



Universidad Autónoma
de Madrid



Escuela
de Doctorado

Tesis Doctoral

Interplay between Complex Formation and Intracellular Compartmentalization in the regulation of the TGF- β Pathway signaling dynamics

Ahmed Naser Fayad

Programa de Doctorado en Biociencias
Moleculares / Facultad de Ciencias

Dirección:

David Míguez Gómez, PhD.

Madrid, 2026



Universidad Autónoma
de Madrid



Escuela
de Doctorado

Tesis Doctoral

Interplay between Complex Formation and Intracellular Compartmentalization in the regulation of the TGF- β Pathway signaling dynamics

Ahmed Naser Fayad

Programa de Doctorado en Biociencias
Moleculares / Facultad de Ciencias

Dirección:

David Míguez Gómez, PhD

Madrid, 2026

RESUMEN / SUMMARY

RESUMEN

Los sistemas vivos dependen de una incompleja y precisa interacción distintos procesos regulatorios. Estos tipos de módulos reguladores pueden ser de naturaleza física o bioquímica. La vía TGF- β es una de las vías de señalización más estudiadas, y combina tanto aspectos físicos (compartimentación) como bioquímicos (modificación postraducciona) para interpretar señales extracelulares. Las R-Smads (Smad2 y Smad3) son los principales efectores de la vía. Esta tesis se centra en la caracterización de la translocación dinámica de las proteínas Smad2 y Smad3 endógenas, así como de las variantes exógenas de las proteínas Smad2 y Smad3 fusionadas con proteínas fluorescentes. Para ello, investigamos cómo el contexto celular influye en la distribución y compartimentación núcleo-citoplasmática de los R-Smads, utilizando una combinación de enfoques experimentales y teóricos.

Mostramos que nuestras Smads de fusión se comportaron de manera similar a las R-Smads endógenas, en términos de localización y capacidad para activarse por fosforilación. Estos resultados sirven como validación de que las proteínas de fusión se pueden utilizar para monitorear la activación de la vía de señalización TGF- β , y que la co-expresión de combinaciones de estas proteínas de fusión se puede utilizar para monitorear la dinámica de formación y translocación de los diferentes complejos de las R-Smads que modulan la activación de los genes diana.

En general, esta tesis está diseñada como los pasos preliminares requeridos para diseñar y caracterizar estas proteínas de fusión como un potencial sensor FRET. También se han establecido líneas de investigación para el trabajo futuro, centrándose en la exploración de la dinámica de la formación de complejos formados por la interacción ente los diferentes R-Smads y su papel en la cascada de señalización aprovechando la herramienta desarrollada.

Palabras clave: Vía de señalización TGF- β , Smad2/Smad3, Translocación núcleo- citoplasmática, Proteínas de fusión y Sensor FRET

SUMMARY

Living systems rely on an intricate interplay of complex and precise layers of regulatory processes. These types of regulatory modules can be physical or biochemical in nature. The TGF- β pathway is one of the most studied signaling pathways, and it combines both physical (compartmentalization) and biochemical (post-translational modification) features to interpret extracellular cues. The R-Smads (Smad2 and Smad3) are the main downstream effectors of the pathway. This thesis focuses on the characterization of the dynamic translocation of both endogenous Smad2 and Smad3 proteins, as well as exogenous fusion variants of Smad2 and Smad3 proteins. To do that, we investigated how cellular context influences Smad nucleocytoplasmic distribution and compartmentalization, using a combination of experimental and theoretical approaches.

We show that our fusion Smads behaved similarly to endogenous R-Smads, in terms of localization and ability to get activated by phosphorylation. These results serve as validation that the fusion proteins can be used to monitor the activation of the TGF- β signaling pathway, and that co-expression of combinations of these fusion proteins can be used to monitor the dynamics of formation and translocation of the different complexes of the R-Smads that modulate the activation of target genes.

Overall, the present thesis is designed as the required preliminary steps to design and characterize these fusion proteins as a potential FRET sensor. Research lines for future work have also been proposed, focusing on exploring the dynamics of Smad complex formation from the interaction between the different R-Smads, and their role in the TGF- β signaling cascade.

Keywords: TGF- β signaling pathway, Smad2/Smad3, Nucleocytoplasmic translocation, Fusion proteins and FRET sensor

INDEX

INDEX

Summary.....	III
Index.....	V
Abbreviations.....	VIII
I. Introduction.....	1
1. The Transforming Growth Factor- β (TGF- β) signaling pathway	1
1.1 Signaling networks in health and disease	1
1.2 The TGF- β signaling pathway	3
1.3 Historical context	5
1.4 TGF- β in Cancer	6
1.5 TGF- β in other human diseases.....	7
1.6 TGF- β Ligands	8
1.7 TGF- β receptors.....	8
1.8 Smad Proteins	10
1.8.1 Historical Perspective.....	10
1.8.2 Structure.....	11
1.8.3 Smad Activation	13
1.8.4 Smad De-activation	13
1.8.5 Smad Interactions at the cytoplasm level.....	14
1.8.6 Smad Complex Formation.....	14
1.8.7 Nuclear entry.....	16
1.8.8 Interaction with DNA.....	16
1.8.9 Nuclear Export.....	17
1.8.10 Nuclear localization as readout of TGF- β activity	17
1.8.11 Previous contributions from our lab in the context of TGF- β activity	18

II. Objectives.....	19
III. Materials and Methods	20
2.1 Materials	20
2.1.1 Cell Lines and Culture ...	20
2.1.2 Plasmids, Lentiviral Vectors and transfection ...	20
2.1.3 Reagents for Activation and Inhibition Assays ...	20
2.1.4 Antibodies	21
2.1.4.1 Primary Antibodies ...	21
2.1.4.2 Secondary Antibodies	21
2.1.5 Immunofluorescence and Staining Reagents	21
2.1.6 Microscopy and Imaging ...	21
2.1.7 FRET and Colocalization Analysis ...	21
2.2 Methods ...	22
2.2.1 Cell culturing	22
2.2.2 Cell infection and generation of stable and clone cell lines	22
2.2.3 Cell immunofluorescence	23
2.2.4 Image acquisition and analysis	23
2.2.5 FRET and colocalization analysis ...	24
IV. Results	25
4.1 Compartmentalization as a mechanism of regulation in the TGF- β pathway	25
4.2 The concept of compartmentalization	25
4.3 R-Smad Nucleocytoplasmic Shuttling as a Modulation of Signal Transduction	27
4.4 Dynamics and Regulation of Export and Import of the R-Smads	27
4.5 Taking Advantage of Nucleocytoplasmic Shuttling to Study the TGF- β Pathway	28
4.6 Smad2 and Smad3 endogenous localization in vitro ...	29
4.7 Smad2 and Smad3 fusion proteins	34
4.8 Mathematical Modeling of Regulatory Compartmentalization ...	39
4.8.1 Mass action law to model nucleocytoplasmic shuttling ...	40

4.8.2 Using the Mass Action Law to Model a Biological System ...	41
4.8.3 Simplification Using the Mass Conservation Law ...	43
4.8.4 Nondimensionalization of the Model ...	44
4.8.5 Pseudo-Steady-State Approximation ...	48
4.8.6 Conclusions from the immunofluorescence and the modeling part ...	50
4.9 Preliminary FRET quantification ...	51
4.9.1 Introduction to the FRET Technique	51
4.9.2 Preliminary experiments	52
V. Discussion	57
VI. Conclusions	60
VII. Bibliography	64
VIII. Appendices ...	87

ABBREVIATIONS LIST

ABBREVIATIONS LIST

- Akt** – Protein Kinase B
- ALK** – Activin Receptor-Like Kinase
- AMH** – Anti-Müllerian Hormone
- BMPs** – Bone Morphogenetic Proteins
- BRAF** – B-Raf Proto-Oncogene, Serine/Threonine Kinase
- BSA** – Bovine Serum Albumin
- CBP** – CREB-Binding Protein
- CCVs** – Clathrin-Coated Vesicles
- CFP** – Cyan Fluorescent Protein
- CO₂** – Carbon Dioxide
- Co-Smad** – Common-mediator Smad
- CRM1** – Chromosome Region Maintenance 1 (Exportin 1)
- DAPI** – 4',6-Diamidino-2-Phenylindole (DNA Stain)
- DMEM** – Dulbecco's Modified Eagle Medium
- DMSO** – Dimethyl Sulfoxide
- Dpp** – Decapentaplegic (Drosophila Homolog of TGF- β)
- ECM** – Extracellular Matrix
- EDTA** – Ethylenediaminetetraacetic Acid
- EGFR** – Epidermal Growth Factor Receptor
- ELF** – Embryonic Liver Fetal Protein
- EMT** – Epithelial-Mesenchymal Transition
- FBS** – Fetal Bovine Serum
- FIJI** – Fiji Is Just ImageJ (Image Processing Software)
- FoxO** – Forkhead Box O
- FRAP** – Fluorescence Recovery After Photobleaching
- FRET** – Förster Resonance Energy Transfer
- GFP** – Green Fluorescent Protein
- ggplot2** – A data visualization package for R
- I-Smads** – Inhibitory Smads
- JAK/STAT** – Janus Kinase / Signal Transducer and Activator of Transcription
- MAPK** – Mitogen-Activated Protein Kinase
- mCherry** – A red fluorescent protein
- MDSCs** – Myeloid-Derived Suppressor Cells

MH1/MH2 – Mad Homology 1 / Mad Homology 2 Domains

Michaelis–Menten – Enzyme kinetics model

NES – Nuclear Export Signal

NIH – National Institutes of Health

NLS – Nuclear Localization Signal

Nup153/Nup214 – Nucleoporins 153 and 214

ODEs – Ordinary Differential Equations

P Smad – Phosphorylated Smad Protein

p300 – E1A-Binding Protein P300

PAGFP – Photoactivatable Green Fluorescent Protein

PBS – Phosphate-Buffered Saline

PFA – Paraformaldehyde

PI3K – Phosphoinositide 3-Kinase

pLvX – Lentiviral Expression Vector

PPXY – Proline-Proline-X-Tyrosine Motif

R – Statistical Computing Software

RFP – Red Fluorescent Protein

R-Smads – Receptor-regulated Smads

Runx – Runt-related Transcription Factor

SARA – Smad Anchor for Receptor Activation

SB-431542 – A selective inhibitor of TGF- β type I receptor kinase

SCP – Small C-terminal Domain Phosphatase

SGF – Sarcoma Growth Factor

TGF- β – Transforming Growth Factor Beta

ToPro3 – A far-red fluorescent nucleic acid stain

Tregs – Regulatory T Cells

T β RI – Transforming Growth Factor-Beta Receptor Type I

T β RII – Transforming Growth Factor-Beta Receptor Type II

T β RIII – Transforming Growth Factor-Beta Receptor Type III (Betaglycan)

VSV-G – Vesicular Stomatitis Virus Glycoprotein

WW – Tryptophan-Tryptophan (Protein Interaction Domain)

YFP – Yellow Fluorescent Protein

μ L – Microliter

I. INTRODUCTION

I. INTRODUCCION

1. The Transforming Growth Factor- β (TGF- β) signaling pathway

1.1 Signaling networks in health and disease

Signaling networks are fundamental to all biological systems, orchestrating a vast array of cellular processes, from development and homeostasis to responses to environmental stimuli. These networks represent intricate systems of communication, allowing cells to perceive and react to both internal and external cues. The complexity of these networks arises from the diverse array of signaling molecules, receptors, and downstream effectors that interact in a highly regulated manner. The emergence of multicellular organisms necessitated mechanisms to regulate interplay between different cells, such as the migratory behaviors of individual cells within a collective organismal context. As a result, sophisticated communication networks between cells evolved (Brunet & King, 2017).

The molecular dissection of these cellular signaling networks has been a cornerstone of biological research for decades. Early investigations focused on identifying individual signaling molecules and their corresponding receptors. The discovery of growth factors, hormones, and their respective signaling pathways, such as the Ras/MAPK and PI3K/Akt pathways, provided foundational insights into the mechanisms governing cell proliferation, differentiation, and survival. As molecular biology tools advanced, researchers focused on the characterization of the components of these networks at the genetic level. This led to a deeper understanding of the intricate regulatory mechanisms that control signal transduction, paving the way for the development of targeted therapies for various diseases (Sabbah et al., 2024).

A comprehensive understanding of cellular signaling pathways is paramount in the context of pharmacology and disease. Dysregulation of these intricate networks is a hallmark of many pathological conditions, including cancer, autoimmune disorders, and neurodegenerative diseases. By elucidating the specific molecular aberrations within these pathways, researchers can identify potential therapeutic targets and develop drugs. For example, the discovery of oncogenic mutations in signaling proteins like EGFR and BRAF has led to the development of highly effective kinase inhibitors used in cancer treatment (Kannaiyan & Mahadevan, 2018). Furthermore, understanding how signaling pathways contribute to drug resistance and off-target effects is crucial for optimizing therapeutic strategies and minimizing adverse reactions. In this direction, the investigation of the complex aspects of cellular signaling is essential for advancing personalized therapeutics and improving drug efficiency (Ji & Tao, 2025).

While the translational potential of signaling network research is evident in drug development, the foundation for these advancements lies in rigorous basic research. Unraveling the

fundamental mechanisms governing signal transduction, including the identification of novel signaling components, their interactions, and the dynamics of pathway activation, is essential for a comprehensive understanding of these complex systems (Nair et al., 2019). Basic research provides the groundwork for identifying potential therapeutic targets by revealing critical vulnerabilities within disease-associated signaling pathways. For instance, studies on the structure and function of kinases have not only elucidated their roles in cellular signaling but have also enabled the rational design of kinase inhibitors (Silnitsky et al., 2023). Moreover, basic research into the regulatory loops and feedback mechanisms within signaling networks can reveal potential mechanisms of drug resistance, informing the development of combination therapies or alternative targeting strategies. Ultimately, these insights gained from basic research provide the conceptual and mechanistic framework necessary for the successful translation of scientific discoveries into effective therapeutic interventions (Kholodenko et al., 2021).

Basic research constitutes the first and arguably the most important step of the drug development pipeline. Computational modeling, or *in silico* analysis, offers a powerful complement to this classical approach to drug discovery, streamline this process and reduce expenses (Marques et al., 2024). By simulating the behavior of signaling pathways and predicting the effects of potential drug candidates, researchers can prioritize promising compounds and eliminate those likely to fail in later stages. For example, network-based modeling can be used to predict drug target interactions, off-target effects, and potential toxicity, allowing for the selection of safer and more effective candidates (Visan & Negut, 2024). Moreover, *silico* models can be used to optimize drug dosing and predict patient responses, potentially reducing the number of costly *in vivo* experiments. By integrating computational approaches into the early stages of drug development, researchers can accelerate the identification of promising therapeutics, reduce the need of animal models, and ultimately lower the cost new drugs (Vora et al., 2023).

This thesis aims to contribute to the advancement of knowledge in signaling pathway biology, by integrating traditional wet-lab experiments using *in vitro* cell culture with advanced microscopy techniques and mathematical modeling. The insights gained from this integrated approach try to provide a robust foundation for identifying potential therapeutic targets, potentially accelerating the identification of next-generation drugs with enhanced efficacy and specificity.

Specifically, this Thesis focuses on the Transforming Growth Factor beta (TGF- β) signaling pathway, a critical regulator of numerous cellular processes. However, rather than simply characterizing individual components of this pathway, this work adopts a systems-level perspective, aiming to derive generalizable conclusions about how signaling is regulated through the integration of physical and biochemical cues. By investigating the interplay between these features, this research seeks to identify fundamental principles that govern signal transduction, that may be extrapolated to other signaling systems and pathways across various biological contexts. Ultimately, the insights gained from this study of the TGF β pathway will contribute to a broader understanding of cellular communication and provide a framework for developing novel therapeutic strategies targeting signaling dysregulation in human diseases.

1.2 The TGF- β signaling pathway

Signaling networks frequently rely on the secretion of polypeptide ligands, a diverse group of molecules ranging from growth factors and cytokines to hormones and chemokines (Yi et al., 2024). These polypeptides act as extracellular messengers, initiating cellular responses by binding to specific membrane-bound receptors. This interaction triggers a cascade of intracellular signaling events, often involving conformational changes in the receptor and the recruitment of adaptor proteins (Su et al., 2024). Downstream signaling pathways, such as the Ras/MAPK, PI3K/Akt, and JAK/STAT pathways, are then activated, leading to the modulation of various cellular processes. Ultimately, these signaling cascades converge on the nucleus, where they influence transcriptional activities by regulating the expression of target genes. This finely tuned control of gene expression allows cells to adapt to changing environmental conditions, coordinate developmental processes, and maintain tissue homeostasis (Steelman et al., 2011). The specificity of polypeptide signaling is ensured by the precise interaction between ligands and their corresponding receptors, as well as by the intricate regulatory mechanisms that govern downstream signaling pathways (Armingol et al., 2021).

The transforming growth factor-beta (TGF- β) signaling pathway represents one of the most versatile and evolutionarily conserved of these types of pathways in metazoans (Lo et al., 2022). Due to its many important roles in the regulation of key biological processes and its involvement in many diseases, it constitutes one of the most studied models of since its discovery in the late 1970s. Research on TGF- β has revolutionized our understanding of cellular communication, embryonic development, tissue homeostasis, and disease pathogenesis (Moses et al., 2016).

The transforming growth factor-beta (TGF- β) superfamily, (encompassing approximately 40 distinct members encoded within the human genome) includes TGF- β isoforms, bone morphogenetic proteins (BMPs), myostatins, nodals, anti-Müllerian hormone (AMH), and activins, among others, and plays a pivotal role in regulating cell division, differentiation, migration, adhesion, and apoptosis (Morikawa et al., 2016; Oshimori & Fuchs, 2012). These ligands are produced by a wide variety of cell types and remain active throughout development and adulthood (Diacou et al., 2022). Smad proteins serve as central signal transducers for the TGF- β superfamily of cytokines and their associated receptors (Sisto et al., 2021). Upon TGF- β binding, a cascade of signaling events is activated, primarily mediated by the receptor-regulated Smads (R-Smads), and the common-mediator Smad (Co-Smad), Smad4. These proteins transduce the signal from the cell membrane to the nucleus, modulating gene expression and subsequent cellular functions (Miyazawa et al., 2024; Nakao et al., 1997; Bourgeois et al., 2013; Wu et al., 2001; Miyazono et al., 2000). The core processes involving the TGF- β signaling pathway are illustrated in Figure 1.1.

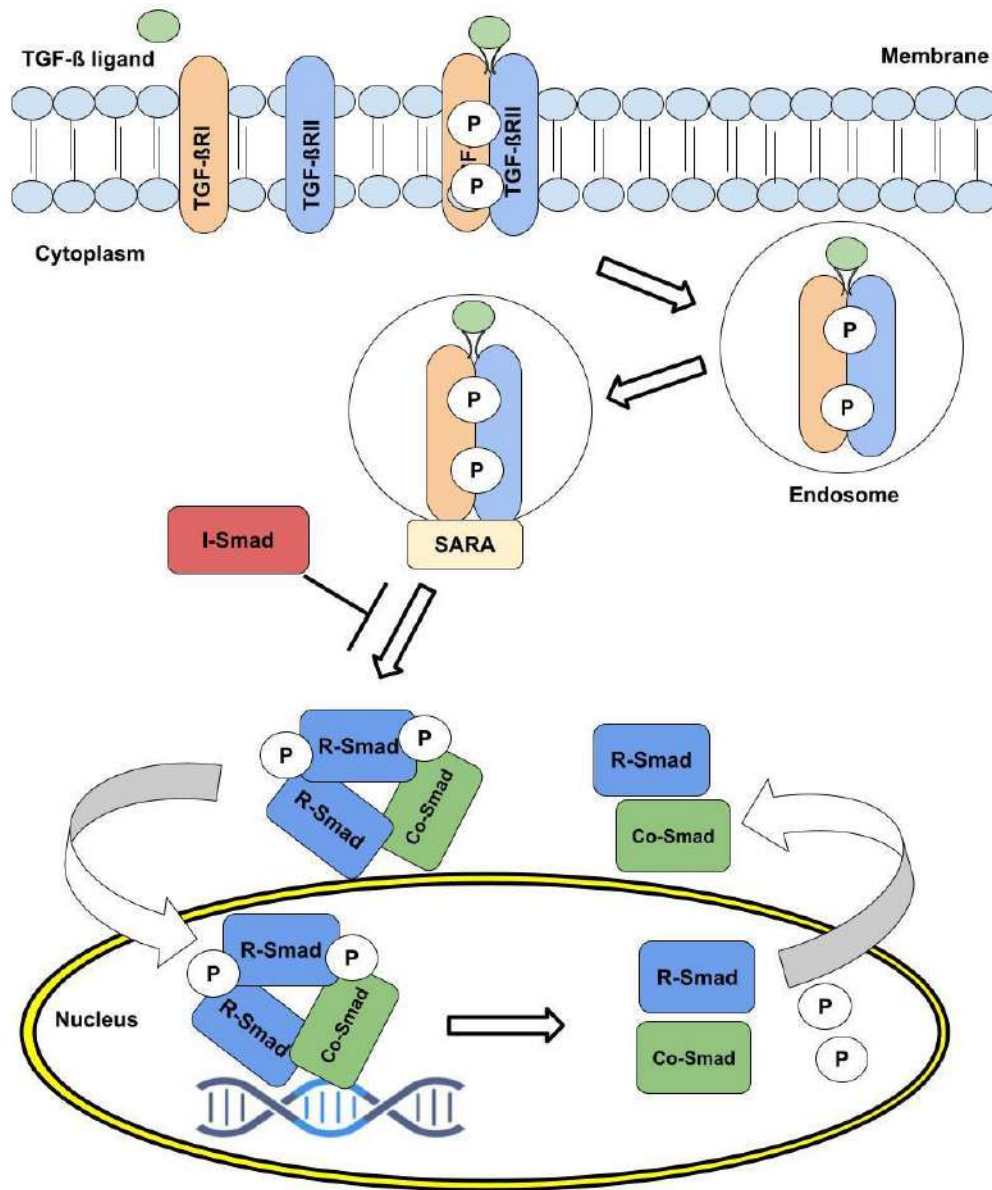


Figure 1.1 Signaling through TGF- β superfamily ligands. The ligand binds to the receptor, which forms a heterotetramer structure of two type-I and two type-II receptors. The receptor complex is endocytosed via clathrin-coated vesicles (CCVs). Type-II receptors phosphorylate the GS segment of type I kinase receptor. The activated type I receptor phosphorylates cytoplasmic R-Smads with the help of SARA. I-Smads compete with R-Smads for binding SARA, acting as a down-regulatory system. R-Smads are then phosphorylated and assembled with one Co-Smad into heterotrimers which are then translocated into the nucleus, where they regulate transcription. R-Smads are dephosphorylated before nuclear export, adapted from Derynck & Zhang (2003).

1.3 Historical context

The TGF- β story began in 1978 when Robert Holley identified a growth-inhibitory factor secreted by African green monkey kidney cells (BSC-1), marking the first hint of TGF- β 's dual role in cellular regulation (Moses et al., 2016). Concurrently, De Larco and Todaro isolated a sarcoma growth factor (SGF) from Moloney sarcoma virus-transformed fibroblasts, which exhibited both epidermal growth factor (EGF)-like activity and transforming potential in vitro (de Larco & Todaro, 1978). By 1981, collaborative efforts resolved the ambiguity: the separation of TGF- α (an EGF receptor agonist) from TGF- β , which operated through distinct receptors (Winkler et al., 1989). This distinction laid the groundwork for recognizing TGF- β as a unique cytokine family.

Next, the purification of TGF- β from platelet-rich serum in 1982 revealed its ubiquitous presence in normal tissues (Deng et al., 2024). Pioneering work by Anita Roberts and colleagues demonstrated TGF- β 's ability to induce fibroblast transformation into soft agar assays, but only in the presence of EGF, highlighting its context-dependent activity (Danielpour, 2024). Notably, TGF- β 's growth-inhibitory effects on epithelial cells emerged in 1984, when Moses's group showed that purified TGF- β suppressed proliferation in AKR-2B fibroblasts (Moses et al., 2016). This bifunctionality—stimulating mesenchymal cell growth while inhibiting epithelial proliferation—became a hallmark of TGF- β biology.

A landmark 1982 study by Joan Massagué identified high-affinity TGF- β receptors through competitive binding assays, distinguishing them from EGF receptors (Massague et al., 1982). Subsequent work in the mid-1980s delineated all three receptor subtypes: type I (T β RI), type II (T β RII), and type III (betaglycan), (López-Casillas et al., 1993). The discovery that T β RI and T β RII form a heteromeric complex essential for signaling provided the first mechanistic framework for TGF- β signal transduction (Gilboa et al., 1998). These findings were complemented by the observation that TGF- β receptors were widely expressed across tissues.

The next step involved the Smad2 and Smad3 as the main effectors of the pathway and direct substrates of T β RI kinase activity (Hata & Chen, 2016). Structural studies further revealed that Smad activation requires precise receptor interplay, with T β RII phosphorylating T β RI, which then phosphorylates Smads (Chaikuad & Bullock, 2016).

Regarding the search for specific functions of the pathway, TGF- β was shown to induce epithelial-mesenchymal transition (EMT) by suppressing E-cadherin—a process critical for embryonic development and cancer metastasis (Hao et al., 2019). Conversely, in keratinocytes, TGF- β promoted differentiation by upregulating cyclin-dependent kinase inhibitors like p15 and p21 (Hasskarl et al., 2000).

Due to these multiple roles, the next phase involves searching for roles of TGF- β in morphogenesis and developmental processes. In this direction, seminal studies in the 1980s–1990s established TGF- β as a master regulator of embryogenesis. Knockout mice lacking TGF-

$\beta 1$ exhibited severe inflammatory phenotypes, revealing its role in immune tolerance (Kulkarni et al., 2002). In developmental contexts, TGF- $\beta 2$ and TGF- $\beta 3$ were found to mediate palate fusion and cardiac septation, respectively, with mutations linked to congenital defects (Azhar et al., 2003). The pathway's involvement in left-right patterning, via nodal signaling, further highlighted its central role in whole embryo morphogenesis.

In the context of adult morphogenesis, TGF- β 's role in wound healing emerged from its ability to stimulate fibroblast recruitment and extracellular matrix (ECM) deposition. Silberstein and Daniel's 1987 experiment, where TGF- β pellets inhibited mammary ductal growth in mice, demonstrated the role of TGF- β signaling in spatial regulation of tissue remodeling (Moses & Barcellos-Hoff, 2011). Several studies by Marti lab and our own lab provided insights into how the pathway regulates neurogenesis in vertebrate embryos (Míguez et al, 2013; Dréau & Martí, 2013). Later work revealed that TGF- β maintains stem cell quiescence in niches such as the intestinal crypt and hematopoietic system, acting as a guardian against uncontrolled proliferation (Liu et al., 2024).

