-304.

Li, X., DiFiglia, M., 2012. The recycling endosome and its role in neurological disorders. Prog Neurobiol 97, 127-141.

Lindsay, M.E., Schepers, D., Bolar, N.A., Doyle, J.J., Gallo, E., Fert-Bober, J., Kempers, M.J., Fishman, E.K., Chen, Y., Myers, L., Bjeda, D., Oswald, G., Elias, A.F., Levy, H.P., Anderlid, B.M., Yang, M.H., Bongers, E.M., Timmermans, J., Braverman, A.C., Canham, N., Mortier, G.R., Brunner, H.G., Byers, P.H., Van Eyk, J., Van Laer, L., Dietz, H.C., Loeys, B.L., 2012. Loss-of-function mutations in *TGFB2* cause a syndromic presentation of thoracic aortic aneurysm. Nat Genet 44, 922-927.

Loeys, B.L., Chen, J., Neptune, E.R., Judge, D.P., Podowski, M., Holm, T., Meyers, J., Leitch, C.C., Katsanis, N., Sharifi, N., Xu, F.L., Myers, L.A., Spevak, P.J., Cameron, D.E., De Backer, J., Hellemans, J., Chen, Y., Davis, E.C., Webb, C.L., Kress, W., Coucke, P., Rifkin, D.B., De Paepe, A.M., Dietz, H.C., 2005. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in *TGFBR1* or *TGFBR2*. Nat Genet 37, 275-281.

Lu, S.L., Kawabata, M., Imamura, T., Akiyama, Y., Nomizu, T., Miyazono, K., Yuasa, Y., 1998. HNPCC associated with germline mutation in the TGF-β type II receptor gene. Nat Genet 19, 17-18.

Lu, S.L., Kawabata, M., Imamura, T., Miyazono, K., Yuasa, Y., 1999. Two divergent signaling pathways for TGF-β separated by a mutation of its type II receptor gene. Biochem Biophys Res Commun 259, 385-390.

Lu, Z., Murray, J.T., Luo, W., Li, H., Wu, X., Xu, H., Backer, J.M., Chen, Y.-G., 2002. Transforming growth factor β activates Smad2 in the absence of receptor endocytosis. J Biol Chem 277, 29363-29368.

Lücke, C.D., Philpott, A., Metcalfe, J.C., Thompson, A.M., Hughes-Davies, L., Kemp, P.R., Hesketh, R., 2001. Inhibiting mutations in the transforming growth factor β type 2 receptor in recurrent human breast cancer. Cancer Res 61, 482-485.

Massague, J., 1998. TGF-β signal transduction. Annu Rev Biochem 67, 753-791.

Massague, J., 2008. TGFβ in Cancer. Cell 134, 215-230.

Massague, J., Blain, S.W., Lo, R.S., 2000. TGFβ signaling in growth control, cancer, and heritable disorders. Cell 103, 295-309.

Massagué, J., Chen, Y.G., 2000. Controlling TGFβ signaling. Genes Dev 14, 627-644.

Massague, J., Wotton, D., 2000. Transcriptional control by the TGF-β/Smad signaling system. Embo J 19, 1745-1754.

Matyas, G., Arnold, E., Carrel, T., Baumgartner, D., Boileau, C., Berger, W., Steinmann, B., 2006. Identification and in silico analyses of novel *TGFBR1* and *TGFBR2* mutations in Marfan syndrome-related disorders. Hum Mutat 27, 760-769.

Maxfield, F.R., McGraw, T.E., 2004. Endocytic recycling. Nat Rev Mol Cell Biol 5, 121-132.

Mitchell, H., Choudhury, A., Pagano, R.E., Leof, E.B., 2004. Ligand-dependent and -independent transforming growth factor-β receptor recycling regulated by clathrin-mediated endocytosis and Rab11. Mol Biol Cell 15, 4166-4178.

Mizuguchi, T., Collod-Beroud, G., Akiyama, T., Abifadel, M., Harada, N., Morisaki, T., Allard, D., Varret, M., Claustres, M., Morisaki, H., Ihara, M., Kinoshita, A., Yoshiura, K., Junien, C., Kajii, T., Jondeau, G., Ohta, T., Kishino, T., Furukawa, Y., Nakamura, Y., Niikawa, N., Boileau, C., Matsumoto, N., 2004. Heterozygous *TGFBR2* mutations in Marfan syndrome. Nat Genet 36, 855-860.

Murphy, S.J., Shapira, K.E., Henis, Y.I., Leof, E.B., 2007. A unique element in the cytoplasmic tail of the type II transforming growth factor-β receptor controls basolateral delivery. Mol Biol Cell 18, 3788-3799.

Nagayama, S., Onodera, H., Toguchida, J., Imamura, M., 2002. Altered expression of the receptor and ligand in the TGF-β signaling pathway in diffusely infiltrating colon carcinoma. Anticancer research 22, 3545-3554.

Neptune, E.R., Frischmeyer, P.A., Arking, D.E., Myers, L., Bunton, T.E., Gayraud, B., Ramirez, F., Sakai, L.Y., Dietz, H.C., 2003. Dysregulation of TGF-β activation contributes to pathogenesis in Marfan syndrome. Nat Genet 33, 407-411.

Nerlich, A.G., Sauer, U., Ruoss, I., Hagedorn, H.G., 2003. High frequency of TGF-β-receptor-II mutations in microdissected tissue samples from laryngeal squamous cell carcinomas. Lab Invest 83, 1241-1251.

Nishimura, R., Hata, K., Ikeda, F., Matsubara, T., Yamashita, K., Ichida, F., Yoneda, T., 2003. The role of Smads in BMP signaling. Front Biosci 8, s275-284.

Pan, C.-L., Baum, P.D., Gu, M., Jorgensen, E.M., Clark, S.G., Garriga, G., 2008. *C. elegans* AP-2 and retromer control Wnt signaling by regulating mig-14/Wntless. Dev Cell 14, 132-139.

Patterson, G.I., Padgett, R.W., 2000. TGF-β-related pathways. Roles in *Caenorhabditis elegans* development. Trends Genet 16, 27-33.

Pelkmans, L., 2005. Secrets of caveolae- and lipid raft-mediated endocytosis revealed by mammalian viruses. Biochim Biophys Acta 1746, 295-304.

Piper, R.C., Katzmann, D.J., 2007. Biogenesis and function of multivesicular bodies. Annu Rev Cell Dev Biol 23, 519-547.

Plotnikov, A., Zehorai, E., Procaccia, S., Seger, R., 2011. The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim Biophys Acta 1813, 1619-1633.

Poniatowski, L.A., Wojdasiewicz, P., Gasik, R., Szukiewicz, D., 2015. Transforming growth factor β family: insight into the role of growth factors in regulation of fracture healing biology and potential clinical applications. Mediators Inflamm 2015, 137823.

Praitis, V., 2006. Creation of transgenic lines using microparticle bombardment methods. Methods Mol Biol 351, 93-107.

Praitis, V., Casey, E., Collar, D., Austin, J., 2001. Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. Genetics 157, 1217-1226.

Proost, D., Vandeweyer, G., Meester, J.A.N., Salemink, S., Kempers, M., Ingram, C., Peeters, N., Saenen, J., Vrints, C., Lacro, R.V., Roden, D., Wuyts, W., Dietz, H.C., Mortier, G., Loeys, B.L., Van Laer, L., 2015. Performant Mutation Identification Using Targeted Next-Generation Sequencing of 14 Thoracic Aortic Aneurysm Genes. Hum Mutat 36, 808-814.

Ramel, M.C., Hill, C.S., 2012. Spatial regulation of BMP activity. FEBS Lett 586, 1929-1941.

Reilly, P.R., 2000. Abraham Lincoln's DNA and Other Adventures in Genetics. Cold Spring Harbor Laboratory Press.

Ren, P., Lim, C.S., Johnsen, R., Albert, P.S., Pilgrim, D., Riddle, D.L., 1996a. Control of *C. elegans* larval development by neuronal expression of a TGF-β homolog. Science 274, 1389-1391.

Ren, P., Lim, C.S., Johnsen, R., Albert, P.S., Pilgrim, D., Riddle, D.L., 1996b. Control of *C. elegans* larval development by neuronal expression of a TGFβ homolog. Science 274, 1389-1391.

Richards, D.M., Endres, R.G., 2014. The mechanism of phagocytosis: two stages of engulfment. Biophys J 107, 1542-1553.

Roberts, B., Haupt, A., Tucker, A., Grancharova, T., Arakaki, J., Fuqua, M.A., Nelson, A., Hookway, C., Ludmann, S.A., Mueller, I.A., Yang, R., Horwitz, R., Rafelski, S.M., Gunawardane, R.N., 2017. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. Mol Biol Cell 28, 2854-2874.

Robinson, P.N., Arteaga-Solis, E., Baldock, C., Collod-Beroud, G., Booms, P., De Paepe, A., Dietz, H.C., Guo, G., Handford, P.A., Judge, D.P., Kielty, C.M., Loeys, B., Milewicz, D.M., Ney, A., Ramirez, F., Reinhardt, D.P., Tiedemann, K., Whiteman, P., Godfrey, M., 2006. The molecular genetics of Marfan syndrome and related disorders. J Med Genet 43, 769-787.

Saharinen, J., Taipale, J., Keski-Oja, J., 1996. Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1. EMBO J 15, 245-253.