1.4 TGF- β in Cancer

All these key functions being regulated by TGF- β signaling suggest a potentially important role in human disease. The first evidence of this was proposed in the 1990s, When TGF- β 's tumor-suppressive function was linked to its ability to arrest the cell cycle. Loss-of-function mutations in T β RII or Smad4, underscored TGF- β 's role as a guardian against malignant transformation (Pardali & Moustakas, 2007). Interestingly, TGF- β shifts to an oncogenic role as tumors progress, by promoting EMT, angiogenesis, and immunosuppression. Studies in prostate cancer models revealed that TGF- $\beta 1$ overexpression correlates with stromal fibrosis, immune cell exclusion, and androgen receptor (AR) pathway activation (Yang et al., 2014). In advanced cancers, TGF- β suppresses cytotoxic T-cell responses while also promoting the recruitment and differentiation of regulatory T cells (Tregs) and the expansion of myeloid-derived suppressor cells (MDSCs). These immunosuppressive cell populations contribute to the establishment of an immunotolerant tumor microenvironment, which facilitates immune evasion by cancer cells (Batlle & Massagué, 2019; Gordon et al., 2017).

In the initial stages of epithelial cancer, the signaling of TGF- β is pivotal in suppressing tumor growth through mechanisms that induce cell cycle arrest and apoptosis. However, in advanced stages, cancer cells may develop resistance to this growth inhibition, effectively bypassing TGF- β mediated control and shifting its role towards tumor promotion (Danielpour, 2024; Seoane & Gomis, 2017). This dual nature of TGF- β in cancer (acting as both a tumor suppressor and promoter) remains incompletely understood. Recent studies highlight significant knowledge gaps regarding the mechanisms driving the switch between these opposing roles. Further investigation is needed to clarify the interplay between Smad-dependent and Smad-independent signaling, as well as the influence of the tumor microenvironment, on TGF- β 's paradoxical functions (Giarratana et al., 2024; Kim et al., 2021; Shi et al., 2022).

Mutations in Smad proteins, key components of the TGF- β signaling pathway, are implicated in various cancers. Smad4, a recognized tumor suppressor gene, is frequently mutated in pancreatic and colorectal cancers (Mehrvarz Sarshekeh et al., 2017; Wan et al., 2021). Inactivating mutations in Smad4 are also found in prostate, ovarian, breast, lung, and skin cancers, albeit less frequently (Valkenburg et al., 2017; Antony et al., 2010; Liu et al., 2014; Tan et al., 2021; Tuncer et al., 2019). Germline mutations in Smad4 cause juvenile polyposis syndrome, increasing the risk of colorectal and gastric cancers (Dal Buono et al., 2022). Similarly, Smad2 mutations are observed in colorectal cancer, highlighting its role in these pathologies (Fleming et al., 2013).

While Smad2 and Smad4 function namely as tumor suppressors, Smad3 appears to mediate the oncogenic effects of TGF- β signaling (Bertrand-Chapel et al., 2022). Loss of Smad3 expression is observed in gastric cancer, potentially contributing to tumorigenesis (Han et al., 2004). Most Smad2 and Smad4 mutations in tumors affect the MH2 domain, while fewer impact the DNA-binding MH1 domain (Wan et al., 2021). These mutations often increase interaction between the MH1 and MH2 domains, hindering Smad complex formation after ligand activation and reducing signaling efficiency (Kuang & Chen, 2004).

Finally, there is increasing scientific evidence suggesting that TGF- β signaling can be used as a critical biomarker for predicting tumor progression. Consequently, this pathway is targeted in molecular strategies for cancer prevention and treatment, reflecting its significant role in these processes (Giaratana et al., 2024).

1.5 TGF- β in other human diseases

On top of its key role in cancer discussed in the previous section, when altered through mutations in the germline or disruptions in its regulation, TGF- β signaling plays a significant role in several developmental disorders and diseases. Such alterations can lead to various pathologies affecting the muscular, skeletal, and cardiovascular systems and associated with autoimmune diseases, fibrosis, and vascular disorders like atherosclerosis (Goumans & Ten Dijke, 2018; Newfeld & O'Connor, 2022; Deng et al., 2024; Massagué & Sheppard, 2023). Additionally, the dysregulation of this pathway has been linked to urogenital diseases (Deng et al., 2024) and mutations tied to hereditary diseases, such as pulmonary arterial hypertension (Sharmin et al., 2021; Derynck et al., 2021).

This key involvement in so many diseases prompted an increasing interest in searching for drugs that can target the TGF- β pathway at different levels of the chain. The development of galunisertib (LY2157299), a T β RI kinase inhibitor, demonstrated proof-of-concept in phase II trials for hepatocellular carcinoma (Herbertz et al., 2015). In addition, monoclonal antibodies designed to neutralize TGF- β isoforms are currently being evaluated in fibrosis and metastatic cancers.

1.6 TGF- β Ligands

Ligands are key regulatory molecules that initiate signaling cascades by binding to specific receptors, thereby orchestrating a wide range of cellular processes. The TGF- β superfamily comprises a diverse group of signaling molecules with far-reaching biological effects. These molecules, including TGF- β s, inhibins, activins, nodal, bone morphogenetic proteins (BMPs), myostatin, anti-Müllerian hormone (AMH), and growth/differentiation factors (GDFs) and have been shown to influence a wide array of cellular processes in various cell types (Morikawa et al., 2016). As mentioned previously, these cytokines often act as growth inhibitors, playing a crucial role in maintaining cellular homeostasis and preventing uncontrolled proliferation. The TGF- β superfamily is broadly divided into two main subfamilies based on their sequence homology and receptor activation:

The **TGF- β /Activin/Nodal branch** plays crucial roles in various processes, including cell growth and differentiation, embryonic development, and immune regulation. TGF- β s are involved in wound healing, angiogenesis, and immune suppression, while activins regulate hormone production and tissue homeostasis. Nodals are essential for mesoderm and endoderm formation during embryogenesis.

The **BMP/GDF/MIS** branch is primarily involved in bone and cartilage development, tissue morphogenesis, and cell fate determination. While BMPs induce bone formation, GDFs regulate tissue differentiation and growth, while MIS (Müllerian inhibiting substance), ligands are crucial for sexual differentiation during development.

These two branches activate distinct downstream pathways via different Smad proteins. The TGF- β /Activin/Nodal branch activates Smad2 and Smad3, whereas the BMP/GDF/MIS branch activates Smad1, Smad5, and Smad8. These activated Smads then form complexes with Smad4 and regulate the expression of target genes involved in various cellular processes, as we will review later.

1.7 TGF- β receptors

Cellular responses and intracellular signaling cascades typically originate at specific receptors located on the plasma membrane upon ligand binding. In the case of TGF- β signaling, this process begins with the interaction between TGF- β ligands and their corresponding receptors, which possess intrinsic serine/threonine kinase activity. This kinase activity is essential for propagating the signal intracellularly (Heldin & Moustakas, 2016). Interestingly, some TGF- β family members also exhibit robust interactions with epidermal growth factor (EGF) receptors, adding another layer of complexity to their signaling mechanisms (Morikawa et al., 2016).

The TGF- β receptor family comprises two primary subfamilies: type I (T β R-I) and type II (T β R-II) receptors. Both subfamilies share structural similarities, featuring small cysteine-rich extracellular domains and intracellular regions predominantly composed of kinase domains (Maruyama, 2014). Upon ligand binding, these receptors typically assemble into a heterotetrameric complex consisting of two T β R-I and two T β R-II molecules (Giarratana et al., 2024). Phosphorylation of this complex is a crucial step in its activation (Tie et al., 2022).

As explained in the previous section, the TGF- β family of ligands comprises approximately 30 molecules, but signaling is mediated by only seven type I and five type II receptors (Nickel et al., 2018). Interestingly, only specific combinations of these receptors are functional, with each combination capable of mediating the signaling of multiple growth factors (Nair et al., 2019). A distinguishing feature of type I receptors is the presence of a glycine and serine-rich (GS) domain near the membrane, which is absent in type II receptors (Gomez-Puerto et al., 2019). For example, the binding of TGF- β 1, 2, and 3 ligands to T β R-II leads to the activation of a specific T β R-I receptor (ALK1, ALK2, or ALK5) through multiple phosphorylations within the GS domain (Derynck & Budi, 2019). This receptor activation model suggests that T β R-I governs the specificity of intracellular signaling, with phosphorylation events altering its conformation to enable binding with R-Smads (Verrecchia et al., 2006).

Two primary models of TGF- β receptor activation have been proposed. The first model suggests that TGF- β initially binds to pre-formed oligomeric T β R-II on the cell surface, which then recruits and activates T β R-I through phosphorylation (Gu et al., 2024; Chen et al., 1995). Alternatively, a sequential interaction model has been proposed suggesting that ligand binding to T β R-II precedes T β R-I recruitment and activation, with evidence supporting the ability of active T β R-I to signal independently of T β R-II (Huang et al., 2014). This activation mechanism is likely conserved in other serine/threonine kinase receptor complexes (Tie et al., 2022). Focusing now on the specificity of each ligand to a particular receptor, TGF- β ligands exhibit varying affinities for different receptors of the superfamily.

TGF- β ligands, such as TGF- β 1, have been shown to bind to TGF- β receptors via two distinct mechanisms (Deng et al., 2024). First, they can bind to the high-affinity receptor ALK5 or ALK1, subsequently recruiting T β R-II into a hetero-oligomeric complex (TGF- β induced signaling complexes: TISC), (Pomeranec et al., 2015). Alternatively, TGF- β can bind directly to preformed hetero-oligomeric complexes (PFC) consisting of one ALK5 or ALK1 receptor and one T β R-II receptor, increasing the complex's stability and activating the Smad pathway (Bertolio et al., 2021).

Beyond receptor oligomerization, endocytosis of TGF- β receptors also plays a crucial role in modulating downstream signaling (Pomeranec et al., 2015). Upon TGF- β binding, receptors are internalized into early endosomes, where signaling is initiated. These receptors can then be recycled back to the plasma membrane, or targeted for proteasomal degradation (Tzavlaki & Moustakas, 2020). One well-established internalization route is clathrin-coated pit (CCP)-mediated endocytosis, which is essential for signal transduction by various transmembrane receptors (Lampe et al., 2016).

Alternatively, TGF- β receptors can be internalized via a clathrin-independent pathway involving caveolae, specialized membrane invaginations enriched in cholesterol and the protein caveolin (Makvandi et al., 2021). Caveolin-1 (cav-1) has been shown to bind and internalize T β R-II, leading to its translocation into the cytosol and subsequent proteasomal degradation. This cav-1-mediated degradation regulates Smad signaling by reducing cellular T β R-II levels (Xie et al., 2011; Huang & Chen, 2012).

1.8 Smad Proteins

1.8.1 Historical Perspective

Central to the TGF- β signaling pathway are the Smad proteins, which act as the primary intracellular mediators of signals from the TGF- β receptors. Studies using model organisms like *Drosophila* and *C. elegans* have significantly advanced our understanding of how signals from serine/threonine kinase receptors are transmitted to the nucleus (Kimble & Nüsslein-Volhard, 2022). For example, in *Drosophila*, the TGF- β receptor homologues are referred as Decapentaplegic (Dpp) type II and type I receptors (Upadhyay et al., 2017). Genetic screens identified Mothers against dpp (Mad) as a key downstream component of the Dpp pathway, with Mad mutants exhibiting similar phenotypes to Dpp mutants (Hudson et al., 1998; Nakagoshi et al., 1998). Similarly, in *C. elegans*, genetic studies revealed that Sma proteins function downstream of the Daf-4 receptor, which is homologous to TGF- β receptors (Greer et al., 2008; Savage et al., 1996). These findings led to the identification of Mad and Sma homologues in vertebrates, including humans, highlighting the evolutionary conservation of this signaling pathway.

To standardize nomenclature, the term "Smad" was adopted to represent the vertebrate homologues of the Sma and Mad proteins (Massagué & Wotton, 2000). Mammals possess eight Smad proteins. Smad2 and Smad3 are receptor-regulated Smads (R-Smads) activated by TGF- β and activin ligands through type I receptors, while Smad1, Smad5, and Smad8 are activated by other TGF- β superfamily members such as BMPs (Hata & Chen, 2016). Smad4 is the sole common-mediator Smad (Co-Smad), participating in TGF- β /activin signal transduction and initially identified as a tumor suppressor gene frequently deleted or mutated in pancreatic carcinomas (Liu et al., 2021; Zhao et al., 2018). The third class comprises inhibitory Smads (I-Smads), namely Smad6 and Smad7, which act as negative regulators of TGF- β signaling (Zhang et al., 2007).

1.8.2 Structure

R-Smads and Co-Smads share two conserved domains: MH1 and MH2. The MH1 domain, located at the N-terminus, is responsible for binding DNA, interacting with transcription factors, and connecting to the cytoskeleton. The MH2 domain, at the C-terminus, is involved in various functions, including phosphorylation, Smad complex formation, interactions with receptors, transcriptional activation, and targeted degradation (Hill, 2016; Gromova et al., 2007; Massagué & Wotton, 2000). Importantly, all R-Smads have a conserved SSXS motif (Ser-Ser-Val/Met-Ser) at their C-terminal end. Upon ligand binding, type I receptors phosphorylate two serine residues within this motif, triggering downstream signal transduction, such as in the TGF- β pathway (Randall et al., 2004).

The linker region connecting the MH1 and MH2 domains in R-Smads also plays a crucial role in Smad function. This region contains consensus sequences that can be phosphorylated by MAP kinases, providing an additional layer of regulation. Furthermore, the linker regions in most R-Smads and I-Smads contain a PPXY sequence, known as the PY motif, which interacts with WW domain-containing proteins. These interactions further diversify the functional roles of Smads

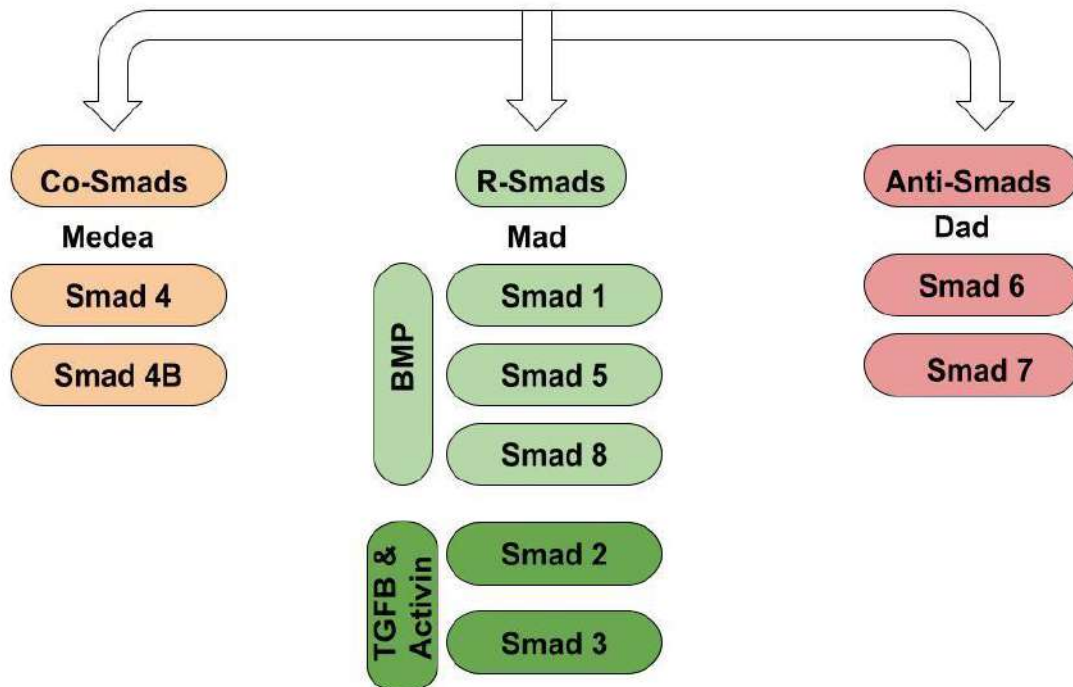


Figure 1.2 The Smad family Dendrogram illustrating the simplified sequence similarity among the three Smad subfamilies. The receptor-regulated Smads (R-Smads) and their cooperating Smads (Co-Smads) and the inhibitory Smads (I-Smads). Adapted from Massagué and Wotton (2000).

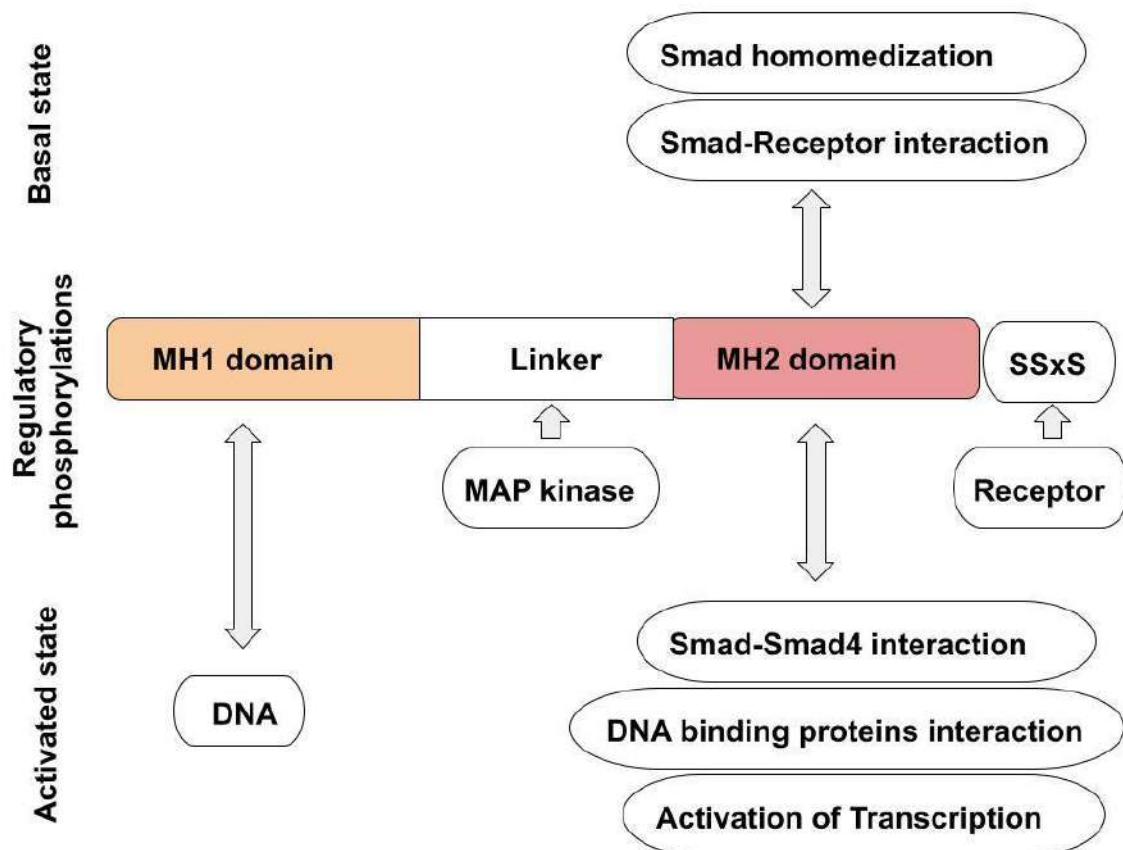


Figure 1.3 The SMAD domains and their respective functions. During the basal state, Smads assemble into homo-oligomers and remain inactive due to the interaction between the MH1 and MH2 domains. Receptor-regulated Smads engage with active type I receptors through the MH2 domain and are activated through receptor-mediated phosphorylation at the C-terminal SS(V/M) S motif. When in the activated state, Smads form associations with Smad4 and DNA-binding proteins through the MH2 domain. The MH1 domain of certain Smads is involved in DNA binding, while the MH2 domain is involved in transcriptional activation. MAP kinases phosphorylate certain Smads at the linker region, adapted from Massagué (1998).

1.8.3 Smad Activation

Phosphorylation, the addition of a phosphate group to a protein, is a common mechanism for activating proteins. In R-Smads, phosphorylation occurs primarily on serine residues within the SSXS motif of the MH2 domain. This phosphorylation is facilitated by the interaction between the R-Smad's L3 loop and the L45 loop of the type I receptor kinase, as well as by anchoring proteins like SARA that localize R-Smads to the plasma membrane (Chaikuad & Bullock, 2016; Tzavlaki & Moustakas, 2020).

Upon phosphorylation by the type I receptor, R-Smads undergo a conformational change that releases the inhibitory effect of the MH1 domain. This allows the R-Smad to dissociate from the receptor and form a complex with the Co-Smad (Smad4), enabling DNA binding and interaction with transcription factors (Hata & Chen, 2016; Miyazawa & Miyazono, 2017; Duan & Derynck, 2019).

Recent studies suggest that phosphorylated R-Smads may remain associated with the type I receptor kinase complex longer than previously thought, potentially influencing the dynamics of intracellular signaling (Deng et al., 2024). This prolonged association suggests that the release of phosphorylated R-Smads from the receptor complex may be a slow process. Furthermore, the presence of released phosphorylated R-Smads could facilitate the dissociation of other phosphorylated R-Smads from the same complex, suggesting a cooperative regulatory mechanism in the TGF- β signaling pathway.

1.8.4 Smad De-activation

Smad activity is tightly regulated not only by phosphorylation but also by dephosphorylation, a process that removes phosphate groups from proteins. Several phosphatases have been identified as negative regulators of Smad activity. For example, pyruvate dehydrogenase phosphatase (PDP) physically interacts with and dephosphorylates R-Smads, inhibiting their signaling (Smith & Hewitson, 2020). Similarly, PPM1A, a nuclear phosphatase, directly dephosphorylates the C-terminal phosphorylated forms of Smad1, Smad2, and Smad3 (Bourgeois et al., 2013).

Interestingly, while phosphorylation of the linker region in R-Smads by MAP kinases inhibits Smad activity, small C-terminal domain phosphatases (SCP1, SCP2, SCP3) can dephosphorylate these linker regions, enhancing TGF- β signaling (Burch et al., 2011; Sun et al., 2015).

1.8.5 Smad Interactions at the cytoplasm level

Multiple processes at the cytoplasm contribute to Smad regulation. Notably, TGF- β receptor endocytosis serves as a principal regulatory mechanism, as ligand binding initiates receptor internalization into early endosomes—a step essential for sustaining Smad-mediated TGF- β signaling (Li et al., 2021). Consequently, receptor-associated proteins may play critical roles in vesicular transport, facilitating TGF- β receptor internalization and Smad recruitment to the receptors (Gu et al., 2024). Multiple protein interactions are thus likely to govern receptor localization at the subcellular level and regulate their availability on the cell surface, ultimately impacting the duration and intensity of Smad phosphorylation and activation (Miyazawa et al., 2024; Runa et al., 2024).

At the cytoplasm, the Smads interact with a variety of proteins, including anchor proteins that modulate their activity. These proteins can directly bind Smads or associate with the receptors to influence Smad-mediated signaling events (Wang et al., 2023). Cytoskeletal components also play pivotal roles in Smad localization and signaling. For example, unphosphorylated R-Smads interact with microtubule filaments, and TGF- β stimulation promotes their dissociation. Disruption of microtubules increases the release of R-Smads and enhances their phosphorylation, emphasizing the importance of cytoskeletal dynamics in regulating Smad activation (Dong et al., 2000). In addition, R-Smads can interact with filamin, a scaffold protein that crosslinks actin, and ELF, a β -spectrin that induces Smad phosphorylation, further integrating cytoskeletal architecture with signaling pathways (Glasgow & Mishra, 2008).

In addition, Smads can also interact with the p53 family of proteins (Elston & Inman, 2012), and in p53-deficient cells, the cytosolic response to TGF- β signaling is impaired (Cordenonsi et al., 2003). Although p53 and Smads bind distinct cis-elements on target promoters, they can interact functionally to synergistically enhance TGF- β -induced transcription (Huang et al., 2023). The p53 protein can directly engage with the MH1 domain of Smad2 in a TGF- β -dependent fashion, intersecting with areas that are crucial for DNA binding and interactions with other transcription factors such as JunD, LEF-1, and Foxo. This interaction has been proposed to influence the dynamic between p53 and Smad2 (Elston & Inman, 2012). While p53 associates with the MH1 domain of Smad2, the C-terminal MH2 domain is available to interact with FAST-1 and Smad4, potentially enhancing the stability and specificity of the DNA-bound p53 complex (Heldin & Moustakas, 2012).

1.8.6 Smad Complex Formation

The formation of protein complexes is a fundamental mechanism that drives a wide range of biological processes, enabling intricate regulatory networks and precise control over cellular functions. After phosphorylation and conformational change, the R-Smads dissociate from the receptor and form homodimers or homotrimers with other R-Smads (Ruiz et al., 2021). These complexes then associate with the Co-Smad, Smad4, forming heterodimers or heterotrimers that translocate to the nucleus to regulate gene expression (Jia & Meng, 2021).

Phosphorylation promotes R-Smad trimerization by inducing interactions between the phosphorylated C-terminal tails and residues within the L3 loop of adjacent Smad molecules (Chacko et al., 2001; Nam, 2023). These heteromeric complexes, including both heterodimers and heterotrimers, exist in various stoichiometries and are found in both the cytoplasm and nucleus, contributing to the complexity of Smad signaling (Chacko et al., 2004).

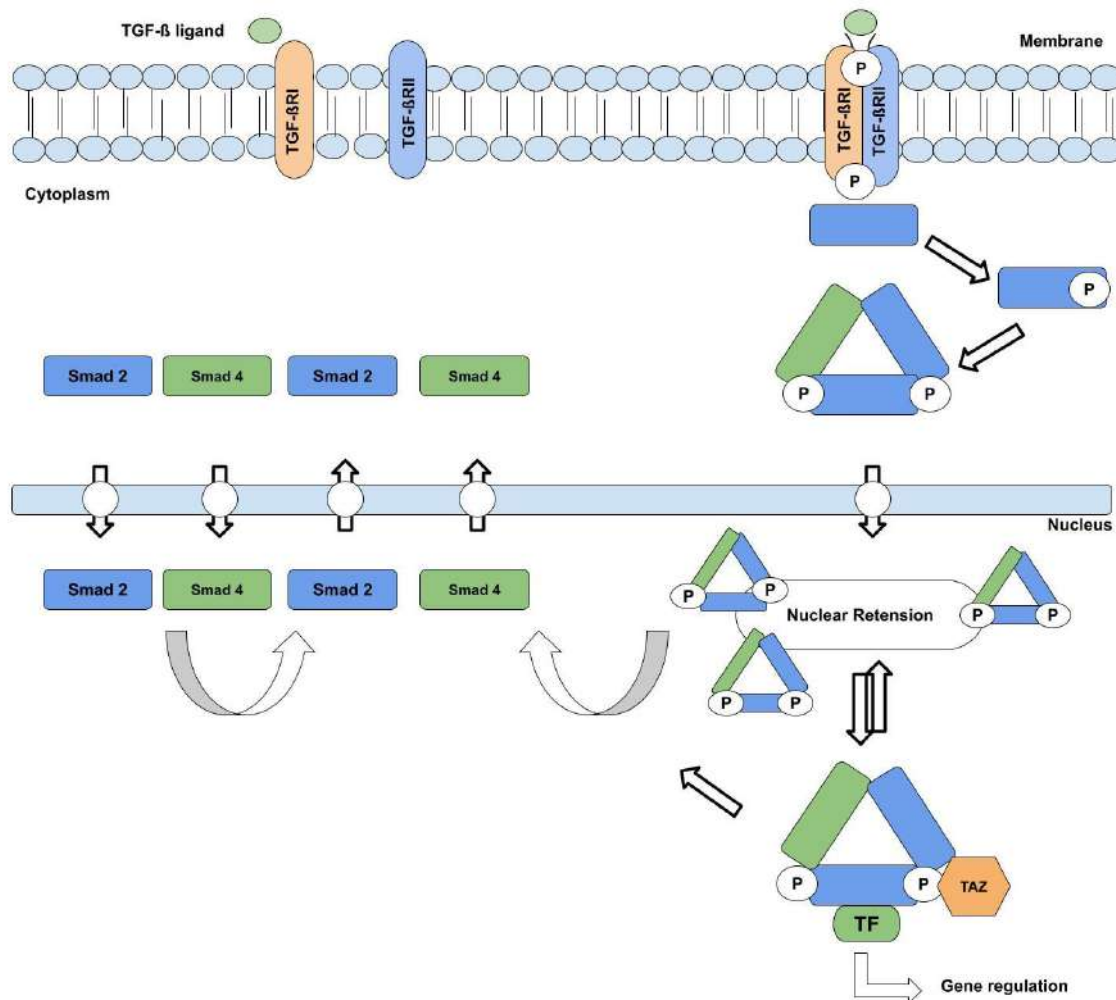


Figure 1.4 model for nucleocytoplasmic shuttling of Smads in uninduced and in TGF- β stimulated cells. Receptor activation by TGF- β ligand binding leads to R-Smad phosphorylation and complex formation with Smad4. In the absence of signaling, Smad2 appears cytoplasmic because its export rate is faster than its import rate. In these conditions Smad4 is distributed approximately equally between the cytoplasm and nucleus. Upon ligand induction Smad complexes accumulate in the nucleus, because of their import being faster than that of monomeric Smads and their export being inhibited, possibly as a result of nuclear retention. The transcriptional regulator TAZ has been shown to be required for ligand-induced Smad nuclear accumulation. Smad complexes regulate transcription in conjunction with other transcription factors (TF). The phosphatase, PPM1A has been proposed to be the Smad2 C-terminal phosphatase, adapted from Schmierer & Hill (2005).

1.8.7 Nuclear entry

The mechanisms regulating Smad nucleocytoplasmic transport have been extensively studied, and it constitutes a very important part of the regulation of TGF- β signaling dynamics. Smad proteins can shuttle passively between the nucleus and cytoplasm, independent of classical nuclear transport factors. This movement is orchestrated by two opposing signals: a nuclear localization signal (NLS) within the MH1 domain and a nuclear export signal (NES) within the MH2 domain (Hill, 2009). Upon ligand-induced activation of TGF- β receptors, Smad heterocomplexes accumulate in the nucleus, likely due to their slow nuclear diffusion and limited export dynamics (Li et al., 2018). Nuclear entry of Smads in response to regulatory cues relies on importins, which recognize NLS motifs and facilitate nuclear import by interacting with nuclear pore components, including the nucleoporins Nup153 and Nup214 (Lu et al., 2021; Nofrini et al., 2016).

1.8.8 Interaction with DNA

Upon entering the nucleus, Smad heterocomplexes regulate gene expression by binding to specific DNA sequences and interacting with various transcription factors. Smad3 and Smad4 directly recognize and bind to Smad-binding elements (SBEs), such as AGAC or GTCT sequences (Morikawa et al., 2013). While Smad2 cannot directly bind DNA, it forms a complex with Smad4 to access SBEs, playing a crucial role in activin and TGF- β signaling (Aragón et al., 2019). Smad complexes regulate a diverse array of target genes involved in various biological processes, including Vent2, BAMBI, Mix2, Smad6, Smad7, Id proteins 1–3, OASIS, Prx2, TIEG, Snail, Hey1, Tcf7, ITF-2, ICSBP, BMP-4, BMP-7, BR-II, Tsg, Msx1, Msx2, GATA2, GATA3, Tlx2, and Tbx2 (Hill, 2016).

Within the nucleus, Smads interact with numerous proteins and complexes, influencing histone modifications and chromatin remodeling to fine-tune gene expression (Ross et al., 2006). The Smads also function as key mediators in transcriptional regulation by interacting with transcriptional co-activators and co-repressors to fine-tune expression of target genes. Among these transcription factors, we have Runx1, Runx2, and Runx3, as well as other transcription factors such as MyoD, OAZ, Vent2, Hoxc-8, Msx1, and transcriptional repressors like SIP1, FoxG1/BF-1 and Menin (Krishnan, 2023; Naito et al., 2005).

1.8.9 Nuclear Export

After regulating target gene expression, Smads are dephosphorylated by specific phosphatases, allowing them to be exported back to the cytoplasm. Once in the cytoplasm, Smads can be recycled for further signaling or targeted for proteasomal degradation. These processes are tightly regulated by various signaling pathways and influenced by the cell cycle stage (Miyazawa et al., 2024).

Nuclear export of Smads involves the general export factor CRM1, which recognizes the NES present in Smad4 and Smad1, but not in Smad2 or Smad3. Inhibition of CRM1 by leptomycin B (LMB) blocks the nuclear export of Smad1 and Smad4, highlighting the crucial role of CRM1 in regulating Smad subcellular localization (Hill, 2009).

1.8.10 Nuclear localization as readout of TGF- β activity

The subcellular localization of Smads is a widely used readout of TGF- β pathway activity, as their translocation from the cytoplasm to the nucleus reflects pathway activation and transcriptional regulation. Several studies have utilized this approach to investigate TGF- β signaling in various contexts:

For example, Altomare et al. (2013) used immunofluorescence and confocal microscopy to demonstrate the nuclear accumulation of Smad2/3 in response to TGF- β stimulation in human keratinocytes. Similarly, Dewidar et al. (2019) showed the nuclear translocation of Smad2 in rat hepatic stellate cells upon TGF- β treatment.

Another example used Subcellular fractionation followed by Western blotting for the quantification of Smad protein levels in different cellular compartments (Luo et al. 2006) to demonstrate the nuclear accumulation of Smad2/3 in response to TGF- β in Mv1Lu mink lung epithelial cells.

In addition, Live-cell imaging with fluorescently tagged Smads was used by Schmierer & Hill. (2005) to track the movement of Smad2 in HaCaT keratinocytes upon TGF- β stimulation, revealing the kinetics of Smad nuclear accumulation.

In addition, time-lapse fluorescence microscopy studies using GFP-tagged Smad2 and Smad4 have demonstrated their continuous shuttling between the cytoplasm and nucleus, regardless of TGF- β stimulation (Gromova et al., 2007). These studies also revealed differences in Smad mobility, with Smad2 being less mobile in the cytoplasm than Smad4 in unstimulated cells, suggesting potential tethering mechanisms.

Recent advances in fluorescence microscopy and labeling techniques have provided more detailed insights into Smad localization and dynamics (Díaz & Malacrida, 2023). Confocal microscopy has been used to examine the subcellular distribution of Smads, explore their nuclear export signals (NES), and investigate the nucleocytoplasmic shuttling of Smad1, Smad2, and Smad3. These studies have revealed that continuous nucleocytoplasmic shuttling is essential for active signaling by R-Smads (Nicolás et al., 2004).

Further investigation into Smad2 dynamics revealed that, in unstimulated cells, Smad2 is predominantly cytoplasmic due to a higher export rate than import rate (Li et al., 2018). Upon TGF- β stimulation, Smad2 accumulates in the nucleus due to decreased export, coinciding with reduced nuclear mobility of both Smad2 and Smad4 (Dai et al., 2009). These findings suggest that TGF- β -induced nuclear accumulation of Smad2 is not caused by release from cytoplasmic retention or increased import, but rather by altered rates of nucleocytoplasmic exchange and mobility, which ultimately govern Smad distribution and activity.

1.8.11 Previous contributions from our lab in the context of TGF- β activity

Our lab has previously studied the role of the TGF- β pathway in the regulation of vertebrate neurogenesis (Miguez et al., 2013). In this context, we combined *in vivo* experiments with a simplified mathematical model to analyze the cooperation and antagonism between Smad2 and Smad3 in regulating their specific targets and influencing neurogenesis. Immunofluorescence experiments revealed that Smad2 and Smad3 are expressed in the ventricular zone of the neural tube, with some enrichment in the transition zone.

We also showed that co-expression of Smad3 with Smad2 decreased activity in reporters of Smad2, suggesting antagonism between Smad2 and Smad3 in regulating Smad2-specific targets. Conversely, co-expression of Smad2 with Smad3 increased Smad3 reporter activity, indicating cooperation between Smad2 and Smad3 in regulating Smad3-specific targets.

In conclusion, the previous studies from our lab suggest that Smad2 and Smad3 cooperate and antagonize simultaneously in the regulation of their specific targets, and that this interplay influences neurogenesis.

II. OBJECTIVES

II. OBJECTIVES

At first glance, signaling via TGF- β family members might appear as a straightforward cascade of biochemical events, in which TGF- β ligands bind to transmembrane serine/threonine kinase receptors, leading to the activation of intracellular Smad proteins that directly regulate gene transcription in the nucleus. Yet, it is now clear that multiple layers of complexity are interwoven into each stage of the TGF- β /Smad pathway—from receptor activation at the membrane to Smad translocation and the intricate modulation of Smad functions within the nucleus. This current work aims to elucidate specific aspects of these complex signaling processes and to address some of the gaps in our understanding of Smad dynamics and function.

The specific aims of this study are:

1. To investigate the translocation of R-Smads in different cell lines during TGF- β pathway activation and inhibition using immunofluorescence, to provide insights into how these Smads behave in different cellular contexts.
2. To analyze the localization of R-Smad when fused to fluorescent proteins: to do this we developed and analyzed R-Smad fusion proteins tagged with fluorescent markers to compare their localization after activation and inactivation of signaling pathways with that of endogenously expressed R-Smads. This analysis helped determine whether the fusion proteins mimic the behavior of endogenous R-Smads during signaling events.
3. To study the translocation of the R-Smads as a physical regulation of the signaling cascade, that operates on top of the well-known biochemical regulation.
4. To understand how the interplay between the biochemical regulation (phosphorylation, degradation...) with the physical layer (compartmentalization) is key to fully understand the dynamics of the TGF- β pathway and its response to activation.
5. To provide the grounds for the development of a future tool based on FRET transfer to study the dimerization of the different R-Smads and how this dimerization depends on the biological context.

These objectives will be approached using a combination of modeling, experiments using tissue culture cells, advanced imaging techniques, with the main final objective of providing detailed insights into the dynamics of R-Smad activation, complex formation, and localization, thus expanding our understanding of the complexities of TGF- β /Smad signaling.

III. MATERIALS AND METHODS

III. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines and Culture

- 1- N2A cells, PC12 cells.
- 2- DMEM supplemented with 10% FBS (Sigma, F7524).
- 3- PBS 10X
- 4- PBS containing 0.05% sodium azide
- 5- P100 dishes (VWR), (Gibco, 16000-044).
- 6- Plate 12 wells (VWR).
- 7- Plate 24 wells (VWR).
- 8- DMSO (VWR)
- 9- Chamber slides (Lab-Tek System 154534).
- 10- Cover slips 24x24mm (Thermo Scientific).
- 11- Eppendorf tube.
- 12- DNA Eppendorf tube.
- 13- Trypsin EDTA (Gibco).
- 14- DNA
- 15- OptiMEM
- 16- ToPro3 (Thermofisher, T-3605, 624/661 nm).
- 17- DAPI (Merck, 268298, 359/461 nm).

2.1.2 Plasmids, Lentiviral Vectors and transfection

- 1- Lentiviral vectors containing Smad 2 and Smad 3 fused with CFP or YFP were developed by our lab in collaboration with Filip Lim.

Plasmids used: pLvX CFP Smad 2, pLvX CFP Smad 3, pLvX YFP Smad 2, and pLvX YFP Smad 3

- 2- Lentiviral transduction was conducted using VSV-G pseudotyped lentiviral particles with reagents provided by Filip Lim (Lim et al., 2013).
- 3- Transfection was done using Lipofectamine LTX 2000 (Thermo Fisher) Plus reagent (ThermoFisher #15338100).

2.1.3 Reagents for Activation and Inhibition Assays

- 1- TGF- β 1 (PeproTech) dissolved in 4 mM HCl and 1 mg/ml with BSA at concentrations of 2 ng/ml and 4 ng/ml.

- 2- Activin A at concentrations of 10 ng/ml, 20 ng/ml, 25 ng/ml, and 40 ng/ml.
- 3- SB-431542 (Sigma Aldrich) dissolved in DMSO at concentrations of 0.15 mM, 0.25 mM, 0.5 mM, and 1 mM.
- 4- Paraformaldehyde (PFA) 4% (Sigma) and 1X PBS (Gibco).

2.1.4 Antibodies

2.1.4.1 Primary Antibodies:

- 1- Rabbit anti-Smad2 (D43B4) XP (Cell Signaling, 5339).
- 2- Rabbit anti-Smad3 (Abcam, ab52903).
- 3- Rabbit anti-P Smad 3 (Thermoscientific, 702292).
- 4- Mouse anti-beta-actin (Thermoscientific, MA5-15739).

2.1.4.2 Secondary Antibodies:

- 1- Alexa Fluor 488 nm (rabbit, ThermoFisher, A21206, 495/519 nm).
- 2- Alexa Fluor 555 (rabbit, ThermoFisher, A21431, 560/576 nm).
- 3- Alexa Fluor 555 (mouse, ThermoFisher, A21137, 560/576 nm).
- 4- Alexa Fluor 594 (mouse, ThermoFisher, A11032, 590/617 nm).

2.1.5 Immunofluorescence and Staining Reagents

- 1- Triton X-100 for cell permeabilization.
- 2- BSA (3%) for blocking reactions.
- 3- Prolong Gold Antifade (Invitrogen) for mounting slides.

2.1.6 Microscopy and Imaging

- 1- Confocal Microscope Nikon A1R+ with Nikon Eclipse Ti-E microscope and NIS Elements 5.20.01 software.
- 2- Objectives: 40X/1.3 oil Plan-Fluor and 60X/1.4 Plan-Apochromatic.
- 3- Fluorescence channels: DAPI, GFP, Texas Red for native cells; Cy5, CFP, YFP for clonal cells.
- 4- Image Analysis: ImageJ software version 1.53 (NIH) and FIJI software version 2.1.0.
- 5- Data Visualization: R software version 4.0.3 (CRAN) and ggplot2 package version 3.3.3.

2.1.7 FRET and Colocalization Analysis

- 1- FRET analysis was conducted using the FRET Analyzer plugin for FIJI.
- 2- Control experiments for donor and acceptor using CFP and YFP fluorophores.
- 3- Five images were captured for each control condition.

2.2 Methods

2.2.1 Cell culturing

N2A cells, PC12 cells were cultured in DMEM supplemented with 10% FBS (Sigma, F7524) in P100 dishes (VWR). Passaging was done using Trypsin EDTA (DMEM and Trypsin EDTA stock solutions). Nuclear staining was conducted using ToPro3 (Thermofisher, T-3605, 624/661 nm), for the clone cells and Dapi (Merck, 268298, 359/461 nm) for the unmodified cells.

2.2.2 Cell infection and generation of stable and clone cell lines

Four expression plasmids encoding Smad 2 or Smad 3 fused to either Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP), (pLvX CFP Smad2, pLvX YFP Smad2, pLvX CFP Smad3, pLvX YFP Smad3) were generated in the Molecular Biology Department of the Sciences Faculty (UAM).

We initially tried to generate stable cell lines by direct transfection of plasmids with Lipofectamine LTX and Plus reagent (ThermoFisher #15338100). Cells were seeded at $2 \cdot 10^3$ cells per well in a 12 well plate (VWR). We diluted 1 µg DNA into 50 µl OptiMEM in an Eppendorf and mixed slowly. We diluted 4 µl of Lipofectamine into 50 µl Optimem and added it by dropping the solution into the DNA Eppendorf and incubated for 20 minutes to form the liposomes. After we replaced the media of cells with 1 ml of OptiMEM. We added 400 µl of OptiMEM into DNA/Lipofectamine solution, added it slowly into the cell wells, and left it for 4 hours at 37°C. After we replaced the media with 2 ml DMEM + 10%FBS and incubated overnight at 37°C and 5% CO₂. Although strong short-term expression (2-3 days) of the fluorescent Smad fusion proteins was observed, we were unable to obtain cells with long-term stable expression.

The plasmids were packaged into VSV-G pseudotyped lentiviral particles following protocols provided by (Dull et al., 1998). Transient cotransfection of 5 µg of the corresponding lentivector plasmid, 5 µg of the packaging plasmid pCMVdR8.74, 38 and 2 µg of the vesicular stomatitis virus G envelope protein plasmid pMD2G (Addgene plasmid 12259) in 10-cm plates of sub confluent 293T cells using Lipofectamine Plus reagent following instructions of the supplier (Invitrogen, Carlsbad, CA). The packaged lentivectors were then used to transduce cells in the following 8 combinations:

- 1)pLvX_CFPSmad2
- 5)pLvX_CFPSmad2_YFPSmad3
- 2)pLvX_YFPSmad2
- 6)pLvX_CFPSmad3_YFPSmad2
- 3)pLvX_CFPSmad3
- 7)pLvX_CFPSmad2_YFPSmad2
- 4)pLvX_YFPSmad3
- 8)pLvX_CFPSmad3_YFPSmad3

We seeded 2×10^3 cells per well in a 12 well plate and 0.5 ml of lentiviral solution (containing approximately 105 lentiviral particles) was added to each well together with 1 ml of DMEM+10%FBS. After 2 days, cells were plated out in 10-cm diameter plates at high dilutions to obtain colonies arising from single cells. All work using lentivectors was performed in the biosafety level II culture room. Lentivectors and Genetically Modified Organisms employed for this project were previously authorized by the UAM Biosafety and Bioethics Committee.

2.2.3 Cell immunofluorescence

Cells were placed in 24-well dishes at 50% confluence with 24x24mm cover slips (Thermo Scientific) and chamber slides from Lab-Tek System 154534. Activation assays were conducted in serum-free media using TGF- β 1 (PeproTech) dissolved in 4 mM HCl and 1 mg/ml BSA at concentrations of 2ng/ml and 4 ng/ml, and Activin A at concentrations of 10 ng/ml, 20 ng/ml, 25 ng/ml, and 40 ng/ml. Inhibition experiments were conducted with SB-431542 (Sigma Aldrich) dissolved in DMSO (VWR) at concentrations of 0.15, 0.25, 0.5, and 1 mM.

Cells were either stimulated or inhibited for 4 hours, then treated with 4% paraformaldehyde (Gibco) and immersed in 1X PBS (Gibco), before undergoing ToPro nuclei staining and being mounted onto slides. Various concentrations of ToPro were evaluated to determine the most effective procedure. Cells were cultured to full capacity in 24-well plates, washed three times, treated with 4% PFA for 30 minutes, and then washed three more times with 1X PBS for 15 minutes at room temperature.

In immunocytochemistry investigations, after fixation, cells were permeabilized by exposing them to 0.1% Triton X-100 in PBS for 10 minutes at 4°C. The cells were subsequently treated with a 3% BSA solution in PBS for 30 minutes at room temperature to prevent further reactions. BSA was removed before adding the primary antibody and incubated overnight. Cells were then washed three times with PBS 10X and incubated with the secondary antibody and nuclear stain for 1 hour. The cells were rinsed three times with PBS and then placed in PBS containing 0.05% sodium azide before being mounted in Prolong (Invitrogen). The primary antibodies utilized were Rabbit anti-Smad2 (D43B4) XP (Cell Signaling, 5339), Rabbit anti-Smad3 (Abcam, ab52903), Rabbit anti-P Smad 3 (Thermoscientific, 702292), Mouse anti-beta-actin (Thermoscientific, MA5-15739). Secondary antibodies utilized were Alexa Fluor 488 nm (rabbit, ThermoFisher, A21206, 495/519 nm), Alexa Fluor 555 (rabbit, ThermoFisher, A21431, 560/576 nm), Alexa Fluor 555 (mouse, ThermoFisher, A21137, 560/576 nm), Alexa Fluor 594 (mouse, ThermoFisher, A11032, 590/617 nm).

2.2.4 Image acquisition and analysis

Fluorescence images were captured using a Confocal Microscope Nikon A1R+ with high sensitivity and fast acquisition speed. The microscope was connected to an inverted Nikon Eclipse Ti-E microscope with NIS Elements 5.20.01 software. Images were recorded with 40X/1.3 oil Plan-Fluor and 60X/1.4 Plan-Apochromatic objectives. Fluorescence images were taken using several channels based on the experiments: GFP, RFP and DAPI with excitation/emission fluorescence at 488/509; 555/584; 395/25, 414/450 respectively for immune fluorescence of native cells; CFP, YFP and Cy5 (Topro3), with excitation/emission

fluorescences at 460/500; 511/16, 529/556; 640/30, 665 respectively for clonal cells.

Distinct laser intensities and gain values were selected for each clone based on their expression level and kept constant throughout the tests. The images were analysed using ImageJ software version 1.53 (NIH) in conjunction with FIJI software version 2.1.0. Intensity was assessed at the nucleus and a cytoplasmic region near the nuclear membrane to determine the overall intensity value for each cell. The data was graphed using R software version 4.0.3 from CRAN and the ggplot2 package version 3.3.3.

2.2.5 FRET and colocalization analysis

We conducted a FRET test on our clone cells to examine fusion protein interaction under various conditions, focusing on Smad2CFP-Smad2YFP and Smad3CFP-Smad3YFP clones. Initially, a donor and an acceptor control are required to determine the fluorescence contribution of CFP and YFP. This analysis should be conducted in a monoclonal cell line, such as Smad2CFP or Smad3YFP, but not with both fusion proteins simultaneously as it would hinder the calculation of contribution coefficients. It is advisable to maintain consistent laser parameters (intensity and gain) throughout all images in the microscope. Therefore, it is suggested to initially employ clone cells containing both fluorophores to establish the microscope settings. Ideally, the values will not excessively saturate cells and reduce background noise. The FRET analyzer plugin for FIJI was used to estimate coefficients, with five photos taken for each fluorophore control. The plugin automatically computes FRET and colocalization images, providing values that may be retrieved by the user.

IV. RESULTS

IV. RESULTS

4.1 Compartmentalization as a mechanism of regulation in the TGF- β pathway

Cells compartmentalize biochemical processes using physical barriers in the form of membranes. Eukaryotes have a wide diversity of membrane-based compartments that can be used in this context, with the main ones being the extracellular membrane which separates the inside from the outside of the cell and the nuclear membrane, (which separates the nucleus from the cytoplasm). The nuclear membrane not only isolates and protects the DNA and the transcription and replication processes from the other processes that are occurring in cytoplasm but also has an active role in the regulation of cellular signaling. The TGF- β pathway achieves compartmentalization using a well-tuned balance between the import and export rates in and out of the nucleus of the active and inactive forms of key proteins. Thus, compartmentalization serves as an additional regulatory mechanism, physically isolating transcription factors from their targets, influencing the dynamics and strength of signal transduction. Here, we focus on this biophysical layer of regulation, using the TGF- β pathway to illustrate the molecular mechanisms underlying this process, as well as the biological consequences of this compartmentalization.

4.2 The concept of compartmentalization

The development and organization of multicellular organisms are mainly regulated by intercellular communication in the form of signaling molecules (Heldin & Moustakas, 2016). These molecules trigger intracellular events that are processed by the cell in the form of signaling pathways, which ultimately lead to changes in the expression, regulation, and dynamics of proteins. Most of these regulatory events are biochemical, e.g., the reversible covalent addition of a phosphate group (phosphorylation), sugar molecules (glycosylation), ubiquitin proteins (ubiquitination), acetyl groups (acetylation), and methyl groups (methylation). These post-translational modifications can alter many aspects of the target proteins: phosphorylation can activate or deactivate enzymes and signaling proteins, acetylation of histones can alter DNA–protein interactions, glycosylation can affect protein folding and stability, methylation can affect the dynamics of protein–protein interactions, and ubiquitination can target a protein for degradation (Zhong et al., 2023).

In addition to these biochemical mechanisms, other types of regulatory mechanisms involve physical constraints. One of the most studied is protein compartmentalization, i.e., intracellular proteins can be directed to specific organelles or regions within the cell, such as the nucleus or the cell membrane. In addition to providing and maintaining organization at the cellular level, this physical separation of processes is highly dynamic (proteins can change location due to specific signals) and has a strong biological regulatory impact (Matschinsky, 2001). For instance, constraining a given enzyme to a specific region results in a localized increase in concentration, enhancing its efficiency. One of the most important cases is the compartmentalization of glucokinase, which can shift location in response to substrate availability (Jetton et al., 2001). In brief, a fraction of the glucokinase can

translocate from the nucleus to the cytoplasm after stimulation, resulting in a reduction in its nuclear to cytoplasmic ratio. Another important protein subjected to compartmentalization is Rubisco, a crucial enzyme in photosynthesis and carbon fixation. The compartmentalization of this protein in high concentrations inside the chloroplasts of bundle-sheath cells or the carboxysomes of cyanobacteria has been shown to affect its catalytic efficiency and, consequently, the overall photosynthetic rate of plants (Erb & Zarzycki, 2018). Interestingly, compartmentalization may restrict the conformational variability in a protein, shaping the evolution of all the living organisms that harvest energy via photosynthesis (Amritkar et al., 2024).