Saito, M., Kurokawa, M., Oda, M., Oshima, M., Tsutsui, K., Kosaka, K., Nakao, K., Ogawa, M., Manabe, R., Suda, N., Ganjargal, G., Hada, Y., Noguchi, T., Teranaka, T., Sekiguchi, K., Yoneda, T., Tsuji, T., 2011. ADAMTSL6beta protein rescues fibrillin-1 microfibril disorder in a Marfan syndrome mouse model through the promotion of fibrillin-1 assembly. J Biol Chem 286, 38602-38613.

Santibanez, J.F., Quintanilla, M., Bernabeu, C., 2011. TGF-β/TGF-β receptor system and its role in physiological and pathological conditions. Clin Sci (Lond) 121, 233-251.

Sato, M., Sato, K., Fonarev, P., Huang, C.J., Liou, W., Grant, B.D., 2005. *Caenorhabditis elegans* RME-6 is a novel regulator of RAB-5 at the clathrin-coated pit. Nat Cell Biol 7, 559-569.

Savage, C., Das, P., Finelli, A., Townsend, S., Sun, C., Baird , S., Padgett , R., 1996. *Caenorhabditis elegans* genes *sma-2*, *sma-3*, and *sma-4* define a conserved family of transforming growth factor β pathway components. Proc Natl Acad Sci U S A 93, 790-794.

Savage-Dunn, C., Maduzia, L.M., Cohen, S., Padgett, R.W., 2000. A genetic screen for body size mutants reveals new components of the TGFβ signaling pathway in *C. elegans*. in preparation.

Schepers, D., Doyle, A.J., Oswald, G., Sparks, E., Myers, L., Willems, P.J., Mansour, S., Simpson, M.A., Frysira, H., Maat-Kievit, A., Van Minkelen, R., Hoogeboom, J.M., Mortier, G.R., Titheradge, H., Brueton, L., Starr, L., Stark, Z., Ockeloen, C., Lourenco, C.M., Blair, E., Hobson, E., Hurst, J., Maystadt, I., Destrée, A., Girisha, K.M., Miller, M., Dietz, H.C., Loeys, B., Van Laer, L., 2015. The SMAD-binding domain of SKI: a hotspot for *de novo* mutations causing Shprintzen-Goldberg syndrome. Eur. J. Hum. Genet. 23, 224-228.

Schepers, D., Tortora, G., Morisaki, H., MacCarrick, G., Lindsay, M., Liang, D., Mehta, S.G., Hague, J., Verhagen, J., van de Laar, I., Wessels, M., Detisch, Y., van Haelst, M., Baas, A., Lichtenbelt, K., Braun, K., van der Linde, D., Roos-Hesselink, J., McGillivray, G., Meester, J., Maystadt, I., Coucke, P., El-Khoury, E., Parkash, S., Diness, B., Risom, L., Scurr, I., Hilhorst-Hofstee, Y., Morisaki, T., Richer, J., Desir, J., Kempers, M., Rideout, A.L., Horne, G., Bennett, C., Rahikkala, E., Vandeweyer, G., Alaerts, M., Verstraeten, A., Dietz, H., Van Laer, L., Loeys, B., 2018. A mutation update on the LDS-associated genes TGFB2/3 and SMAD2/3. Hum Mutat 39, 621-634.

Schiemann, W.P., Rotzer, D., Pfeifer, W.M., Levi, E., Rai, K.R., Knaus, P., Kadin, M.E., 2004. Transforming growth factor-β (TGF-β)-resistant B cells from chronic lymphocytic leukemia patients contain recurrent mutations in the signal sequence of the type I TGF-β receptor. Cancer detection and prevention 28, 57-64.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676-682.

Schrodinger, LLC, 2015. The PyMOL Molecular Graphics System, Version 2.1.
Schweinsberg, P.J., Grant, B.D., 2013. *C. elegans* gene transformation by microparticle bombardment. WormBook, 1-10.

Seaman, M.N., 2004. Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. J Cell Biol 165, 111-122.

Seaman, M.N., 2012. The retromer complex - endosomal protein recycling and beyond. J Cell Sci 125, 4693-4702.

Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H., Gelbart, W.M., 1995. Genetic characterization and cloning of *Mothers against dpp*, a gene required for *decapentaplegic* function in *Drosophila melanogaster*. Genetics 139, 1347-1358.
Shapira, K.E., Gross, A., Ehrlich, M., Henis, Y.I., 2012. Coated pit-mediated endocytosis of the type I transforming growth factor-β (TGF-β) receptor depends on a di-leucine family signal and is not required for signaling. Journal of Biological Chemistry 287, 26876-26889.

Shi, Y., Massague, J., 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113, 685-700.

Shi, Y., Wang, Y.F., Jayaraman, L., Yang, H., Massague, J., Pavletich, N.P., 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-β signaling. Cell 94, 585-594.