Some proteins are known to change function depending on the compartment to which they are confined. For instance, ERp57, a protein that belongs to the protein disulfide isomerase (PDI) family (Brittain & Gulnik, 2017) regulates protein folding while in the cytoplasm, (Oliver et al., 1999; Kimura et al., 2005). When the protein is inside the nucleus, its main function is as a transcription factor (Grillo et al., 2007; Grindel et al., 2011). This switch illustrates the importance of localization and its impact in function, and it probably affects many other proteins (Scott et al., 2005; Binder et al., 2014).

In our laboratory, we have focused on this concept of compartmentalization in the context of dimensionality reduction (Ruiz-Herrero et al., 2013; Míguez, 2010). This concept refers to the transition from moving in a three-dimensional space (the cytoplasm) to a two-dimensional space where molecules are embedded or associated with the cell membrane (Míguez, 2010). This shift from 3D to 2D movement results in an important change in local concentration, increasing the probability of molecular encounters and interactions by orders of magnitude and, consequently, the rate of biochemical reactions (Doldán-Martelli et al., 2013).

This way, the association of a protein or ligand to a receptor in the membrane catalyzes subsequent reactions by restricting the movement of the ligand to the membrane surface, resulting in an effective increase in its local concentration because the volume in which the molecules can move is significantly reduced. This mechanism of action drives the dynamics of activation of erythropoietin (EPO) (Míguez, 2010). In addition, it is the basis of our design of chimeric drugs that can selectively affect tumor cells versus normal cells (Ruiz-Herrero et al., 2013; Doldán-Martelli et al., 2013).

The bidirectional movement of transcription factors between the nucleus and cytoplasm in eukaryotic cells is a common example of intracellular compartmentalization. This exchange of proteins that are dynamically excluded from the cytoplasm or the nucleus is essential for various cellular functions, including gene expression, signal transduction, and cell cycle regulation. The dysregulation of nuclear translocation is directly involved in a variety of pathological conditions, with a critical role in maintaining cellular homeostasis.

One of the most important pathways that take advantage of this nucleocytoplasmic shuttling to modulate signal transduction is the transforming growth factor beta (TGF- β) pathway. Here, we focus on the effect of transcription factor compartmentalization in the context of the TGF- β pathway and how this physical regulation is utilized to modulate signal transduction.

4.3 R-Smad Nucleocytoplasmic Shuttling as a Modulation of Signal Transduction

As mentioned in the introduction of the present thesis, the R-Smads constitute the direct substrates of TGF- β family receptors and are the key mediators of the TGF- β pathway, translating receptor activation into changes in gene expression levels. One of the main characteristics of R-Smads is their ability to translocate between the cytoplasm to the nucleus, depending on their phosphorylation state and organization as monomers or complexes. The sequence of events in this well-characterized process, commonly referred to as Smad nucleocytoplasmic shuttling, occurs as follows: (a) unphosphorylated R-Smads are mainly located in the cytoplasm as monomers; (b) after activation by the type-I receptor, two R-Smads typically assemble with one Co-Smad in the form of heterotrimers, also known as the activated Smad complex (the complex is stabilized by the phosphorylated serines at the C-terminal); (c) these complexes then translocate into the nucleus where they act as transcription factors (Xu et al., 2016; Bai et al., 2017) ; (d) these complexes are then dephosphorylated via interaction with PPM1A and other phosphatases (Schmierer & Hill 2005), and this de-phosphorylation has been linked to the destabilization of the trimer complex (Hill, 2009); (e) once dephosphorylated, the complexes disassemble; (f) finally, the monomeric R-Smads and Smad4 are exported separately back to the cytoplasm by distinct mechanisms (Hill, 2009).

The two main processes involved in this shuttling are nuclear import and nuclear export through the nuclear pore complex (NPC), a large protein structure embedded in the nuclear envelope that serves as a gateway for the selective transport of molecules between the nucleus and cytoplasm. In brief, the NPCs act as hydrophobic channels (Otsuka & Ellenberg, 2018; Paci et al., 2021) that allow proteins smaller than 20–30 kDa to pass via diffusion, while larger proteins require active transport mediated by importin and exportin receptors (Timney et al., 2016; Yang et al., 2023).

4.4 Dynamics and Regulation of Export and Import of the R-Smads

In recent years, several studies have used different levels of R-Smad protein in the nucleus or cytoplasm to study the mechanisms that regulate this process (Schmierer & Hill 2005). As a result of this complex regulation (review as part of the introduction), the R-Smads in the cytoplasm are mainly in the form of unphosphorylated monomers, while, after activation, they primarily locate inside the nucleus, mostly in the form of complexes. The concentration of R-Smas monomers or complexes in each compartment is determined by the differences in their import and export rates. This way, a higher concentration in the cytoplasm of unphosphorylated R-Smad monomers results from a slower import rate and a faster export rate, compared to the complexes (Song et al., 2022). Each of these rates depends on the ability of R-Smads to interact with NPCs, importins, and exportins in monomer or complex configuration (Song et al., 2022; Pierreux et al., 2000).

4.5 Taking Advantage of Nucleocytoplasmic Shuttling to Study the TGF- β Pathway

As described previously, one advantage of these differential R-Smad levels in cellular compartments is that they can be used as simplified readouts of changes in TGF- β pathway activation, measurable by microscopy. This type of study relies on the direct visualization of the R-Smads or Co-Smads with fluorescence or photoactivatable fusion proteins (Li et al., 2018) or the use of highly reliable and specific antibodies that can be used to measure changes in relative levels and compare the experimental conditions in fixed samples. For instance, the seminal contribution by the laboratory of Caroline Hill (Pierreux et al., 2000) used FLAG-tagged Smad4 and antibodies against Smad2 and Smad3 to show that the inhibition of CRM1-mediated nuclear export results in very fast Smad4 nuclear accumulation, while endogenous Smad2 and Smad3 remain completely unaffected.

The same laboratory (Schmierer & Hill, 2005; Nicolás et al., 2004) developed a fusion of Smad2 with GFP to follow the difference in translocation in unstimulated versus TGF- β - stimulated cells in vivo. This study showed that the nuclear accumulation of the active form of Smad2 is due to a decrease in the rate of the nuclear export of the protein rather than an increase in import (Schmierer & Hill, 2005).

Moreover, it suggested that the import rates of phosphorylated R-Smads (in complex configuration) and unphosphorylated R-Smads (mainly in monomer configuration) are similar, indicating that import proteins act similarly in the activated and deactivated states. If this is correct, the import rate of R-Smads as complexes should be twice as fast as that of the R-Smads as monomers, since each complex is composed of two molecules of R-Smad. On the other hand, the fact that the rate of export of the phosphorylated form of R-Smads is much smaller than the rate of export when unphosphorylated suggests that the configuration as complexes prevents binding with exportin 4.

Another important example that utilizes nucleocytoplasmic shuttling was reported by Liu et al. (Zi et al., 2011), where they studied how variable dose levels of ligands are translated into intracellular signaling dynamics and how continuous ligand doses can be translated into discontinuous cellular fate decisions. In this study, they found that the pathway processes continuous and pulsating TGF- β ligand levels differently, displaying varied sensitivities to short and long pulses, also finding that it displays different sensitivities in response to short and long pulses of ligand. These two types of dynamic responses may be crucial for the role of the TGF- β pathway in mediating stem cell differentiation.

The differential concentration of R-Smads as a readout was also used (Sorre et al., 2014) to study the temporal signal by the TGF- β pathway and its implications for embryonic

patterning. This time, the authors took advantage of the nuclear accumulation dynamics of a GFP- Smad4 fusion protein to measure TGF- β pathway activity in response to different types of ligand stimulation. The same fusion protein was used to show pulsatile behaviors in Smad4 nuclear localization (Warmflash et al., 2012). Similarly, quantitative measurements of Smad2/3 nuclear localization illustrated that the TGF- β pathway exhibits fold-change detection properties (Frick et al., 2017), i.e., cells respond to relative rather than absolute levels of TGF- β . In another context, the accumulation of R-Smads in the nucleus was the main readout used to study the crosstalk between oncogenic Ras and TGF- β signaling (Kretzschmar et al., 1999), since Ras activation inhibits the TGF- β -induced nuclear accumulation of Smad2 and Smad3.

In our laboratory, we have studied how different R-Smad accumulations influence the role of the TGF- β signaling cascade during vertebrate spinal cord formation (Míguez et al., 2013; Sanz & Míguez, 2014). In these studies, we quantified the changes in the nuclear localization of the two R-Smads to explore the effects of the interplay between Smad2 and Smad3. We showed that the heterotrimers Smad2–Smad3–Smad4 induce cooperation and antagonism in the activation of the targets of the two R-Smads and that this dual role strongly influences the balance between neural stem cell proliferation and differentiation (Míguez et al., 2013). Using immunofluorescence and confocal imaging, we visualized and quantified R-Smad expression patterns at different developmental stages, showing pathway activation (i.e., R-Smads become more nuclear) in the transition zone (where stem cells cycle and perform interkinetic nuclear migration) and the differentiated zone (where differentiated neurons migrate after exiting the cell cycle). The study was complemented by numerical simulations that allowed us to interpret the dual antagonism and cooperation as a consequence of Smad heterotrimer assembly (Míguez et al., 2013).

4.6 Smad2 and Smad3 endogenous localization in vitro

To characterize the state of activation of the TGF- β pathway in tissue culture cells, we take advantage of this translocation of the active complexes of R-Smads and measure using confocal microscopy and immunofluorescence the localization of both Smad2 and Smad3 using specific antibodies. We also include immunostaining with antibodies that recognize phosphorylated forms of both Smads, to test if localization correlates with activation, as suggested by the literature, and reviewed in the introduction. The protocols for tissue culture, immunofluorescence, image acquisition, and image quantification are detailed in the Methods section.

Since we are ultimately interested in the dynamics of activation and deactivation of the pathway during neurogenesis, we choose for these experiments N2A cells (also known as Neuro2a cells), a fast-growing mouse neuroblastoma cell line. The N2a cell line has a neuronal and amoeboid stem cell morphology, and they can differentiate in response to

environmental factors. The differentiated cells have many properties of neurons, including neurofilaments.

Examples of the images taken are shown as part of Figure 4.1. We can see that endogenous Smad2 is mainly cytoplasmic. Based on the previous research that correlates activity of the pathway with localization, this configuration will fit with a situation where Smad2 is mainly in monomer configuration, and the TGF- β signaling is low. This configuration seems to not change in the conditions tested, since activin A and the inhibitor SB do not modify this balance. Interestingly, when we look at the images of the Phospho-Smad2 (second row), we observe weak intensity levels that would correlate with the situation of low pathway activation. These levels seem to increase after Activin A treatment (middle slide), and several cells with high levels of Phospho-Smad2 can be observed. Interestingly, these cells appear more rounded and with the chromatin condensed, a typical configuration of M-Phase. The fact that we observe such high levels of phosphorylation of Smad2 in cells undergoing mitosis, suggests an important correlation of Smad2 activation with the cell cycle. This makes sense, since the antibody recognizes a Thr8-phosphorylation site that has been shown in previous studies to be a target of the MAPK pathway.

On the other hand, the rest of the cells appear also with slightly higher levels of immunofluorescence, indicating that there is some crosstalk between the MAPK and the TGF- β (Activin A interacts with the receptors of the TGF- β pathway) at the level of this phosphorylation site. Inhibition of the pathway using SB-431542 (right panel) slightly reduces again the phosphorylation, to levels similar to the control (left panel). This correlates with the localization of Smad2 (upper row): since Smad2 is mainly cytoplasmic in control conditions, inhibiting more the pathway should not result in change in this nuclear/cytoplasmic ratio.

On the other hand, Smad 3 in control conditions (left panel) displays higher heterogeneity in its cellular localization, with some cells showing nuclear localization and others showing cytoplasmic localization. This suggests that despite having Smad2 localization that correlates with pathway inactivation, Smad3 correlates with a situation of variable pathway activation. This further suggests that there are strong differences in the activation levels of the two R-Smads, despite being assumed to be activated by the same set of membrane receptors.

Moreover, in response to activation with Activin A (central panel), the levels of nuclear Smad3 are clearly increased, suggesting that the pathway is clearly responding to activation. There is also some heterogeneity, suggesting that not all cells respond equally, despite being clonally equivalent. This heterogeneity could also suggest a dynamic localization of the Smad3, or even a correlation of the localization with the cell cycle. This hypothesis needs further exploration.

Finally, the inhibition of Smad3 with SB-431542 also shows a clear phenotype, with cells showing a decreased nuclear/cytoplasmic ratio. This change correlates well with the

previous literature, and with the assumed organization of the signaling mediated by TGF- β (inhibition should reduce the number of complexes, and the monomers have a preference to remain in the cytoplasm).

Based on the current model for the TGF- β pathway, the amount of phosphorylated Smad3 in the cytoplasm should be very low, (phosphorylated R-Smads form trimers with Smad4 and move to the nucleus, where they drive transcription). This scheme does not correlate well with our findings after immunostaining with phospho-Smad3 antibody (Figure 4.1, lower panel), where we see in control conditions (left panel) that most Phospho-Smad3 is at the cytoplasm, in the majority of cells. Phospho-levels seem to increase after Activin A stimulation, and move towards the nuclei, as expected, suggesting that the antibody is working as expected.

Finally, the inhibition (right panel) translocates the phosphorylated Smad3 back to the cytoplasm as expected. Altogether, these results based on the phospho-Smad3 staining challenge the previous understanding of how the TGF- β signaling operates, suggesting that complexes can be located preferentially at the cytoplasm, or that not all phosphorylated Smad3 is forming complexes (Hough et al., 2012; Kamato et al., 2013). Validation of these hypotheses needs further analysis.

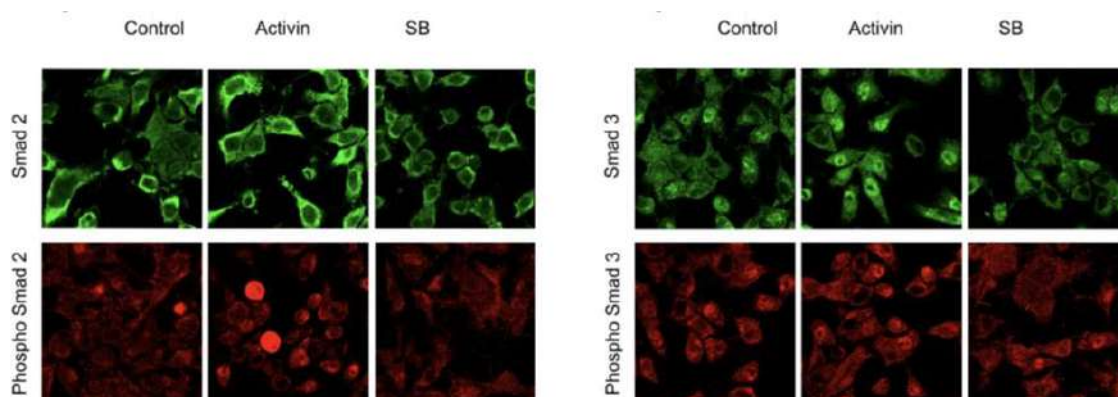


Figure 4.1: Endogenous Smad2 and Smad3 in N2A Cells. First row: Confocal images of endogenous Smad 2 and Smad3 in N2A cells under different conditions. control (left), activation using Activin A (center) and inhibition using SB-431542 (right). Second row: Immunofluorescence using phospho-Smad2 (left) and phospho-Smad3 antibodies (see methods) in the same three conditions. control (left), activation using Activin A (center) and inhibition using SB-431542 (right).

To test if these results are statistically relevant, and to obtain a detailed quantitative characterization of the observations, we perform measurements of the nuclei intensity and cytoplasmic intensity in around 100 cells per condition. Several sets of these images are then used to compute the average nuclear and cytoplasmic intensities in different conditions. Results of this quantification are shown as bar plots in Figure 4.2

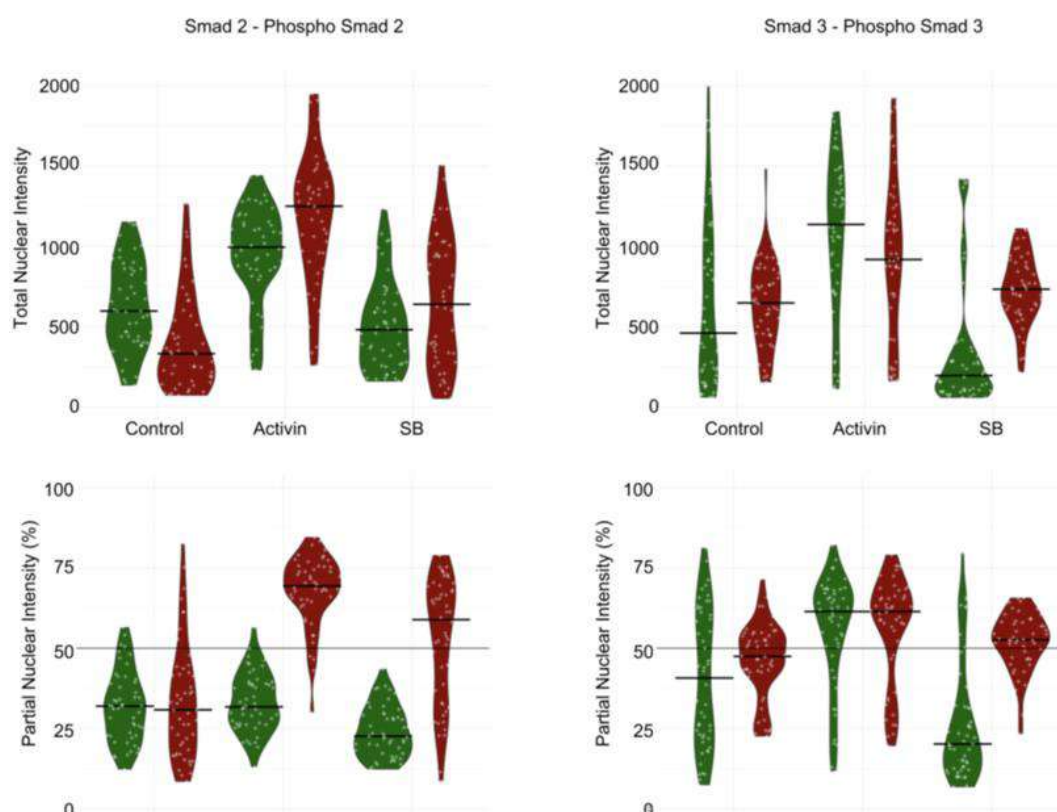


Figure 4.2: Analysis of Smad 2 (left) and Smad3 (right) for several images (around 100 cells per condition) of N2A cells. Panels show the intensity levels in the nuclei of the endogenous protein (green column) and Phosphorylated (red column) for the three conditions (nuclear intensity levels are considered to correlate with pathway activation).

Results of the quantification correlate well with the visual observations. The concentration of both Smad2 and Smad3 (green columns) increase when the pathway is activated and decrease to levels similar to control when the pathway is inhibited. This will suggest that, or the inhibitor is not working as expected, or that the pathway is mainly inactive in control conditions (so, it is insensitive to further inhibition). On the other hand, the levels of the phosphorylation increase as well, following the same trend and the levels of the endogenous protein. This suggests that there is a direct correlation between nuclei intensity and phosphorylation levels, in agreement with the current model of the TGF- β signaling dynamics.

To validate if these results are general or depend on the cell line used, we proceed to characterize the same endogenous levels of Smad2, Smad, and their relative phosphorylation in PC12 cells. The PC-12 cell line has been derived from a transplantable rat pheochromocytoma. It is one of the most commonly used cell line in neuroscience research, including studies on neurotoxicity, neuroprotection, neurosecretion, neuroinflammation, and synaptogenesis, due to its extreme versatility for pharmacological manipulation, ease of culture, and the large amount of information relative to their proliferation and differentiation. Images obtained using a confocal microscope after immunofluorescence staining are shown in Figure 4.3

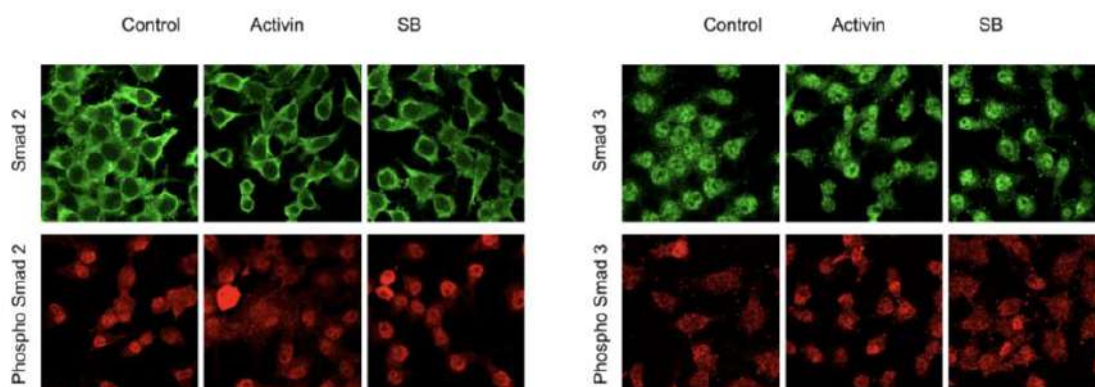


Figure 4.3: Endogenous Smad2 and Smad3 in PC12 Cells. First row: Confocal images of endogenous Smad 2 and Smad3 in PC12 cells under different conditions. control (left), activation using Activin A (center) and inhibition using SB-431542 (right). Second row: Immunofluorescence using phospho-Smad2 (left) and phospho-Smad3 antibodies (see methods) in the same three conditions. control (left), activation using Activin A (center) and inhibition using SB-431542 (right). Fluorescence images were taken using the 40x oil objective.

Visual observation of the Smad2 levels (figure 4.3, upper left) show a localization of endogenous Smad2 that is more cytoplasmic, compared to the N2A cells in Figure 4.1. No change in this configuration after activation or inhibition is observed. The lower panel shows again that the phosphorylation levels increase in cells undergoing mitosis, suggesting again that the TGF- β pathway is involved in the process also in this cell line. No clear increase of the total levels of phosphorylation of Smad2 observed after inhibition or activation, contrary to the N2A cells (Figure 4.1).

Focusing on the Smad3 protein (Figure 4.3, right panel), we see again that the immunofluorescence levels show a higher nuclear intensity, evidencing that this part of the signaling pathway is more active. Again, this does not correlate with the current model of how TGF- β signaling operates, since the nuclear concentrations are supposed to correlate with activation levels, and both R-Smads are assumed to be targets of the same receptor complexes.

A potential explanation of this discrepancy is that the Smad2 antibody is able to enter cytoplasm, but not the nuclei of the cells (more evidence by other studies in the lab suggests that this is the case, as we will see later when we introduce the R-Smad fusion proteins).

On the other hand, the immunostaining using an antibody that recognizes the phosphorylated form of Smad3 shows a staining that is both cytoplasmic and nuclear in control conditions. This result also does not correlate with the hypothesis that phosphorylated Smad3 should translocate to the nucleus. When the pathway is inactive, the intensity levels of phosphorylated Smad3 increase and translocate to the nucleus, as expected. Inhibition (right panel) results in levels of phosphorylation similar to control conditions.

Next, similarly to the N2A cells, we performed a quantitative characterization of these levels in around 100 cells per condition, measuring the intensity of the signal in the nuclei using FIJI. Results shown in Figure 4.4. show that now the treatment is not having the response

expected. In fact, Activin A now does not result in an increase in nuclear levels, and also in phosphorylation levels. On the other hand, inhibition is reducing the intensity levels in the nuclei, as expected. We hypothesize that the TGF- β pathway in this cell line is behaving aberrantly, perhaps due to a mutation or a problem upstream of the R-Smads.

Comparison of the response of the pathway in PC12 versus N2A suggest that the dynamics of the pathway is very dependent on the type of cell used. Therefore, if we want to develop tools based on fusion proteins of the R-Smads, we should be testing their function and behavior in a cell line that does behave as expected. In this particular case, the N2A cell line seems better suited for testing the fusion proteins, since it responds closer to the expected behavior (despite showing much higher phosphorylation levels in the cytoplasm than expected). In the next section, we will test fusion proteins of the R-Smads with different fluorescent proteins and compare their performance and dynamics with the response shown by the endogenous R-Smads in the N2A cell line.

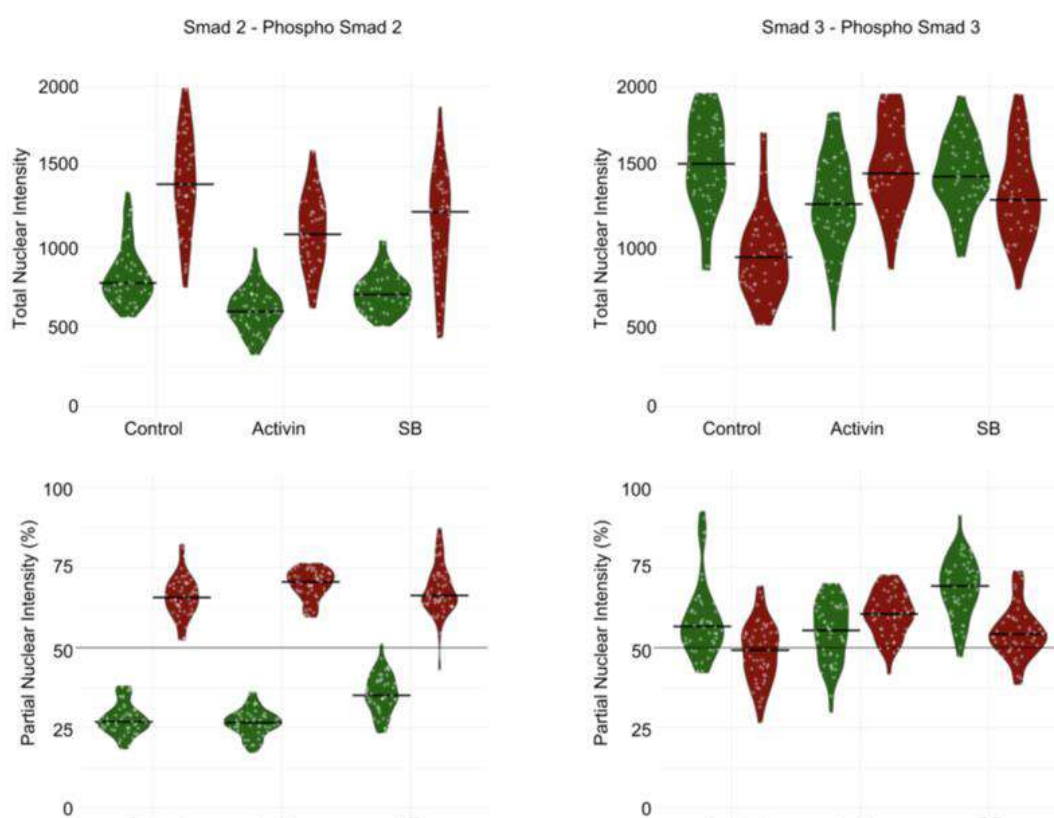


Figure 4.4: Analysis of Smad 2 (left) and Smad3 (right) for several images (around 100 cells per condition) of PC12 cells. Panels show the intensity levels in the nuclei of the endogenous protein (green column) and Phosphorylated (red column) for the three conditions (nuclear intensity levels are considered to correlate with pathway activation).

4.7 Smad2 and Smad3 fusion proteins

The use of molecular constructs where two proteins are linked via a peptide linker—has revolutionized the study of cellular dynamics, enabling real-time visualization and quantification of protein behavior. By fusing proteins of interest with fluorescent markers

quantification of protein behavior. By fusing proteins of interest with fluorescent markers such as green fluorescent protein (GFP) or its variants, researchers gain unprecedented spatial and temporal resolution to track localization, interactions, and conformational changes.

One of the most relevant examples of this approach involves p53 fusion to GFP to the C-terminus to show the importance of its localization in its function as tumor suppressor (Stommel et al., 1999). Also, a key contribution of this type of fusion protein was used to show how Calreticulin deficiency resulted in nuclear accumulation of p53-EGFP due to impaired Mdm2-mediated export (Mesaeli & Phillipson, 2004). Another very important protein involved in more than 50% of all advanced cancers is PTEN, a lipid phosphatase antagonizing PI3K/Akt signaling.

In this case, authors fused PTEN with cyan and yellow fluorescent proteins (CFP/YFP) to study changes in PTEN activity when in a membrane-bound state (Kagan et al., 2025). In the context of development, the fusion of Hh-GFP was used to study morphogen diffusion, distribution and signaling range (Hatori & Kornberg, 2020).

In the context of TGF- β , photoactivatable GFP (PAGFP)-Smad fusions were used to independent tracking of nuclear import and export (Schmierer & Hill, 2005). In uninduced cells, Smad2 export exceeded import, resulting in cytoplasmic predominance. TGF- β stimulation reduced nuclear export rates by 60%, shifting the equilibrium toward nuclear retention. The N-terminal MH1 domain was critical for this regulation, as its deletion abolished nuclear imports.

This strategic fusion of Smad proteins with fluorescent markers has provided unparalleled insights into TGF- β signaling dynamics. Beyond foundational studies on Smad2 and Smad4, recent innovations in biosensor design and imaging methodologies have uncovered novel regulatory mechanisms across multiple Smad family members. For instance, GFP-Smad4 fusions were used to study protein mobility under varying signaling conditions (Nicolás et al., 2004). In addition, a tripple tagging system (Smad2-Cerulean, Smad3-Venus, Smad4-mCherry) revealed higher-order complex dynamics (Nakao et al., 1997). In addition, building on earlier PAGFP-Smad2 studies, a dual photoactivatable system (Smad3-PAmCherry/Smad4-PAGFP) enabled simultaneous tracking of two Smad species (Schmierer & Hill, 2005), illuminated post-translational regulation mechanisms of Smad3, showing that its stability is highly compartment-dependent—a feature undetectable without fluorescent fusion tags.

In this direction, our lab has developed fusion proteins of the R-Smads to be used as tools to test the dynamics of the TGF- β pathway signaling in the context of neurogenesis. Here, we proceed to perform experiments designed to validate them as reporters that can be used to track the state of activation of the pathway. Figure 4.4 shows two images taken with a confocal microscope of N2A infected with lentivirus containing fusion proteins of CFP-Smad2 (left) and YFP-Smad2.

Interestingly, and despite the very high levels of protein expression, cells seem healthy and proliferate normally (an important point if we want to use these proteins as readouts of the pathway activation). Second, the localization of Smad3-YFP seems similar to the endogenous

Smad3, suggesting that their localization is not affected by the fusion protein being linked. Interestingly, when we use mCherry as a fusion protein, Smad2 does not translocate as expected. (see Figure 4.8). On the other hand, the Smad2 fusion protein localization does not correlate with immunofluorescence data (see Figure 4.5 compared to Figure 4.1)

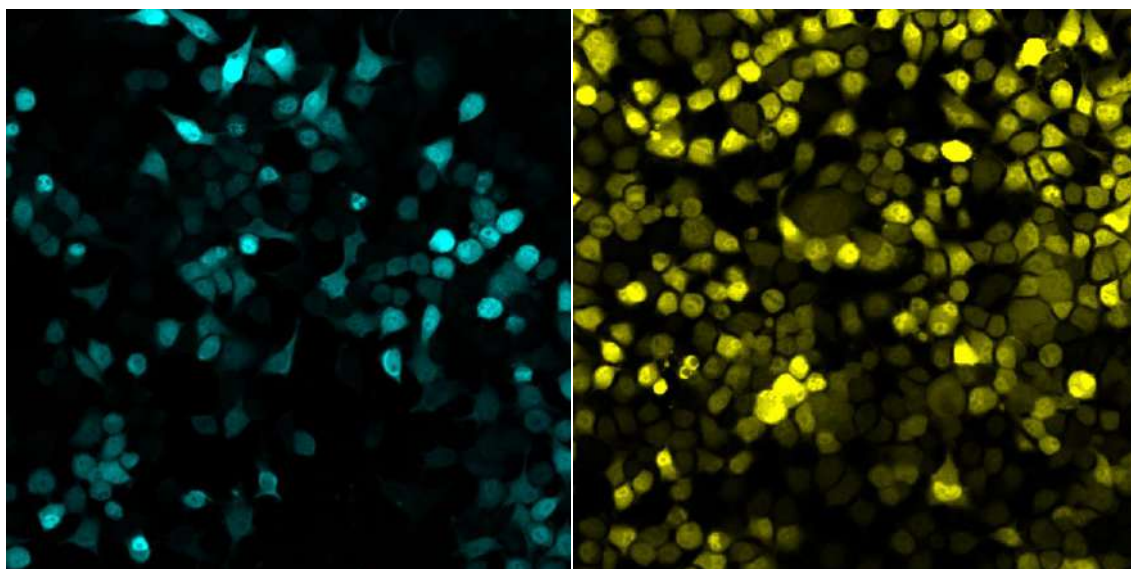


Figure 4.5: Examples of fusion proteins of CFP-Smad2 (left) and YFP-Smad2 (right) in N2A cells. The localization of Smad2 is more cytoplasmic than the endogenous levels monitored by immunofluorescence against endogenous proteins. This, combined with the fact that there is no clear change in the localization of endogenous Smad2 after Activin A treatment. Despite seeing a clear increase in nuclear levels of the phosphorylation of Smad2, it suggests that the antibody against Smad2 may not be penetrating in the nuclei.

This, combined with the fact that there is no clear change in the localization of Smad2 after Activin A treatment. Despite showing a clear increase in nuclear levels of the phosphorylation of Smad2, it suggests that the antibody may not be penetrating in the nuclei. To test this, we performed an additional experiment where we performed immunofluorescence against Smad2 using the same antibody as before, but now over these cells are infected with Smad2-YFP. Results are shown in Figure 4.6.

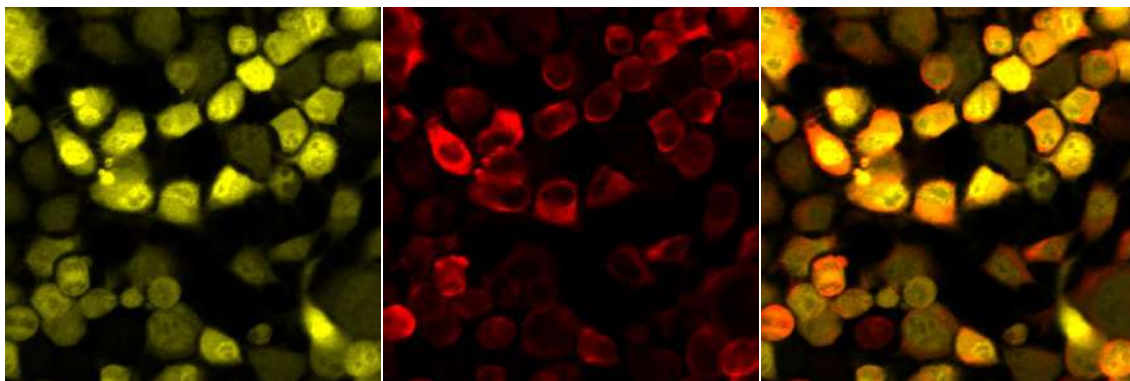


Figure. 4.6: Snapshot showing N2A cells showing Smad2-YFP (left), immunostaining against Smad2 (center) and overlay of both channels (right). The antibody recognizes the exogenous protein (cells with higher yellow intensity appear also as more red), but the nuclear/cytoplasmic pattern is very different.

If antibody staining is performing as expected, we should see a similar profile by immunofluorescence and by the fusion protein imaging. Also, if the antibody recognizes the exogenous protein, we should see an increase in antibody staining in cells that express higher levels of the fusion protein. Although this seems to be the case (cells that are more yellow are more red), the patterns of the immunofluorescence and the fusion protein are clearly different (as shown by the overlay of both channels). Next, we test if the antibody against phosphorylated Smad2 recognizes the exogenous protein. This experiment is illustrated in Figure 4.7.

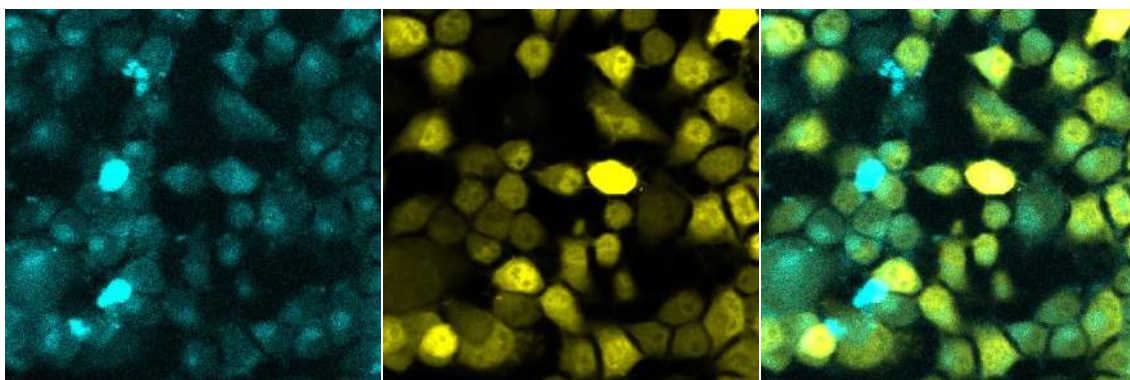


Figure 4.7: Staining after immunofluorescence using the phospho-Smad2 antibody (left). Confocal image of the YFP channel, showing the location of the YFP-Smad2 protein (center). Overlay of both channels (right) shows uncorrelated staining, suggesting that the antibody does not recognize the phosphorylated form of the exogenous protein.

If the antibody recognizes the phosphorylated form of the exogenous protein, we should see a certain correlation between the intensity levels of both channels of the image. On the contrary, cells with higher levels of YFP-Smad2 do not show higher levels of Phospho-Smad2. This can be explained because the phosphorylation levels detected by the antibody are highly dependent on the cell cycle state (close to background in all stages of the cell cycle, but in M-phase, where levels appear increased).

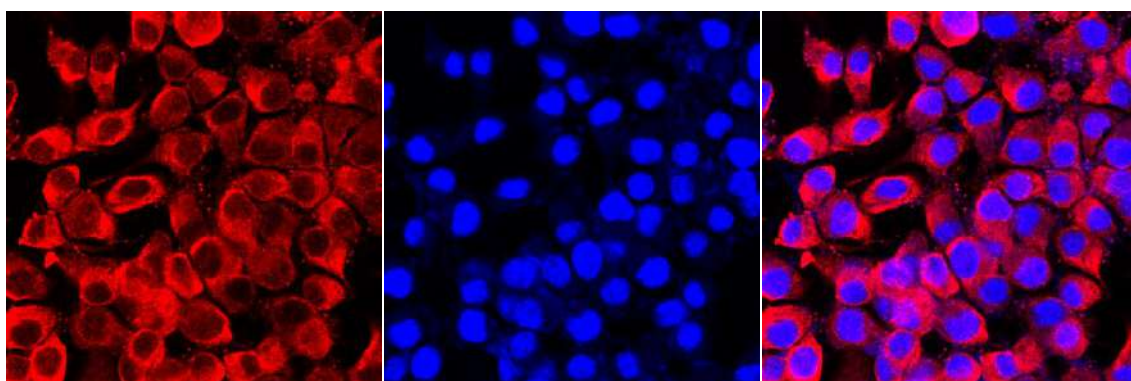


Figure 4.8: N2A cells infected with the Smad2-mCherry fusion protein showing mainly a purely cytoplasmic configuration, suggesting that this fusion inhibited the correct localization of the Smad2, contrary to what happens with Smad2-YFP and Smad2-CFP.

Now let's focus on Smad3 fusion proteins. To do that we infect N2A cells with a fusion protein of Smad3 fused to CFP and let the cells grow several passages, to ensure that the DNA has been incorporated stably into the DNA. Comparison of growth rate of the cells and the aid type is similar, showing that the over expression of these fusion proteins does not damage the cell in a manner that can impact the cell cycle. Then we perform immunostaining against the phosphorylated form of Smad3 using the same antibody as in the characterization of the endogenous proteins. Results of this experiment are shown in Figure 4.9.

Focusing first on the localization of the fusion protein, we see that it is similar to the localization of the endogenous protein. This is important since previous attempts to generate a fusion protein of Smad3 with a fluorescence protein by other laboratories failed to reproduce the dynamics and translocation shown by endogenous.

Focusing now on the ability to get phosphorylated, we see that there is a clear correlation between the levels of immunostaining against phospho-Smad3 (red, central panel) and the levels of our endogenous protein Smad3-CFP (left channel). This correlation (shown also as an overlay of the two channels, right panel) suggests that the exogenous protein is being phosphorylated similarly to the endogenous. These results suggest that the Smad3 fusion protein can be used as a readout of pathway activation similarly to the endogenous Smad3.

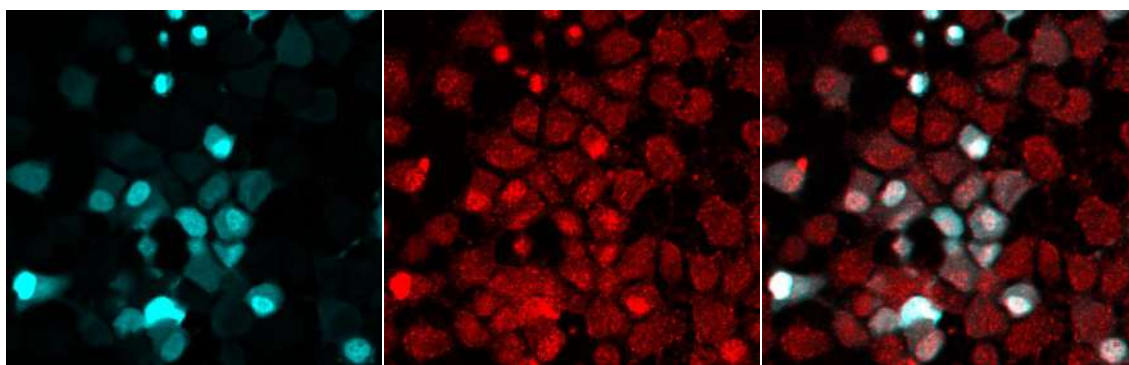


Figure 4.9: N2A cells infected with the Smad3-CFP fusion protein and stained with immunostaining against phosphorylated form of Smad3. The staining shows that the Smad3 fusion protein is being phosphorylated and recognized by the antibody.

4.8 Mathematical Modeling of Regulatory Compartmentalization

Mathematical modeling in molecular biology is a complementary approach to the more time-consuming and expensive experimental approach, which can help explain results, make predictions, and test hypotheses that complement *in vivo* and *in vitro* studies. In the context of TGF- β signaling, several key contributions have combined modeling approaches with experimental results to try to understand the different aspects of the regulation of compartmentalization as a physical regulation of gene expression (Zi et al., 2011; Míguez et al., 2013; Schmierer et al., 2008).

One of the earliest approaches to studying the TGF- β pathway dynamics using numerical simulations focused on the dynamics in experiments of fluorescent recovery after photobleaching (FRAP) (Schmierer et al., 2008). The results support the hypothesis that there is a dynamic maintenance of Smad nuclear accumulation during signaling (Schmierer et al., 2008). In another study, the authors developed a model that shows how cells can integrate repeated pulses and maintain the phosphorylated Smad2 concentration (Zi et al., 2011). The main conclusion derived from the model is that the pathway is able to respond with different sensitivities to different ligand doses and time scales (Zi et al., 2011).

One main caveat of these early approaches is that the models used are composed of around 20 ordinary differential equations, making them very difficult to interpret. More recent examples have opted for more simplified but less general mathematical formulations, for instance, using the “pseudo-steady-state” approximation or “rapid equilibrium assumption” to reduce the network signaling reactions based on an analysis of the different time scales of the individual reactions (Khatibi et al., 2017). This model predicted a critical role of potential positive and delayed negative feedback loops on the regulation of the TGF- β signaling system.

In line with simplified models, composed of the minimal set of equations required for explaining experimental observations, we developed a mathematical model based on differential equations to support the hypothesis that the heterotrimer Smad2–Smad3–Smad4 is formed during vertebrate neurogenesis and that its formation could explain the fact that

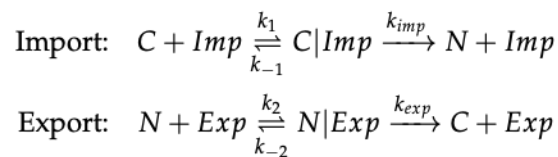
Smad2 and Smad3 antagonize in some targets while cooperate in activating the transcription of others (Míguez et al., 2013). The model prediction was then validated using immunoprecipitation (Míguez et al., 2013).

4.8.1 Mass action law to model nucleocytoplasmic shuttling

In this section, we propose, using the same philosophy of simple modeling approaches, a very simple yet powerful mathematical approach to study the dynamics of this nucleocytoplasmic shuttling. The model was designed with ordinary differential equations derived using the mass action law and was used to reproduce the dynamics of a R-Smad protein in and out of the nuclei. Using this simplified system, it was easy to study the main mechanisms and parameters that affect this dynamic equilibrium without needing to perform complicated calculations or simulations.

To build the model, let us consider an R-Smad protein inside of a cell that can enter and exit the nucleus. These two potential states of the R-Smad protein are represented in the model as C (for cytoplasmic) and N (for nuclear). Based on the molecular details of the process reviewed above, we know that the processes crossing the NPCs are actively mediated by interactions with other proteins, which we call Imp (for import) and Exp (for export). This way, to enter into the nucleus, a cytoplasmic molecule C must be linked to the import enzyme Imp and forms a complex C/Imp . This complex is then allowed to enter the nucleus. Once in the nucleus, the C/Imp complex is disassembled into one molecule N (since the R-Smad molecule is now inside the nucleus) and the Imp enzyme.

Similarly, the export process follows the same rationale: The nuclear molecule N binds to the export enzyme Exp , forming the N/Exp complex. This complex is now able to cross the NPCs and translocate to the cytoplasm. Once in the cytoplasm, the complex is disassembled, giving one C (our molecule is now in cytoplasmic configuration again) and the enzyme Exp . The rates of the nuclear import and export processes for C/Imp and N/Exp complexes are k_{imp} and k_{exp} , respectively. The formation rates of C/Imp and N/Exp are k_1 and k_2 , respectively. We included the fact that complex formation is a reversible process in the model, which means that the complex can spontaneously disassemble before crossing the NPCs. The dissociation rates of C/Imp and N/Exp are labeled as k_{-1} and k_{-2} , respectively. Moreover, the dissociation of each complex once it crosses the NPCs is considered to be instantaneous, and Imp and Exp transport enzymes that can translocate freely between the nucleus and cytoplasm (they are not limiting reagents) (Chen et al., 2010; Hangos & Szederkényi, 2011). The whole system of interactions can be illustrated using a chemical reaction formalism as



The scheme of the interaction is illustrated in Figure 4.10.

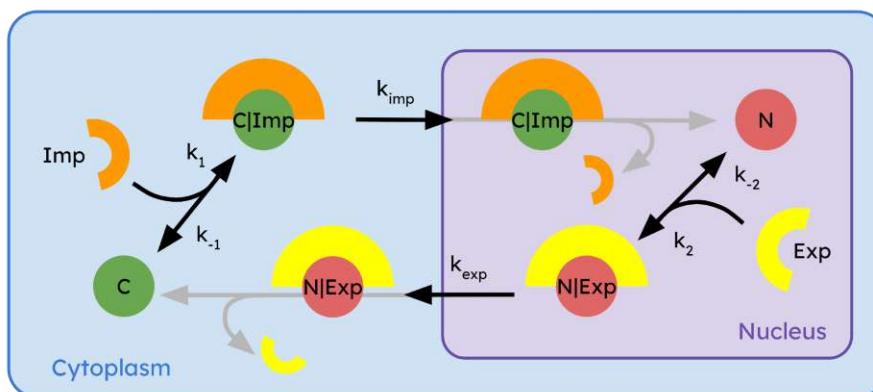


Figure 4.10 Schematic representation of the nuclear import–export process of a molecule inside a cell. The molecule in the cytoplasm is represented by C and in the nucleus by N . To be imported inside the nucleus, one C molecule must bind one molecule of the import enzyme Imp . Then, the complex $C|Imp$ can enter the nucleus, where it disassembles into N (former C) and Imp . The export process is analogous, involving the formation of an $N|Exp$ complex (one molecule of N and one export enzyme Exp) and then the disassembly of this complex in the cytoplasm yielding one molecule of C and one molecule of Exp .

4.8.2 Using the Mass Action Law to Model a Biological System

To translate this system of interactions into differential equations, we use mass action law formalism, a fundamental principle in the mathematical modeling of chemical and biological systems. In brief, the law of mass actions states that the rate of change of a species involved in a chemical interaction with other species is directly proportional to the product of the masses of the reactants, each raised to a power equal to the coefficient used in the chemical equation. Initially proposed for chemical systems by Cato Guldberg and Peter Waage in 1864 (Guldberg & Waage, 1879) based on previous work by Claude Louis Berthollet (Berthollet, 1803) and further clarified by Jacobus van't Hoff in 1877 (Hoff, 1877), it also provides a simplified yet effective framework for quantifying biological interactions.

The set of differential equations that corresponds to these interactions is (step-by-step derivation is shown in the Annex of this thesis)

$$\begin{aligned}
\frac{d[C]}{dt} &= -k_1[C][Imp] + k_{-1}[C|Imp] + k_{exp}[N|Exp] \\
\frac{d[Imp]}{dt} &= -k_1[C][Imp] + k_{-1}[C|Imp] + k_{imp}[C|Imp] \\
\frac{d[C|Imp]}{dt} &= k_1[C][Imp] - k_{-1}[C|Imp] - k_{imp}[C|Imp] \\
\frac{d[N]}{dt} &= -k_2[N][Exp] + k_{-2}[N|Exp] + k_{imp}[C|Imp] \\
\frac{d[Exp]}{dt} &= -k_2[N][Exp] + k_{-2}[N|Exp] + k_{exp}[N|Exp] \\
\frac{d[N|Exp]}{dt} &= k_2[N][Exp] - k_{-2}[N|Exp] - k_{exp}[N|Exp]
\end{aligned}$$

The numerical solution of this set of ODEs, starting from a given initial species concentration and parameter values (e.g., rate constants for each reaction), provides the temporal evolution of the concentration of all involved species. One important point to take into account is the units of the rate constants: if the time is measured in seconds (s) and the concentrations in mol/L (molar, M), each equation provides the speed of change of the reactants in units of M/s. Therefore, for the equations to be consistent in terms of dimensions, the units of the rate constants k_{-1} , k_{-2} , k_{imp} , and k_{exp} must be in s^{-1} (first-order reactions), whereas k_2 and k_1 are measured in $(M \cdot s)^{-1}$ (second-order reactions in a thermodynamical sense).

Figure 4.11 A–C illustrates three different numerical solutions of the system for an equal set of initial conditions but using the parameter values listed as sets 1, 2, and 3 in Table 1, respectively. In all cases, the concentrations of the species changes during the first seconds until they reach a steady state. Both [C] and [N] decrease while [C|Imp] and [N|Exp] increase (complexes are forming). The enzymes [Imp] and [Exp] also decrease due to complex formation. Because of the different parameter values used, three different steady states are reached: In Figure 4.11 A, the final concentration of [C] in the equilibrium is larger than the concentration of [N], meaning that most protein is located at the cytoplasm for this value of the rate constant. The opposite scenario is shown in Figure 4.11 C, where the nuclear concentration is now higher.

Figure 4.11 B corresponds to an intermediate situation, where the parameters were tuned to obtain an equilibrium condition where the concentrations at the steady state in the nucleus and in the cytoplasm are equivalent. If we imagine a hypothetical situation where our molecule can be seen using immunofluorescence or by direct fusion with a fluorescence protein, case (A) would correspond to a cell that is brighter in the cytoplasm, case (C) would correspond to a cell with a brighter nuclei, and case (B) would correspond to a cell with homogeneous brightness. Note that the different cases were obtained by varying only the values of k_{imp} and k_{exp} , meaning that the efficiency of the nuclear import and export processes ultimately controls the balance between the nuclear and cytoplasmic configuration.