Shin, I., Bakin, A.V., Rodeck, U., Brunet, A., Arteaga, C.L., 2001. Transforming growth factor β enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. Mol Biol Cell 12, 3328-3339.

Siegert, A.M., Serra-Peinado, C., Gutierrez-Martinez, E., Rodriguez-Pascual, F., Fabregat, I., Egea, G., 2018. Altered TGF-β endocytic trafficking contributes to the increased signaling in Marfan syndrome. Biochim Biophys Acta Mol Basis Dis 1864, 554-562.

Suzuki, Y., Yandell , M., Roy, P., Krishna, S., Savage-Dunn, C., Ross, R., Padgett, R., Wood, W., 1999. A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in *Caenorhabditis elegans*. Development 126, 241-250.

Takei, K., Haucke, V., 2001. Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol 11, 385-391.

Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature 395, 854.

Van Laer, L., Dietz, H., Loeys, B., 2014. Loeys-Dietz syndrome. Adv Exp Med Biol 802, 95-105.

Vowels, J.J., Thomas, J.H., 1992. Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*. Genetics 130, 105-123.

Wang, D., Kanuma, T., Mizunuma, H., Takama, F., Ibuki, Y., Wake, N., Mogi, A., Shitara, Y., Takenoshita, S., 2000. Analysis of specific gene mutations in the transforming growth factor-β signal transduction pathway in human ovarian cancer. Cancer Res 60, 4507-4512.

Wang, J., Tokarz, R., Savage-Dunn, C., 2002. The expression of TGFβ signal transducers in the hypodermis regulates body size in *C. elegans*. Development 129, 4989-4998.

Weiss, A., Attisano, L., 2013. TGFβ superfamily signaling pathway. Wiley Interdisciplinary Reviews: Develop Biology 2, 17.

Wooderchak-Donahue, W., VanSant-Webb, C., Tvrdik, T., Plant, P., Lewis, T., Stocks, J., Raney, J.A., Meyers, L., Berg, A., Rope, A.F., Yetman, A.T., Bleyl, S.B., Mesley, R., Bull, D.A., Collins, R.T., Ojeda, M.M., Roberts, A., Lacro, R., Woerner, A., Stoler, J., Bayrak-Toydemir, P., 2015. Clinical utility of a next generation sequencing panel assay for Marfan and Marfan-like syndromes featuring aortopathy. Am. J. Med. Genet. 167, 1747-1757.

Wrana, J.L., Attisano, L., 1996. MAD-related proteins in TGF-β signalling. Trends Genet 12, 493-496.

Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., Massague, J., 1994. Mechanism of activation of the TGF-β receptor. Nature 370, 341-347.

Yakovich, A.J., Huang, Q., Du, J., Jiang, B., Barnard, J.A., 2010. Vectorial TGFβ signaling in polarized intestinal epithelial cells. J Cell Physiol 224, 398-404.

Yin, X., Kang, J.H., Andrianifahanana, M., Wang, Y., Jung, M.Y., Hernandez, D.M., Leof, E.B., 2017. Basolateral delivery of the type I transforming growth factor β receptor is mediated by a dominant-acting cytoplasmic motif. Mol Biol Cell 28, 2701-2711.

Yin, X., Murphy, S.J., Wilkes, M.C., Ji, Y., Leof, E.B., 2013. Retromer maintains basolateral distribution of the type II TGF-β receptor via the recycling endosome. Mol Biol Cell 24, 2285-2298.

Zangwill, S.D., Brown, M.D., Bryke, C.R., Cava, J.R., Segura, A.D., 2006. Marfan syndrome type II: there is more to Marfan syndrome than fibrillin 1. Congenit Heart Dis 1, 229-232.

Zhang, Z., Zhou, X., Shen, H., Wang, D., Wang, Y., 2009. Phosphorylated ERK is a potential predictor of sensitivity to sorafenib when treating hepatocellular carcinoma: evidence from an in vitro study. BMC Med 7, 41.

Zhang, Z.C., Zwick, S., Loew, E., Grimley, J.S., Ramanathan, S., 2018. Embryo geometry drives formation of robust signaling gradients through receptor localization.

Zhou, Y., Scolavino, S., Funderburk, S.F., Ficociello, L.F., Zhang, X., Klibanski, A., 2004. Receptor internalization-independent activation of Smad2 in activin signaling. Mol Endocrinol 18, 1818-1826.

Zwaagstra, J.C., El-Alfy, M., O'Connor-McCourt, M.D., 2001. Transforming growth factor (TGF)-β 1 internalization: modulation by ligand interaction with TGF-β receptors types I and II and a mechanism that is distinct from clathrin-mediated endocytosis. J Biol Chem 276, 27237-27245.