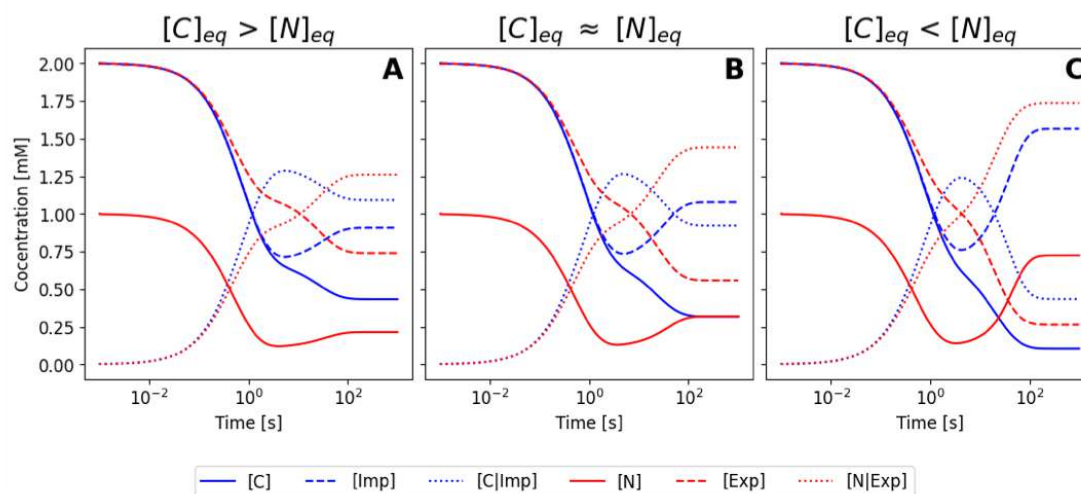


Figure 4.11. Simulation of the ODE system. Three different sets (1, 2, and 3 from Table 1) of rate constants are used for the different cases (A), (B) and (C), respectively. The initial conditions for the species are $[C](0) = [Imp](0) = [Exp](0) = 0.002$ M, $[N](0) = 0.001$ M, and $[C|Imp](0) = [N|Exp](0) = 0$ M in all cases. The concentrations change during the first seconds until they reach a steady state: $[C]$ in equilibrium is bigger (A), equivalent (B), or smaller than $[N]$ in equilibrium. In general, $[C]$, $[N]$, $[Imp]$, and $[Exp]$ tend to decrease initially as $[C|Imp]$ and $[N|Exp]$ form.

Table 1. Values of the constant rates for the different sets used in the simulations.

Rate	Set 1	Set 2	Set 3	Set 4
k_1	$500 \text{ (M}\cdot\text{s)}^{-1}$	$500 \text{ (M}\cdot\text{s)}^{-1}$	$500 \text{ (M}\cdot\text{s)}^{-1}$	$50 \text{ (M}\cdot\text{s)}^{-1}$
k_{-1}	0.15 s^{-1}	0.15 s^{-1}	0.15 s^{-1}	1.5 s^{-1}
k_2	$0.03 \text{ (M}\cdot\text{s)}^{-1}$	$0.036 \text{ (M}\cdot\text{s)}^{-1}$	$0.04 \text{ (M}\cdot\text{s)}^{-1}$	$0.03 \text{ (M}\cdot\text{s)}^{-1}$
k_{-2}	1000 s^{-1}	1000 s^{-1}	1000 s^{-1}	1 s^{-1}
k_{imp}	0.1 s^{-1}	0.1 s^{-1}	0.1 s^{-1}	0.3 s^{-1}
k_{exp}	0.026 s^{-1}	0.023 s^{-1}	0.01 s^{-1}	0.3 s^{-1}

4.8.3 Simplification Using the Mass Conservation Law

The next step in any process of modeling a biological system is to try to simplify and reduce the set of equations. As such, it is often very useful to use the mass conservation law (Weiss, 1943), stated by Antoine Lavoisier in 1789 (Schummer, 2006).

In the context of biological models of signal transduction, it is quite common to obtain redundant equations where the dynamics of one variable can be simply related to the dynamics of another variable in the system. In this condition, the simplification of the system is direct and results in a more transparent set of equations that is easier to interpret. In this particular case of protein translocation, the mass conservation allowed us to reduce the system of six differential equations to four (step-by-step general application is illustrated in the Annex).

As shown in the Annex, we obtained three conservation laws for our system. The first conservation corresponds to the fact that the total amount of Smad in all possible configurations (nuclear, cytoplasmic, alone, or in complex) must remain constant. Two more conservation laws illustrate the fact that the total amount of Imp and Exp proteins must remain constant. One additional simplification can be introduced, taking into account that at the initial time point, there are no complexes, since we start with all proteins as monomers and monitor their interaction.

In this case, since we often focus on the amount of R-Smads and not on the amount of Imp and Exp proteins, we can use the mass conservation to organize the relationships in such a way that we eliminate the variables [Imp] and [Exp], giving us the set of four ODEs:

$$\begin{aligned}
 \frac{d[C]}{dt} &= -k_1[C]([Imp](0) - [C|Imp]) + \dots \\
 &\quad + k_{-1}[C|Imp] + k_{exp}[N|Exp] \\
 \frac{d[C|Imp]}{dt} &= k_1[C]([Imp](0) - [C|Imp]) - \dots \\
 &\quad - k_{-1}[C|Imp] - k_{imp}[C|Imp] \\
 \frac{d[N]}{dt} &= -k_2[N]([Exp](0) - [N|Exp]) + \dots \\
 &\quad + k_{-2}[N|Exp] + k_{imp}[C|Imp] \\
 \frac{d[N|Exp]}{dt} &= k_2[N]([Exp](0) - [N|Exp]) - \dots \\
 &\quad - k_{-2}[N|Exp] - k_{exp}[N|Exp]
 \end{aligned}$$

Numerical integration of this set gives us the temporal evolution of [C], [N] and the complexes [C|Imp] and [N|Exp]. If needed, the temporal evolution of the enzymes can be simply calculated as [Imp](t) = [Imp](0) - [C|Imp](t) and [Exp](t) = [Exp](0) - [N|Exp](t). This means that we can calculate the concentration of all six species over time by solving just four ODEs instead of the six that we obtained initially.

4.8.4 Nondimensionalization of the Model

Another common approach to increase the predictive capability of a model is to remove the dimensions of the concentrations and the time, expressing both of them as a factor on an appropriate scale for the system under study. In this type of dimensionless system, it is easier to compare parameter values and to interpret the results from the ODEs, since they show relative changes from a maximum to a minimum value instead of absolute values.

In order to make the concentrations dimensionless, we rewrite the model in terms of new variables with no units. This is achieved by dividing each variable by a scaling factor (with concentration units) in such a way that now the concentration of the species [X] (dimensional) is now represented by [X~] (dimensionless), related via the scaling factor X_0 (Chen et al., 2010):

$$[X](t) = [\tilde{X}](t) \cdot X_0 \quad (1)$$

The scaling factor for each species is chosen as its maximum value. This way, the dimensionless species concentration $[X\tilde{}]$ associated falls in the range $[0, 1]$. The maximum amount of molecules in the cytoplasm that we can have been $[C](0)$, and, as one molecule of C can only produce one molecule of N, the maximum value for the latter is $[C](0)$ as well (supposing that $[N](0) = 0$). In the case of enzymes, the maximum concentration for each one is also its initial concentration. For the scaling factor of the complexes C/Imp and N/Exp , we cannot use their initial concentration as it is zero. Therefore, we must find which is the maximum concentration that they can reach over time ($[C|Imp]_{max}$ and $[N|Exp]_{max}$), which happens at a particular time point $t = t_{max}$ (this time value can be different for $[C|Imp]$ and $[N|Exp]$):

$$\begin{aligned} [C|Imp]_{max} &= \frac{k_1 [C](t_{max}) [Imp](0)}{k_1 [C](t_{max}) + k_{-1} + k_{imp}} \\ [N|Exp]_{max} &= \frac{k_2 [N](t_{max}) [Exp](0)}{k_2 [N](t_{max}) + k_{-2} + k_{exp}} \end{aligned}$$

Next, these expressions can be further simplified by making some assumptions. As the dynamics of the complexes are very fast, k_2 and k_1 are much larger than the other rate constants. Considering that, we can assume that $k_{-1} = k_{imp} = k_{-2} = k_{exp} = 0$; therefore,

$$\begin{aligned} [C|Imp]_{max} &\approx [Imp](0) \\ [N|Exp]_{max} &\approx [Exp](0) \end{aligned}$$

Then, the new dimensionless variables are defined as

$$\begin{aligned} [\tilde{C}] &= \frac{[C]}{[C](0)} \\ [I\tilde{m}p] &= \frac{[Imp]}{[Imp](0)} \\ [C|\tilde{I}mp] &= \frac{[C|Imp]}{[Imp](0)} \\ [\tilde{N}] &= \frac{[N]}{[N](0)} \\ [E\tilde{x}p] &= \frac{[Exp]}{[Exp](0)} \\ [N|\tilde{E}xp] &= \frac{[N|Exp]}{[Exp](0)} \end{aligned}$$

Substituting these values into the ODE system, we obtain the new partially dimensionless ODE system:

$$\begin{aligned}
\frac{d[\tilde{C}]}{dt} &= k_1[\tilde{C}][Imp](0)([C|\tilde{Imp}] - 1) + \dots \\
&\quad + k_{-1} \frac{[Imp](0)}{[C](0)} [C|\tilde{Imp}] + k_{exp} \frac{[Exp](0)}{[C](0)} [N|\tilde{Exp}] \\
\frac{d[C|\tilde{Imp}]}{dt} &= k_1[\tilde{C}][C](0)(1 - [C|\tilde{Imp}]) - \dots \\
&\quad - (k_{-1} + k_{exp})[C|\tilde{Imp}] \\
\frac{d[\tilde{N}]}{dt} &= k_2[\tilde{N}][Exp](0)([N|\tilde{Exp}] - 1) + \dots \\
&\quad + k_{-2} \frac{[Exp](0)}{[N](0)} [N|\tilde{Exp}] + k_{imp} \frac{[Imp](0)}{[N](0)} [C|\tilde{Imp}] \\
\frac{d[N|\tilde{Exp}]}{dt} &= k_2[\tilde{N}][N](0)(1 - [N|\tilde{Exp}]) - \dots \\
&\quad - (k_{-2} + k_{imp})[N|\tilde{Exp}] \\
[\tilde{Imp}](t) &= 1 - [C|\tilde{Imp}](t) \\
[\tilde{Exp}](t) &= 1 - [N|\tilde{Exp}](t)
\end{aligned}$$

It is important to highlight that the mass conservation law in this case is different from the dimensional system. The calculations for this case are not shown, but they are analogous to those in the previous case.

To remove the dimension of time, a scaling factor t_0 is required in order to define a new dimensionless time variable τ :

$$\tau = \frac{t}{t_0}$$

In any system, we have several reaction rate constants that have different values. To obtain the scaling factor, one option is to choose the fastest one, as the dimensionless time is then a factor indicating “how many times the fastest reaction has taken place”. In any case, we can rewrite our ODE system, taking into account that

$$\frac{d}{dt} = \frac{1}{t_0} \frac{d}{d\tau}$$

And obtain:

$$\begin{aligned}
\frac{1}{t_0} \frac{d[\tilde{C}]}{d\tau} &= k_1[\tilde{C}][Imp](0)([C|\tilde{Imp}] - 1) + \dots \\
&\quad + k_{-1} \frac{[Imp](0)}{[C](0)} [C|\tilde{Imp}] + k_{exp} \frac{[Exp](0)}{[C](0)} [N|\tilde{Exp}] \\
\frac{1}{t_0} \frac{d[C|\tilde{Imp}]}{d\tau} &= k_1[\tilde{C}][C](0)(1 - [C|\tilde{Imp}]) - \dots \\
&\quad - (k_{-1} + k_{exp})[C|\tilde{Imp}] \\
\frac{1}{t_0} \frac{d[\tilde{N}]}{d\tau} &= k_2[\tilde{N}][Exp](0)([N|\tilde{Exp}] - 1) + \dots \\
&\quad + k_{-2} \frac{[Exp](0)}{[N](0)} [N|\tilde{Exp}] + k_{imp} \frac{[Imp](0)}{[N](0)} [C|\tilde{Imp}] \\
\frac{1}{t_0} \frac{d[N|\tilde{Exp}]}{d\tau} &= k_2[\tilde{N}][N](0)(1 - [N|\tilde{Exp}]) - \dots \\
&\quad - (k_{-2} + k_{imp})[N|\tilde{Exp}] \\
[Imp](\tau) &= 1 - [C|\tilde{Imp}](\tau) \\
[Exp](\tau) &= 1 - [N|\tilde{Exp}](\tau)
\end{aligned}$$

In our example, let us suppose that k_2 is the fastest rate (which is a biologically plausible assumption). As k_2 is measured in $(M \cdot s)^{-1}$, it cannot be the scaling factor itself because t will have dimensions of $M \cdot s^2$. Looking at the simplified ODE system using the mass conservation law, we can see that this rate k_2 is multiplied by a constant, $[Exp](0)$, measured in M . The product $[Exp](0) \cdot k_2$ is measured in s^{-1} , so we can choose our scaling factor:

$$t_0 = \frac{1}{k_2 \cdot [Exp](0)} \rightarrow \tau = k_2 \cdot [Exp](0) \cdot t$$

Numerical integration of this dimensionless version for the same parameters is now shown in Figure 4.12, where the vertical axis now moves from 0 to 1, and the horizontal axis represents multiples of the t_0 value.

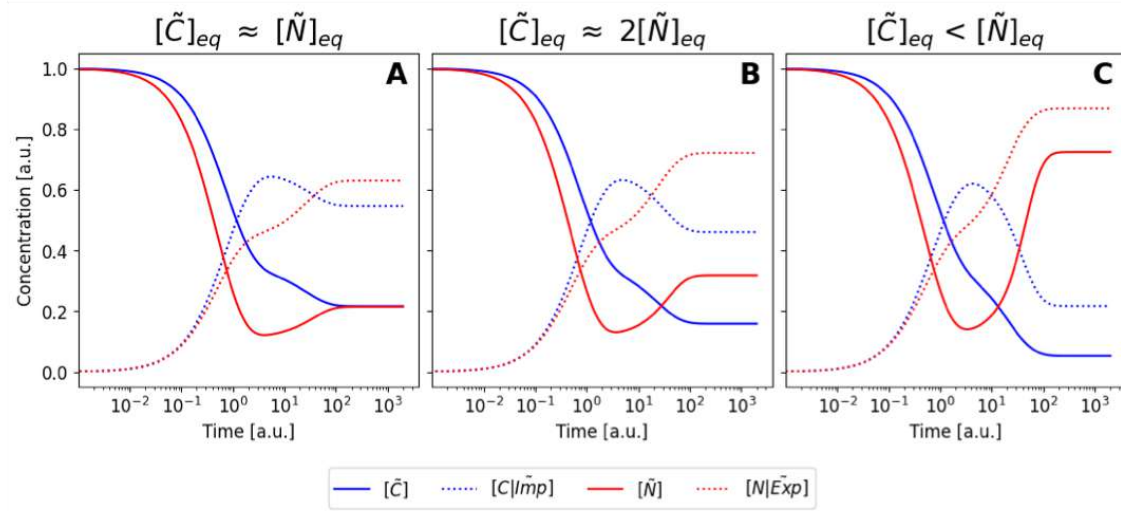


Figure 4.12. Simulation of the ODE system after being made dimensionless. The same rate constants and initial conditions as in Figure 4.11 were used for all the panels. Note that the concentrations range within $[0, 1]$, and, during the simulation, the curves indicate the present fraction of the concentration of each species in the system (normalized by its scaling factor). In the case of $[C^*]$ and $[N^*]$, the curves show the present fraction of their initial condition. As in Figure 4.11, the concentrations change during the first seconds until they reach a steady state: $[C]$ in equilibrium is bigger (A), equivalent (B), or smaller than $[N]$ in equilibrium; which for the dimensionless variables (under the conditions for this simulation) means that $[N^*]$ in equilibrium is equivalent (A), double (B) or greater (C) than $[C^*]$.

4.8.5 Pseudo-Steady-State Approximation

So far, we have an ODE system with 4 coupled equations. Although, in this case the numerical solution of this system is relatively easy to achieve, this might not be the case for any other system. To further simplify our mathematical model, a common approach is to take advantage of the so-called pseudo-steady-state approximation.

A biologically acceptable approximation is considering that the dynamics of the intermediate complexes are very fast compared with the rest of the species. This means that the translocation processes ($C|Imp$ and $N|Exp$ moving from one compartment to another via diffusion or another active process, plus their disassembly) are slow compared to the association and dissociation reactions ($C|Imp$ and $N|Exp$ formation and disassembly).

Mathematically, this translates into k_{imp} and k_{exp} being much smaller than the rest of the rate constants, and thus we can consider that

$$\frac{d[C|Imp]}{dt} = \frac{d[N|Exp]}{dt} = 0$$

This assumption is known as the Michaelis–Menten approximation (Chen et al., 2010) in which the concentration of the enzymes and complexes are considered to be in equilibrium while $[C]$ and $[N]$ change rapidly. With this mathematical condition, we can then obtain the concentration of the complexes over time as

$$\begin{aligned} [C|Imp](t) &= \frac{k_1[C](t)[Imp](0)}{k_1[C](t) + k_{-1} + k_{imp}} \\ [N|Exp](t) &= \frac{k_2[N](t)[Exp](0)}{k_2[N](t) + k_{-2} + k_{exp}} \end{aligned}$$

With this definition, the new simplified system that gives the concentration of all the species over time is

$$\begin{aligned} \frac{d[C]}{dt} &= -k_1[C][Imp](0) + \dots \\ &\quad + (k_1[C] + k_{-1}) \frac{k_1[C][Imp](0)}{k_1[C] + k_{-1} + k_{imp}} + \dots \\ &\quad + k_{exp} \frac{k_2[N][Exp](0)}{k_2[N] + k_{-2} + k_{exp}} \\ \frac{d[N]}{dt} &= -k_2[N][Exp](0) + \dots \\ &\quad + (k_2[N] + k_{-2}) \frac{k_2[N][Exp](0)}{k_2[N] + k_{-2} + k_{exp}} + \dots \\ &\quad + k_{imp} \frac{k_1[C][Imp](0)}{k_1[C] + k_{-1} + k_{imp}} \end{aligned}$$

which has just two coupled ODEs. Lastly, using the definition of the Michaelis–Menten constant for each of the two processes ($C|Imp$ and $N|Exp$), we can rewrite the ODEs as

$$\begin{aligned} \frac{d[C]_{MM}}{dt} &= k_{exp} \frac{[Exp](0)[N]}{K_N + [N]} - k_{imp} \frac{[Imp](0)[C]}{K_C + [C]} \\ \frac{d[N]_{MM}}{dt} &= k_{imp} \frac{[Imp](0)[C]}{K_C + [C]} - k_{exp} \frac{[Exp](0)[N]}{K_N + [N]} \end{aligned}$$

With

$$\begin{aligned} K_C &= \frac{k_{imp} + k_{-1}}{k_1} \\ K_N &= \frac{k_{exp} + k_{-2}}{k_2} \end{aligned}$$

Now, we can compare the initial system with the pseudo-steady-state approximation system that we just obtained. As shown in Figure 4.13, the approximation is not valid for the values of the rates of set 1 (Table 1), but it is for the values of set 4 (Table 1). The rate constants for the system must fulfill the approximation assumptions in order to be able to use the approximation.

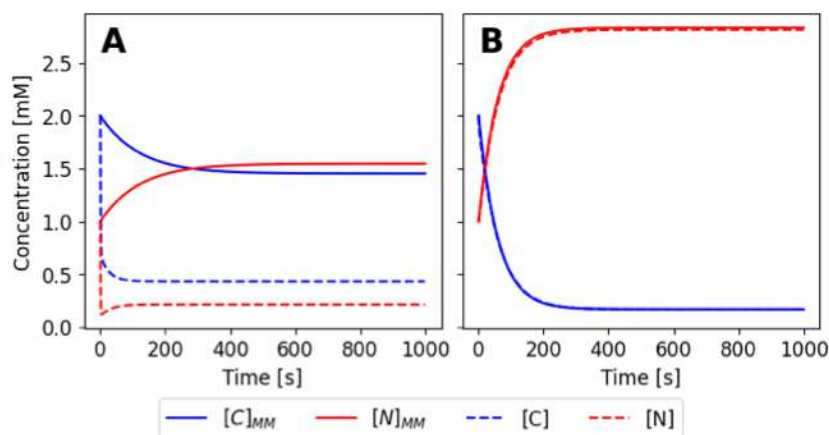


Figure 4.13. Comparison of the raw model and the pseudo-steady-state approximation model. The concentrations of the C and N molecules are shown as dashed lines for the original ODEs ([C] and [N]) and as solid lines for the pseudo-steady-state approximation (Michaelis–Menten, [C]_{MM} and [N]_{MM}). In both insets, the initial concentration values are the same as in Figure 4.11. The rate constants used in (A, B) are sets 1 and 4 (from Table 1), respectively. For case (A), there is a considerable discrepancy between the approximation (solid curves) and the original system (dashed curves). When the rate constants are chosen in such a way that the approximation assumptions are fulfilled (set 4), the steady-state approximation becomes valid and can be used to simplify the analysis of the system.

4.8.6 Conclusions from the immunofluorescence and the modeling part

The TGF- β pathway is often viewed as an almost linear cascade, where events at the receptor level correspond to the direct phosphorylation and activation of transcription factors. This oversimplified view leaves out important processes that can fully dictate the response, such as regulation via compartmentalization of the R-Smads, which are actively maintained in and out of the nucleus depending on their activation state. In principle, inactive unphosphorylated R-Smads do not form complexes and do not bind to DNA, so the fact that there is an extra layer that keeps these inactive proteins from biological responses, and some are still perhaps waiting to be discovered and studied. For instance, living organisms are open systems by definition, and this property is key to understanding their unique properties, such as adaptation and morphogenesis (Míguez et al., 2006; Renati, 2023). Physics and biophysics have already helped to understand many of the most important aspects of how living systems operate, and they will play a central role in future discoveries entering the nucleus seems unnecessary. But we know from experience that everything that happens inside the cells often serves many purposes. Therefore, biophysical regulation at least adds an extra safety layer that prevents such important proteins from wrongly activating the expression of their targets

when not needed. We believe that this is just one of the many ways that the physical aspects of cells and organelles influence.

4.9 Preliminary FRET quantification

4.9.1 Introduction to the FRET Technique

Förster Resonance Energy Transfer (FRET) is a distance-dependent phenomenon where energy is transferred non-radiatively from an excited donor fluorophore to an acceptor fluorophore through long-range dipole-dipole interactions. FRET efficiency is typically measured by the ratio of acceptor to donor emission upon donor excitation, providing a metric proportional to their proximity. FRET occurs if the donor's emission spectrum overlaps with the acceptor's excitation spectrum. Using two fluorescent proteins with compatible spectral properties allows monitoring inter-fluorophore distances within living cells. Under appropriate conditions, non-radiative energy transfer occurs from the donor to the acceptor fluorophore when they are in close proximity, a scheme of the FRET system as Figure 4.14.

The characteristic distance at which 50% energy transfer occurs, known as the Förster distance, is well-defined for specific donor-acceptor pairs, such as CFP/YFP (Lim et al., 2022). The CFP-YFP pairing stands out as one of the most commonly and effectively used donor-acceptor combinations. In FRET experiments, when the donor is excited and the acceptor is properly positioned nearby, the donor's energy transfers non-radiatively to the acceptor. This process makes FRET a sensitive tool for measuring intermolecular distances and assessing conformational changes (Verma et al., 2023).

Another approach for confirming FRET involves measuring donor emission before and after acceptor photobleaching. Eliminating the acceptor by photodestruction releases the donor's energy, increasing donor fluorescence emission. An observed increase in donor fluorescence following acceptor photobleaching validates the presence of FRET (Van Munster et al., 2005).

Although FRET is a valuable tool for studying protein interactions in living cells, it's a complex technique with several challenges. Accurate FRET measurements depend on significant spectral overlap between the donor and acceptor fluorophores, but incomplete spectral separation can lead to crosstalk between channels, making data analysis difficult. Photobleaching of the fluorophores and direct excitation of the acceptor can also affect FRET efficiency and lead to inaccurate measurements. Additionally, FRET efficiency is sensitive to the relative orientation of the fluorophores and environmental factors like pH and temperature. Importantly, FRET only indicates the proximity of fluorophores, not necessarily direct physical interaction. To address these challenges, careful experimental design and controls are essential. These controls typically include donor-only and acceptor-only controls, negative and positive controls with known interacting and non-interacting proteins, and photobleaching correction methods. By carefully considering these factors and implementing appropriate controls, researchers can minimize the potential pitfalls of FRET and obtain reliable data on protein interactions.

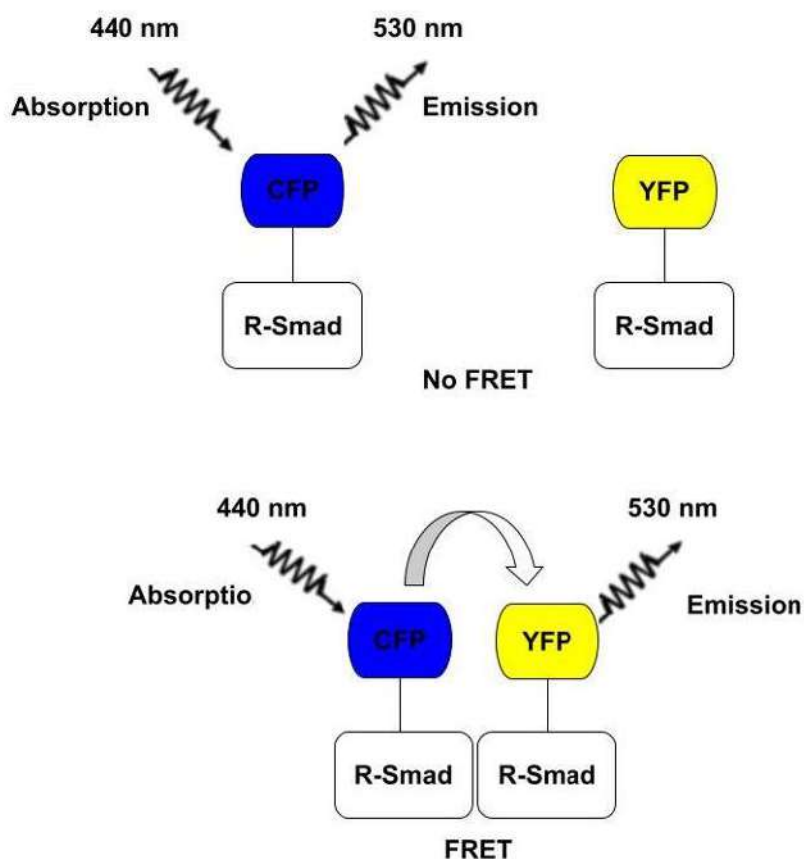


Figure 4.14 Schematic representation of FRET basis. If the donor and acceptors are sufficiently close (under 10 nm), the excited donor (CFP) can transfer energy via a non-radiative process (indicated by a dashed arrow) to the acceptor (YFP), resulting in fluorescence, adapted from Ishikawa-Ankerhold et al., (2012).

4.9.2 Preliminary experiments

As a preliminary test of the FRET pair, we infected N2A cells simultaneously with Smad3-CFP and Smad2-YFP, to see if cells can be successfully infected to express both versions of the proteins and remain healthy. An image of this experiment is shown in Figure 4.15

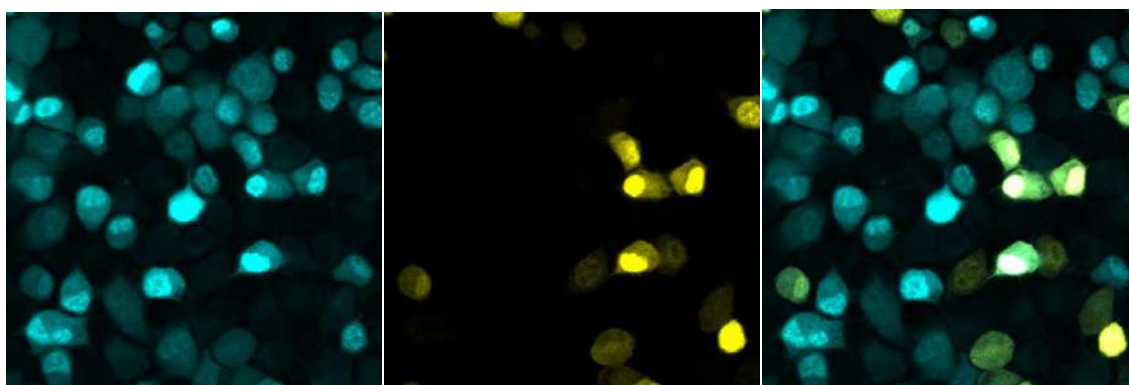


Figure. 4.15: N2A cells infected with the Smad3-CFP (left), Smad2-YFP (center) and overlay (right). Now the localization levels resemble more the profile shown by immunofluorescence, suggesting that the antibody of Smad2 does not penetrate in the nucleus, as expected.

Finally, we quantified if the fusion proteins translocate after activation and inhibition, similarly to the previous analysis of the endogenous levels. Results are shown in Figure 4.16. We can see that increasing the activation of the pathway increases the nuclear accumulation of both Smad2 (left) and Smad3 (right) fusion proteins. It will also increase the level of phospho-Smad2 and phospho-Smad3, as expected. These results suggest that the co-expression of both proteins does not interfere with their ability to translocate to the nucleus after activation.

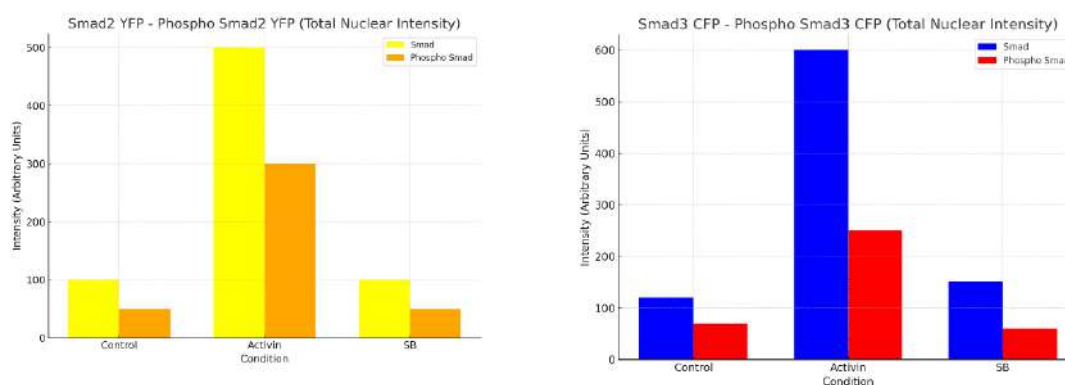


Figure. 4.16: N2A cells infected with the Smad2-YFP (left), Smad3-CFP (right) in different conditions (control, Activin A and inhibition) stained with the Phospho-Smad2 and phospho-Smad3 antibody.

We present in this section preliminary attempts to test if there is some possibility of obtaining FRET interaction using the fusion proteins developed. As a starting point, a FRET test was conducted in two homodimeric clones (Smad2-CFP and Smad2-YFP) and one heterodimeric clone (Smad3-CFP and Smad2-YFP) and analyze the variations in FRET and colocalization

percentages under different settings. We realize that to obtain more conclusive results, the proper controls should be quantified carefully.

Measurements of the FRET index and the localization were automatically obtained by the software of the microscope, combined with Fiji. The following figures can be interpreted as follows: (a) the first row corresponds to the image of the CFP channel, i.e., the amount of protein tagged with CFP. (b) the second row corresponds to the image of the YFP channel, i.e., the amount of protein tagged with CFP. (c) the third row corresponds to the YFP channel when imaging using the CFP laser (FRET channel, the amount of photons captured at the YFP channel when the sample is imaged using the CFP laser). (d) The fourth row estimates the colocalization based on the FRET values (third row) taking into account corrections applied based on the intensity of CFP and YFP, and the amount of bleed through of the CFP into the YFP (i.e., the number of photons captured in the YFP channel cells when illuminating the sample with the CPF but using cells expressing only CFP).

The first experiment estimates the potential co-localization using Smad2-CFP and Smad2-YFP in N2A cells in different conditions (to estimate if Smad2 homodimers are being formed). Results are shown in Figure 4.17.

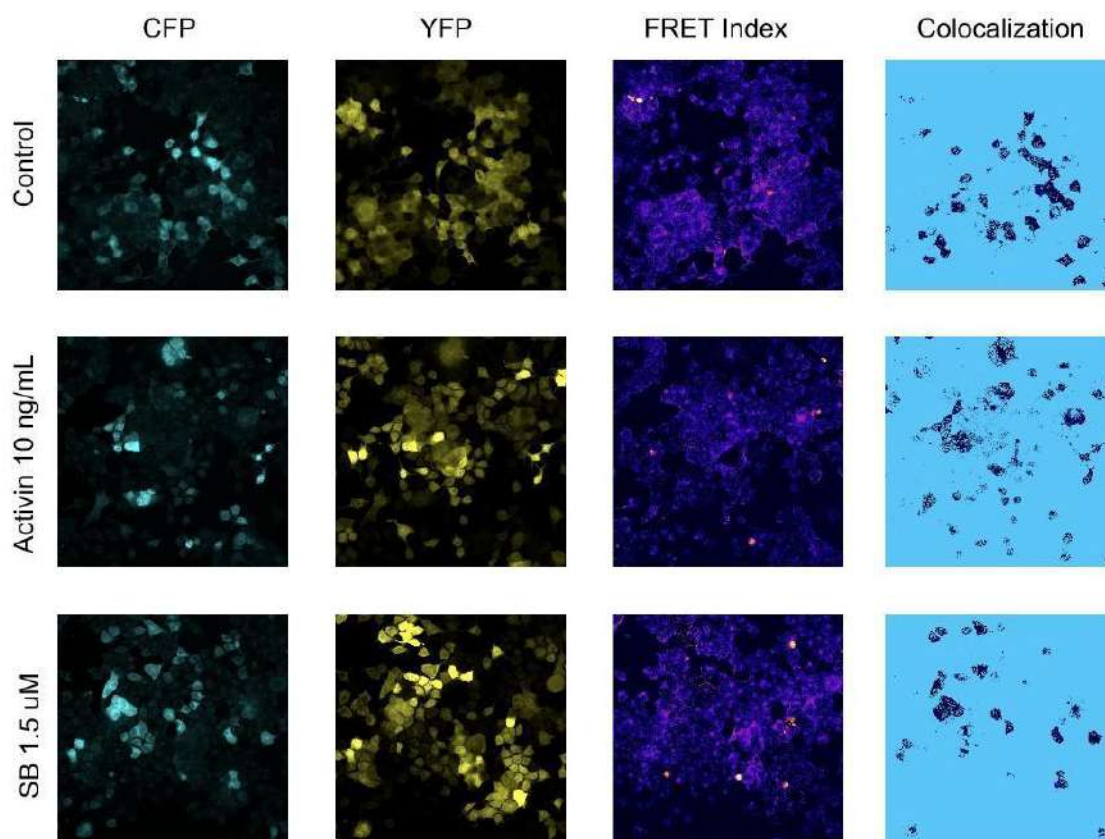


Figure 4.17: Quantification of FRET in N2A cells stably expressing Smad2-CFP and Smad2-YFP.

We see that colocalization can be observed in all conditions (right column), while it seems slightly higher in control. We also observe a small reduction in the number of cells that show positive co-localization between Smad2-Smad2. Only cells that express high levels of both proteins show co-localization.

Next, we test the colocalization of Smad3 with Smad3 (formation of homodimers of Smad3) by infection cells with both variants of the Smad3 fusion protein (fused to CFP and to YFP). Results are shown in Figure 4. 18. Now we see that the number of cells showing colocalization increases in the conditions of the activated pathway (using Activin A). This promising result needs to be further validated using other methods, using proper controls and accompanied by a well-designed measurement of statistical significance (the software used only provides the images, not the statistical analysis).

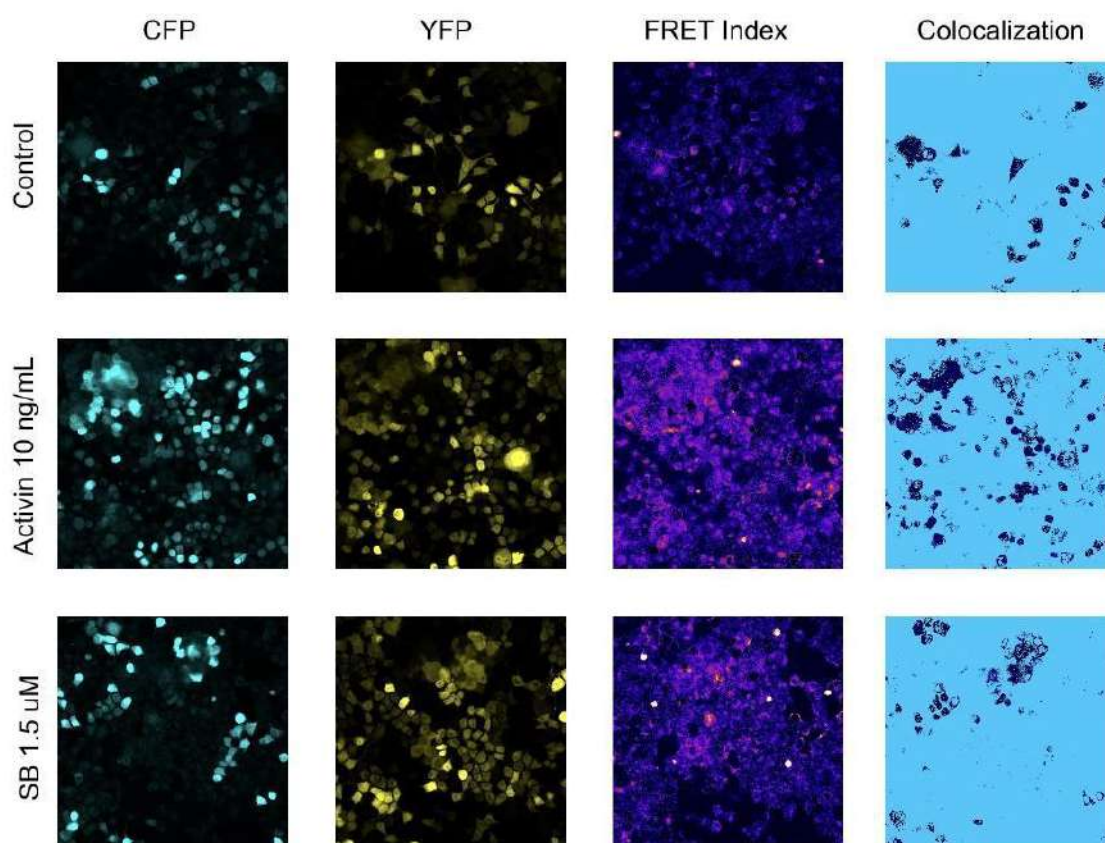


Figure 4.18: Quantification of FRET in N2A cells stably expressing Smad3-CFP and Smad3-YFP.

Finally, to test if there is formation of the heterotrimer complex (smad2, Smad3 and Smad4, the complex reported as more abundant and important in the context of neurogenesis by previous results from our lab), we infect cells with lentivirus containing the fusion Smad3-CFP simultaneously with lentivirus containing Smad2-YFP. Results are shown in Figure 4.19. We can see that in this condition, some colocalization can be detected, but in lower levels of the other two conditions tested. This suggests that the heterotrimer is not as abundant as expected (but needs further validation). We also don't see any changes after activation or inhibition of the pathway.

These sets of experiments are designed to be a first approach to estimate the formation of the complexes that work as transcriptional regulators in response to TGF- β pathway activation. We believe that these types of measurements are important in the sense that they can provide real-time quantification of the type of complexes being formed in different scenarios. To our knowledge this FRET tool is the first ready to provide such values, and we believe will be important for the community, since the TGF- β pathway plays such an important role in the regulation of a large number of biological processes.

Although these data should be treated as preliminary, we already see some interesting results, such as a clear increase in co-localization in Smad3-CFP and Smad3-YFP when Activin A is added. This suggests that the tool could be used to monitor the state of activation and the location of the formation of these complexes *in vivo* and in real time, providing very important insights on the dynamics of the TGFB signaling in several processes, such as during vertebrate neurogenesis.

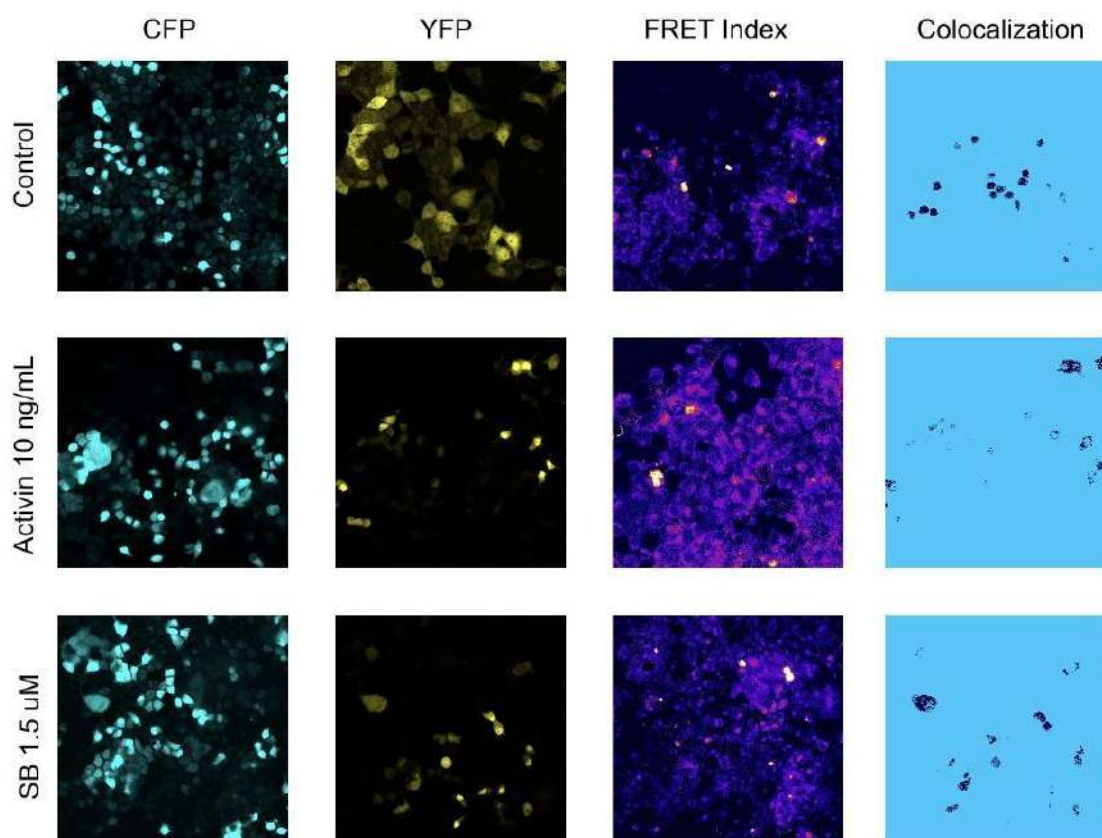


Figure 4.19: quantification of FRET in N2A cells stably expressing Smad3-CFP and Smad2-YFP.

V. DISCUSSION

V. DISCUSSION

Interdisciplinary research has become increasingly vital in advancing the field of Molecular Biology. By integrating diverse perspectives and methodologies from various disciplines, researchers can tackle complex biological questions that transcend the boundaries of individual fields. This collaborative approach fosters innovation, enabling the development of novel techniques, the generation of more comprehensive datasets, and the creation of more robust models. Ultimately, interdisciplinary efforts accelerate the pace of discovery and lead to a deeper and more integrated understanding of biological systems.

The present thesis takes advantage of this interdisciplinary spirit by converging techniques and knowledge from various research areas to investigate the intricate dynamics of cellular signaling. In brief, it integrates tools from biophysics, through the study of physical compartmentalization, with molecular biology techniques such as cloning and lentivirus infection for creating fusion proteins. These experimental approaches are further combined with advanced microscopy techniques, such as FRET, to visualize and quantify protein interactions with high spatial resolution. Moreover, mathematical modeling is employed to provide a theoretical framework for interpreting experimental data and generating testable predictions.

Historically, studies focusing on TGF- β signaling have often emphasized biochemical or pharmacological perspectives. While these approaches have yielded valuable insights, we believe that a more interdisciplinary strategy, like the one used in this thesis, will be able to provide a more powerful and comprehensive understanding of this complex signaling pathway.

Characterizing dynamic changes in protein localization in real-time is crucial since protein complexes can assemble and reorganize rapidly in response to cellular signals. These dynamic changes enable cells to trigger fast and appropriate responses to various stimuli. Traditional biochemical assays often provide a static snapshot of protein interactions, failing to capture the dynamic nature of these events. On the other hand, techniques that can monitor protein localization and interactions in living cells with high temporal resolution, (such as those employed in this thesis), are essential for understanding the dynamic regulation of signaling pathways.

Tools based on FRET, and related techniques like FLIM (Fluorescence Lifetime Imaging Microscopy) and FRAP (Fluorescence Recovery After Photobleaching), are indeed crucial for advancing our understanding of signaling cascade regulation. These techniques allow researchers to go beyond static snapshots and directly examine the dynamic aspects of signaling processes within living cells. In essence, FRET, FLIM, and FRAP provide complementary approaches to dissect the intricate spatial and temporal regulation of signaling cascades, offering a more nuanced understanding of how cells process and respond to information.

This thesis represents a preliminary step toward developing a FRET-based tool to characterize the dynamics of TGF- β signaling activation and deactivation. By utilizing the dimerization of different R-Smads fused with fluorescent proteins, this approach aims to provide a dynamic readout of Smad complex formation in response to TGF- β pathway activation. This tool holds significant potential for characterizing the dynamics of TGF- β signaling during vertebrate neurogenesis. Specifically, it can be used to monitor in real-time when and where different Smad complexes (Smad2/Smad2/Smad4, Smad3/Smad3/Smad4, or Smad2/Smad3/Smad4) form in response to TGF- β stimulation. This will allow for a more detailed understanding of how these complexes are dynamically regulated and how their formation correlates with specific biological events.

The FRET approach proposed here involves overexpression of the proteins involved, in this case, the R-Smads. This overexpression is often assumed to place a significant burden on the cells' protein assembly machinery, potentially leading to increased energy demands and altered cellular functionality (Borkowski et al., 2018). Interestingly, despite this burden, the nuclear distribution of Smad 2 and Smad 3 remained consistent across various clones and fluorophores, suggesting that the overexpression plasmids may be adaptable for use in other cell types (Marvin et al., 2022). This limitation should be addressed in future studies by optimizing expression levels or that more closely mimic physiological conditions.

Nucleocytoplasmic shuttling of R-Smads was explored under different conditions, revealing that at lower expression levels, R-Smads remained predominantly cytoplasmic, whereas higher levels were found in the nucleus. These patterns were consistent across different experiments, in addition, the proportion of Smad3 that is nuclear is significantly higher than for Smad2, suggesting distinct activation level and distinct import/export mechanisms for Smad 2 and Smad 3. These findings align with previous studies that highlight the independence of these mechanisms, supporting the notion that Smad 3 may be more actively translocated into the nucleus compared to Smad 2 (Li, et al., 2016).

The experimental results also indicate that PC12 cells may not be an ideal model for studying TGF- β signaling, since they did not exhibit the expected nucleocytoplasmic translocation dynamics. On the other hand, N2A cells responded well to Activin A treatment. This suggests that N2A cells may be better suited for investigating the TGF- β pathway, particularly when studying translocation dynamics and cellular responses to activin treatment.

Our findings align well with previous studies, such as those by Hough et al. (2012) and Kamato et al. (2013), which emphasize the importance of phosphorylation in Smad signaling and nuclear translocation. Our results further support the notion that phosphorylation plays a key role in regulating Smad 2 and Smad 3, particularly in how they respond to pathway activation and cellular stimuli.

The analysis also points to the broader implications of Smad signaling in the cell cycle. Elevated levels of phosphorylated Smad 2 in M-phase cells suggest a role for Smad 2 in regulating cell division, potentially linking TGF- β signaling to cell cycle control mechanisms. This connection requires further investigation, as it could provide insights into how TGF- β signaling contributes to cell proliferation and differentiation in different biological contexts.

It is important to acknowledge that the model used in this study is a simplification of the complex biological system. As such, it may not fully capture the intricate dynamics and regulatory mechanisms governing TGF- β signaling. While more complex models with numerous parameters could potentially provide a more detailed representation, they can also become as challenging to interpret as the experimental data itself. Therefore, these simplified models can be more useful for providing conceptual insights and generating testable predictions, offering a balance between accuracy and interpretability. The model builds on a previous model proposed by our lab that despite its simplicity, it allowed us to explain the dual cooperativity and antagonism between the R-Smads in the context of the vertebrate neurogenesis (Miguez et al., 2013).

Our findings also raise questions about the functional relevance of phosphorylated Smads in the cytoplasm. The presence of phosphorylated Smad 3 and Smad2 outside the nucleus in non-negligible levels, even under control conditions, suggests that these forms may have roles beyond the classical pathway activation. Future research should explore whether these phosphorylated Smads can have other regulatory functions, such as interacting to cyclins or other proteins to coordinate or regulate cell cycle, perhaps in a context-dependent manner.

In conclusion, our results represent a preliminary characterization of these fusion proteins in a cellular context, in an effort to develop a reliable FRET tool that can be used to obtain a deeper understanding of the differential roles of Smad 2 and Smad 3 in TGF β signaling. Future studies from our lab will build on top of these findings by exploring the mechanisms underlying Smad behavior in different cell types using the FRET approach to study the dynamics of TGF- β signaling during vertebrate neurogenesis.

VI. CONCLUSIONES / CONCLUSIONS

VI. CONCLUSIONES

Las principales conclusiones derivadas de esta tesis son:

- 1- Comenzamos con una caracterización exhaustiva de la organización intracelular y el estado de fosforilación de Smad2 y Smad3 endógenos en tres circunstancias diferentes de la vía: control, activada e inhibida.
- 2- La localización de Smad2 y Smad3 endógenos, basada en el análisis de tinción por inmunofluorescencia, sigue el patrón esperado de aumento en el núcleo después del tratamiento con activina A.
- 3- Los niveles de Smad2 y Smad3 fosforilados aumentan en las células N2A después del tratamiento con activina A, pero no en las células PC12. Esto sugiere que la línea celular N2A es adecuada para investigar la dinámica de las R-Smads.
- 4- Los niveles de Smad2 fosforilado muestran una fuerte correlación con la etapa del ciclo celular, aumentando significativamente en las células en fase M. Esto sugiere una posible interacción entre la señalización de TGF- β y los reguladores del ciclo celular, y un posible papel en la regulación de la división celular.
- 5- El anticuerpo comúnmente utilizado en muchos estudios previos para monitorear la localización y los niveles de expresión de Smad2 muestra predominantemente una organización citoplasmática. Esto sugiere que Smad2 se organiza principalmente como monómeros y que la vía TGF- β está en gran medida inactiva bajo las condiciones observadas. Sin embargo, la expresión exógena de una versión de Smad2 marcada fluorescentemente contradice este análisis de inmunofluorescencia.
- 6- La combinación de la sobreexpresión de Smad2 marcado fluorescentemente con la tinción con el anticuerpo revela que el anticuerpo no penetra en el núcleo. Basándonos en este hallazgo, sugerimos que muchos estudios que utilizaron previamente este anticuerpo deberían ser reevaluados.
- 7- Las proteínas de fusión de Smad3 (Smad3-CFP y Smad3-YFP, pero no Smad3-mCherry) exhibieron una distribución similar a las proteínas endógenas, lo que indica que la etiqueta de fluorescencia adjunta (YFP o CFP) no impide su translocación correcta a través del complejo del poro nuclear.
- 8- Ambas proteínas de fusión de las R-Smads (con YFP o CFP) se fosforilan después del tratamiento con activina A, como lo demuestra la correlación entre la intensidad de CFP o YFP y la tinción por inmunofluorescencia utilizando anticuerpos específicos para las formas fosforiladas de las dos R-Smads. Esto sugiere que las proteínas de fusión reflejan con precisión la dinámica de fosforilación de las proteínas endógenas y pueden utilizarse con éxito para estudiar la dinámica de activación de la vía TGF- β .

- 9- Utilizando un enfoque de modelado matemático, investigamos el papel de la compartimentación de las R-Smads y su preferencia por la localización nuclear o citoplasmática dependiendo de su estado de fosforilación, como una nueva capa de regulación física dentro de la vía. Esta compartimentación física interactúa con la regulación bioquímica para determinar la respuesta de la señalización de TGF- β en términos de niveles de expresión de los genes diana de la vía.
- 10- Los experimentos preliminares que utilizan las proteínas de fusión como sensores FRET sugieren su potencial para estudiar la dinámica de diferentes formaciones de complejos (homodímeros o heterodímeros). Aunque todavía en las primeras etapas, esta investigación presenta oportunidades prometedoras para futuros experimentos y la utilización de Smads de fusión para explorar las complejidades de la vía TGF- β .

En conclusión, nuestros resultados contribuyen a una comprensión más profunda de los distintos roles de Smad2 y Smad3 en la señalización de TGF- β . Los estudios futuros podrían basarse en estos hallazgos explorando los mecanismos subyacentes a la dinámica de las R-Smads en diferentes tipos de células y optimizando los experimentos utilizando modelos animales. Además, investigar los roles no canónicos de las Smads no fosforiladas, como su influencia en las etapas del ciclo celular, podría proporcionar una comprensión más completa de la señalización de TGF- β y sus implicaciones para la función celular y la enfermedad.

VI. CONCLUSIONS

The main conclusions derived from this thesis are:

- 1- We begin with a thorough characterization of the intracellular organization and phosphorylation status of endogenous Smad2 and Smad3 under three different pathway conditions: control, activated, and inhibited.
- 2- The localization of endogenous Smad2 and Smad3, based on immunofluorescence staining analysis, follows the expected pattern of increased nuclear localization after Activin A treatment.
- 3- Levels of phosphorylated Smad2 and Smad3 increase in N2A cells after Activin A treatment, but not in PC12 cells. This suggests that the N2A cell line is suitable for investigating the dynamics of R-Smads.
- 4- Phosphorylated Smad2 levels show a strong correlation with the cell cycle stage, increasing significantly in M-phase cells. This suggests a potential interplay between TGF- β signaling and cell cycle regulators and a possible role in regulating cell division.
- 5- The antibody commonly used in many previous studies to monitor the localization and expression levels of Smad2 predominantly shows a cytoplasmic organization. This suggests that Smad2 is mainly organized as monomers and that the TGF- β pathway is largely inactive under the observed conditions. However, the exogenous expression of a fluorescently tagged version of Smad2 contradicts this immunofluorescence analysis.
- 6- Combining the overexpression of the fluorescently tagged Smad2 with the antibody staining reveals that the antibody is failing to penetrate into the nucleus. Based on this finding, we suggest that many studies that previously used this antibody should be re-evaluated.
- 7- Fusion proteins of Smad3 (Smad3-CFP and Smad3-YFP, but not Smad3-mCherry) exhibited a similar distribution to the endogenous proteins, indicating that the attached fluorescence tag (YFP or CFP) does not impede its correct translocation across the nuclear pore complex.
- 8- Both fusion proteins of the R-Smads (with YFP or CFP) are phosphorylated after Activin A treatment, as demonstrated by the correlation between the CFP or YFP intensity and the immunofluorescence staining using antibodies specific to the phosphorylated forms of the two R-Smads. This suggests that the fusion proteins accurately reflect the phosphorylation dynamics of the endogenous proteins and can be successfully used to study the activation dynamics of the TGF- β pathway.

- 9- Using a mathematical modeling approach, we investigated the role of R-Smad compartmentalization and its preference for nuclear or cytoplasmic localization depending on its phosphorylation state, as a novel layer of physical regulation within the pathway. This physical compartmentalization interacts with biochemical regulation to determine the response of TGF- β signaling in terms of target gene expression levels.
- 10- Preliminary experiments using the fusion proteins as FRET sensors suggest their potential for studying the dynamics of different complex formations (homodimers or heterodimers). Although still in the early stages, this research presents promising opportunities for future experiments and the utilization of fusion Smads to explore the complexities of the TGF- β pathway.

In conclusion, our results contribute to a deeper understanding of the distinct roles of Smad2 and Smad3 in TGF- β signaling. Future studies could build upon these findings by exploring the mechanisms underlying R-Smad dynamics in different cell types and by optimizing experiments using animal models. Furthermore, investigating the non-canonical roles of unphosphorylated Smads, such as their influence on cell cycle stages, could provide a more comprehensive understanding of TGF- β signaling and its implications for cellular function and disease.

VII. BIBLIOGRAPHY

VII-BIBLIOGRAPHY

- Altomare, D., Velidandla, R., Pirisi, L., & Creek, K. E. (2013). Partial loss of Smad signaling during in vitro progression of HPV16-immortalized human keratinocytes. *BMC cancer*, 13, 424. <https://doi.org/10.1186/1471-2407-13-424>
- Amritkar, K., Cuevas, B., & Kacar, B. (2024). Ancestral structure prediction reveals the conformational impact of the RuBisCO small subunit across time. *bioRxiv (Cold Spring Harbor Laboratory)*. <https://doi.org/10.1101/2024.06.06.597628>
- Antony, M. L., Nair, R., Sebastian, P., & Karunagaran, D. (2010). Changes in expression, and/or mutations in TGF-beta receptors (TGF-beta RI and TGF-beta RII) and Smad 4 in human ovarian tumors. *Journal of cancer research and clinical oncology*, 136(3), 351–361. <https://doi.org/10.1007/s00432-009-0703-4>
- Aragón, E., Wang, Q., Zou, Y., Morgani, S. M., Ruiz, L., Kaczmarska, Z., Su, J., Torner, C., Tian, L., Hu, J., Shu, W., Agrawal, S., Gomes, T., Márquez, J. A., Hadjantonakis, A. K., Macias, M. J., & Massagué, J. (2019). Structural basis for distinct roles of SMAD2 and SMAD3 in FOXH1 pioneer-directed TGF- β signaling. *Genes & development*, 33(21-22), 1506–1524. <https://doi.org/10.1101/gad.330837.119>
- Armingol, E., Officer, A., Harismendy, O., & Lewis, N. E. (2021). Deciphering cell-cell interactions and communication from gene expression. *Nature reviews. Genetics*, 22(2), 71–88. <https://doi.org/10.1038/s41576-020-00292-x>
- Azhar, M., Schultz, J. elJ., Grupp, I., Dorn, G. W., 2nd, Meneton, P., Molin, D. G., Gittenberger-de Groot, A. C., & Doetschman, T. (2003). Transforming growth factor beta in cardiovascular development and function. *Cytokine & growth factor reviews*, 14(5), 391–407. [https://doi.org/10.1016/s1359-6101\(03\)00044-3](https://doi.org/10.1016/s1359-6101(03)00044-3)
- Bai, H., Lester, G. M. S., Petishnok, L. C., & Dean, D. A. (2017). Cytoplasmic transport and nuclear import of plasmid DNA. *Bioscience reports*, 37(6), BSR20160616. <https://doi.org/10.1042/BSR20160616>
- Batlle, E., & Massagué, J. (2019). Transforming Growth Factor- β Signaling in Immunity and Cancer. *Immunity*, 50(4), 924–940. <https://doi.org/10.1016/j.immuni.2019.03.024>

- Berthollet, C.L. (1803). *Essai de Statique Chimique*; A Paris: Chez Firmin Didot . . .; Nabu Press: New York, NY, USA,.
- Bertolio, M. S., La Colla, A., Carrea, A., Romo, A., Canziani, G., Echarte, S. M., Campisano, S., Barletta, G. P., Monzon, A. M., Rodríguez, T. M., Chisari, A. N., & Dewey, R. A. (2021). A Novel Splice Variant of Human TGF- β Type II Receptor Encodes a Soluble Protein and Its Fc-Tagged Version Prevents Liver Fibrosis *in vivo*. *Frontiers in cell and developmental biology*, 9, 690397. <https://doi.org/10.3389/fcell.2021.690397>
- Bertrand-Chapel, A., Caligaris, C., Fenouil, T., Savary, C., Aires, S., Martel, S., Huchedé, P., Chassot, C., Chauvet, V., Cardot-Ruffino, V., Morel, A. P., Subtil, F., Mohkam, K., Mabrut, J. Y., Tonon, L., Viari, A., Cassier, P., Hervieu, V., Castets, M., Mauviel, A., ... Bartholin, L. (2022). SMAD2/3 mediate oncogenic effects of TGF- β in the absence of SMAD4. *Communications biology*, 5(1), 1068. <https://doi.org/10.1038/s42003-022-03994-6>
- Binder, J. X., Pletscher-Frankild, S., Tsafou, K., Stolte, C., O'Donoghue, S. I., Schneider, R., & Jensen, L. J. (2014). COMPARTMENTS: unification and visualization of protein subcellular localization evidence. *Database : the journal of biological databases and curation*, 2014, bau012. <https://doi.org/10.1093/database/bau012>
- Borkowski, O., Bricio, C., Murgiano, M., Rothschild-Mancinelli, B., Stan, G. B., & Ellis, T. (2018). Cell-free prediction of protein expression costs for growing cells. *Nature communications*, 9(1), 1457. <https://doi.org/10.1038/s41467-018-03970-x>
- Bourgeois, B., Gilquin, B., Tellier-Lebègue, C., Östlund, C., Wu, W., Pérez, J., El Hage, P., Lallemand, F., Worman, H. J., & Zinn-Justin, S. (2013). Inhibition of TGF- β signaling at the nuclear envelope: characterization of interactions between MAN1, Smad2 and Smad3, and PPM1A. *Science signaling*, 6(280), ra49. <https://doi.org/10.1126/scisignal.2003411>
- Brittain, G. C., & Gulnik, S. (2017). A rapid method for quantifying cytoplasmic versus nuclear localization in endogenous peripheral blood leukocytes by conventional flow cytometry. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 91(4), 351–363. <https://doi.org/10.1002/cyto.a.23103>

- Brunet, T., & King, N. (2017). The Origin of Animal Multicellularity and Cell Differentiation. *Developmental cell*, 43(2), 124–140. <https://doi.org/10.1016/j.devcel.2017.09.016>
- Burch, M. L., Zheng, W., & Little, P. J. (2011). Smad linker region phosphorylation in the regulation of extracellular matrix synthesis. *Cellular and molecular life sciences : CMLS*, 68(1), 97–107. <https://doi.org/10.1007/s00018-010-0514-4>
- Chacko, B. M., Qin, B. Y., Tiwari, A., Shi, G., Lam, S., Hayward, L. J., De Caestecker, M., & Lin, K. (2004). Structural basis of heteromeric smad protein assembly in TGF-beta signaling. *Molecular cell*, 15(5), 813–823. <https://doi.org/10.1016/j.molcel.2004.07.016>
- Chacko, B. M., Qin, B., Correia, J. J., Lam, S. S., de Caestecker, M. P., & Lin, K. (2001). The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. *Nature structural biology*, 8(3), 248–253. <https://doi.org/10.1038/84995>
- Chaikuad, A., & Bullock, A. N. (2016). Structural Basis of Intracellular TGF- β Signaling: Receptors and Smads. *Cold Spring Harbor perspectives in biology*, 8(11), a022111. <https://doi.org/10.1101/cshperspect.a022111>
- 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-beta receptors. *The Journal of biological chemistry*, 270(20), 12235–12241. <https://doi.org/10.1074/jbc.270.20.12235>
- Chen, W. W., Niepel, M., & Sorger, P. K. (2010). Classic and contemporary approaches to modeling biochemical reactions. *Genes & development*, 24(17), 1861–1875. <https://doi.org/10.1101/gad.1945410>
- Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C., & Piccolo, S. (2003). Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell*, 113(3), 301–314. [https://doi.org/10.1016/s0092-8674\(03\)00308-8](https://doi.org/10.1016/s0092-8674(03)00308-8)
- Dai, F., Lin, X., Chang, C., & Feng, X. H. (2009). Nuclear export of Smad2 and Smad3 by RanBP3 facilitates termination of TGF-beta signaling. *Developmental cell*, 16(3), 345–357. <https://doi.org/10.1016/j.devcel.2009.01.022>

- Dal Buono, A., Gaiani, F., Poliani, L., & Laghi, L. (2022). Juvenile polyposis syndrome: An overview. *Best practice & research. Clinical gastroenterology*, 58-59, 101799. <https://doi.org/10.1016/j.bpg.2022.101799>
- Danielpour D. (2024). Advances and Challenges in Targeting TGF- β Isoforms for Therapeutic Intervention of Cancer: A Mechanism-Based Perspective. *Pharmaceuticals (Basel, Switzerland)*, 17(4), 533. <https://doi.org/10.3390/ph17040533>
- De Larco, J. E., & Todaro, G. J. (1978). Growth factors from murine sarcoma virus-transformed cells. *Proceedings of the National Academy of Sciences of the United States of America*, 75(8), 4001–4005. <https://doi.org/10.1073/pnas.75.8.4001>
- Deng, Z., Fan, T., Xiao, C., Tian, H., Zheng, Y., Li, C., & He, J. (2024). TGF- β signaling in health, disease, and therapeutics. *Signal transduction and targeted therapy*, 9(1), 61. <https://doi.org/10.1038/s41392-024-01764-w>
- Derynck, R., & Budi, E. H. (2019). Specificity, versatility, and control of TGF- β family signaling. *Science signaling*, 12(570), eaav5183. <https://doi.org/10.1126/scisignal.aav5183>
- Derynck, R., & Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature*, 425(6958), 577–584. <https://doi.org/10.1038/nature02006>
- Derynck, R., Turley, S. J., & Akhurst, R. J. (2021). TGF β biology in cancer progression and immunotherapy. *Nature reviews. Clinical oncology*, 18(1), 9–34. <https://doi.org/10.1038/s41571-020-0403-1>
- Dewidar, B., Meyer, C., Dooley, S., & Meindl-Beinker, A. N. (2019). TGF- β in Hepatic Stellate Cell Activation and Liver Fibrogenesis-Updated 2019. *Cells*, 8(11), 1419. <https://doi.org/10.3390/cells8111419>
- Diacou, R., Nandigrami, P., Fiser, A., Liu, W., Ashery-Padan, R., & Cvekl, A. (2022). Cell fate decisions, transcription factors and signaling during early retinal development. *Progress in retinal and eye research*, 91, 101093. <https://doi.org/10.1016/j.preteyeres.2022.101093>

- Díaz, M., & Malacrida, L. (2023). Advanced Fluorescence Microscopy Methods to Study Dynamics of Fluorescent Proteins In Vivo. *Methods in molecular biology (Clifton, N.J.)*, 2564, 53–74. https://doi.org/10.1007/978-1-0716-2667-2_3
- Doldán-Martelli, V., Guantes, R., & Míguez, D. G. (2013). A mathematical model for the rational design of chimeric ligands in selective drug therapies. *CPT: pharmacometrics & systems pharmacology*, 2(2), e26. <https://doi.org/10.1038/psp.2013.2>
- Dong, C., Li, Z., Alvarez, R., Jr, Feng, X. H., & Goldschmidt-Clermont, P. J. (2000). Microtubule binding to Smads may regulate TGF beta activity. *Molecular cell*, 5(1), 27–34. [https://doi.org/10.1016/s1097-2765\(00\)80400-1](https://doi.org/10.1016/s1097-2765(00)80400-1)
- Dréau, G. L., & Martí, E. (2013). The multiple activities of BMPs during spinal cord development. *Cellular and Molecular Life Sciences*, 70(22), 4293–4305. <https://doi.org/10.1007/s00018-013-1354-9>
- Duan, D., & Derynck, R. (2019). Transforming growth factor- β (TGF- β)-induced up-regulation of TGF- β receptors at the cell surface amplifies the TGF- β response. *The Journal of biological chemistry*, 294(21), 8490–8504. <https://doi.org/10.1074/jbc.RA118.005763>
- Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., & Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *Journal of virology*, 72(11), 8463–8471. <https://doi.org/10.1128/JVI.72.11.8463-8471.1998>
- Elston, R., & Inman, G. J. (2012). Crosstalk between p53 and TGF- β Signalling. *Journal of signal transduction*, 2012, 294097. <https://doi.org/10.1155/2012/294097>
- Erb, T. J., & Zarzycki, J. (2018). A short history of RubisCO: the rise and fall (?) of Nature's predominant CO₂ fixing enzyme. *Current opinion in biotechnology*, 49, 100–107. <https://doi.org/10.1016/j.copbio.2017.07.017>
- Fleming, N. I., Jorissen, R. N., Mouradov, D., Christie, M., Sakthianandeswaren, A., Palmieri, M., Day, F., Li, S., Tsui, C., Lipton, L., Desai, J., Jones, I. T., McLaughlin, S., Ward, R. L., Hawkins, N. J., Ruskiewicz, A. R., Moore, J., Zhu, H. J., Mariadason, J. M., Burgess, A. W., ... Sieber, O. M. (2013). SMAD2, SMAD3 and SMAD4 mutations in colorectal cancer. *Cancer research*, 73(2), 725–735. <https://doi.org/10.1158/0008-5472.CAN-12-2706>

- Frick, C. L., Yarka, C., Nunns, H., & Goentoro, L. (2017). Sensing relative signal in the Tgf- β /Smad pathway. *Proceedings of the National Academy of Sciences of the United States of America*, 114(14), E2975–E2982. <https://doi.org/10.1073/pnas.1611428114>
- Giarratana, A. O., Prendergast, C. M., Salvatore, M. M., & Capaccione, K. M. (2024). TGF- β signaling: critical nexus of fibrogenesis and cancer. *Journal of translational medicine*, 22(1), 594. <https://doi.org/10.1186/s12967-024-05411-4>
- Gilboa, L., Wells, R. G., Lodish, H. F., & Henis, Y. I. (1998). Oligomeric structure of type I and type II transforming growth factor beta receptors: homodimers form in the ER and persist at the plasma membrane. *The Journal of cell biology*, 140(4), 767–777. <https://doi.org/10.1083/jcb.140.4.767>
- Glasgow, E., & Mishra, L. (2008). Transforming growth factor-beta signaling and ubiquitinators in cancer. *Endocrine-related cancer*, 15(1), 59–72. <https://doi.org/10.1677/ERC-07-0168>
- Gomez-Puerto, M. C., Iyengar, P. V., García de Vinuesa, A., Ten Dijke, P., & Sanchez-Duffhues, G. (2019). Bone morphogenetic protein receptor signal transduction in human disease. *The Journal of pathology*, 247(1), 9–20. <https://doi.org/10.1002/path.5170>
- Gordon, S. R., Maute, R. L., Dulken, B. W., Hutter, G., George, B. M., McCracken, M. N., Gupta, R., Tsai, J. M., Sinha, R., Corey, D., Ring, A. M., Connolly, A. J., & Weissman, I. L. (2017). PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature*, 545(7655), 495–499. <https://doi.org/10.1038/nature22396>
- Goumans, M. J., & Ten Dijke, P. (2018). TGF- β Signaling in Control of Cardiovascular Function. *Cold Spring Harbor perspectives in biology*, 10(2), a022210. <https://doi.org/10.1101/cshperspect.a022210>
- Greer, E. R., Pérez, C. L., Van Gilst, M. R., Lee, B. H., & Ashrafi, K. (2008). Neural and molecular dissection of a C. elegans sensory circuit that regulates fat and feeding. *Cell metabolism*, 8(2), 118–131. <https://doi.org/10.1016/j.cmet.2008.06.005>
- Grillo, C., D'Ambrosio, C., Consalvi, V., Chiaraluce, R., Scaloni, A., Maceroni, M., Eufemi, M., & Altieri, F. (2007). DNA-binding activity of the ERp57 C-terminal domain is related to a redox-dependent conformational change. *The Journal of biological chemistry*, 282(14), 10299–10310. <https://doi.org/10.1074/jbc.M700966200>

- Grindel, B. J., Rohe, B., Safford, S. E., Bennett, J. J., & Farach-Carson, M. C. (2011). Tumor necrosis factor- α treatment of HepG2 cells mobilizes a cytoplasmic pool of ERp57/1,25D₃-MARRS to the nucleus. *Journal of cellular biochemistry*, 112(9), 2606–2615. <https://doi.org/10.1002/jcb.23187>
- Gromova, K. V., Friedrich, M., Noskov, A., & Harms, G. S. (2007). Visualizing Smad1/4 signaling response to bone morphogenetic protein-4 activation by FRET biosensors. *Biochimica et biophysica acta*, 1773(12), 1759–1773. <https://doi.org/10.1016/j.bbamcr.2007.09.007>
- Gu, S., Derynck, R., Chen, Y. G., & Feng, X. H. (2024). New progress in roles of TGF- β signaling crosstalks in cellular functions, immunity and diseases. *Cell regeneration (London, England)*, 13(1), 11. <https://doi.org/10.1186/s13619-024-00194-x>
- Guldborg, C.M. & Waage, P. (1879). Ueber die chemische Affinität. § 1. Einleitung," *Journal Für Praktische Chemie*, 19 (1), 69–114. <https://doi.org/10.1002/prac.18790190111>
- Hangos, K.M. & Szederkényi, G. (2011). Mass action realizations of reaction kinetic system models on various time scales, *Journal of Physics Conference Series*, 268, 012009. <https://doi.org/10.1088/1742-6596/268/1/012009>
- Han, S. U., Kim, H. T., Seong, D. H., Kim, Y. S., Park, Y. S., Bang, Y. J., Yang, H. K., & Kim, S. J. (2004). Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer. *Oncogene*, 23(7), 1333–1341. <https://doi.org/10.1038/sj.onc.1207259>
- Hao, Y., Baker, D., & Ten Dijke, P. (2019). TGF- β -Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis. *International journal of molecular sciences*, 20(11), 2767. <https://doi.org/10.3390/ijms20112767>
- Hasskarl, J., Butz, K., Whitaker, N., Ullmann, A., Dürst, M., & Hoppe-Seyler, F. (2000). Differential cell cycle response of nontumorigenic and tumorigenic human papillomavirus-positive keratinocytes towards transforming growth factor-beta1. *Journal of molecular medicine (Berlin, Germany)*, 78(2), 94–101. <https://doi.org/10.1007/s001090000083>
- Hata, A., & Chen, Y. G. (2016). TGF- β Signaling from Receptors to Smads. *Cold Spring Harbor perspectives in biology*, 8(9), a022061. <https://doi.org/10.1101/cshperspect.a022061>

- Hatori, R., & Kornberg, T. B. (2020). Hedgehog produced by the *Drosophila* wing imaginal disc induces distinct responses in three target tissues. *Development (Cambridge, England)*, 147(22), dev195974. <https://doi.org/10.1242/dev.195974>
- Heldin, C. H., & Moustakas, A. (2012). Role of Smads in TGF β signaling. *Cell and tissue research*, 347(1), 21–36. <https://doi.org/10.1007/s00441-011-1190-x>
- Heldin, C. H., & Moustakas, A. (2016). Signaling Receptors for TGF- β Family Members. *Cold Spring Harbor perspectives in biology*, 8(8), a022053. <https://doi.org/10.1101/cshperspect.a022053>
- Herbertz, S., Sawyer, J. S., Stauber, A. J., Gueorguieva, I., Driscoll, K. E., Estrem, S. T., Cleverly, A. L., Desai, D., Guba, S. C., Benhadji, K. A., Slapak, C. A., & Lahn, M. M. (2015). Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway. *Drug design, development and therapy*, 9, 4479–4499. <https://doi.org/10.2147/DDDT.S86621>
- Hill C. S. (2009). Nucleocytoplasmic shuttling of Smad proteins. *Cell research*, 19(1), 36–46. <https://doi.org/10.1038/cr.2008.325>
- Hill C. S. (2016). Transcriptional Control by the SMADs. *Cold Spring Harbor perspectives in biology*, 8(10), a022079. <https://doi.org/10.1101/cshperspect.a022079>
- Hoff, J.H.V. (1877). Die Grenzebene, ein Beitrag zur Kenntniss der Esterbildung, *Berichte Der Deutschen Chemischen Gesellschaft*, 10(1), 669–78. <https://doi.org/10.1002/cber.187701001185>
- Hough, C., Radu, M., & Doré, J. J. (2012). TGF β eta induced Erk phosphorylation of smad linker region regulates smad signaling. *PloS one*, 7(8), e42513. <https://doi.org/10.1371/journal.pone.0042513>
- Huang, D., Zhao, D., Li, M., Chang, S. Y., Xue, Y. D., Xu, N., Li, S. J., Tang, N. N., Gong, L. L., Liu, Y. N., Yu, H., Li, Q. S., Li, P. Y., Liu, J. L., Chen, H. X., Liu, M. B., Zhang, W. Y., Zhao, X. M., Lang, X. Z., Li, Z. D., ... Cai, B. Z. (2023). Crosstalk between PML and p53 in response to TGF- β 1: A new mechanism of cardiac fibroblast activation. *International journal of biological sciences*, 19(3), 994–1006. <https://doi.org/10.7150/ijbs.76214>

- Huang, F., & Chen, Y. G. (2012). Regulation of TGF- β receptor activity. *Cell & bioscience*, 2, 9. <https://doi.org/10.1186/2045-3701-2-9>
- Huang, T., Schor, S. L., & Hinck, A. P. (2014). Biological activity differences between TGF- β 1 and TGF- β 3 correlate with differences in the rigidity and arrangement of their component monomers. *Biochemistry*, 53(36), 5737–5749. <https://doi.org/10.1021/bi500647d>
- Hudson, J. B., Podos, S. D., Keith, K., Simpson, S. L., & Ferguson, E. L. (1998). The Drosophila Medea gene is required downstream of dpp and encodes a functional homolog of human Smad4. *Development (Cambridge, England)*, 125(8), 1407–1420. <https://doi.org/10.1242/dev.125.8.1407>
- Ishikawa-Ankerhold, H. C., Ankerhold, R., & Drummen, G. P. (2012). Advanced fluorescence microscopy techniques--FRAP, FLIP, FLAP, FRET and FLIM. *Molecules (Basel, Switzerland)*, 17(4), 4047–4132. <https://doi.org/10.3390/molecules17044047>
- Jetton, T. L., Shiota, M., Knobel, S. M., Piston, D. W., Cherrington, A. D., & Magnuson, M. A. (2001). Substrate-induced nuclear export and peripheral compartmentalization of hepatic glucokinase correlates with glycogen deposition. *International journal of experimental diabetes research*, 2(3), 173–186. <https://doi.org/10.1155/edr.2001.173>
- Ji, R. L., & Tao, Y. X. (2025). Biased signaling in drug discovery and precision medicine. *Pharmacology & therapeutics*, 268, 108804. <https://doi.org/10.1016/j.pharmthera.2025.108804>
- Jia, S., & Meng, A. (2021). TGF β family signaling and development. *Development (Cambridge, England)*, 148(5), dev188490. <https://doi.org/10.1242/dev.188490>
- Kagan, T., Gabay, M., Meenakshisundaram, A., Levi, Y., Eid, S., Malchenko, N., Maman, M., Nitzan, A., Ravotto, L., Zaidel-Bar, R., Eickholt, B. J., Gal, M., & Laviv, T. (2025). Genetically encoded biosensor for fluorescence lifetime imaging of PTEN dynamics in the intact brain. *Nature methods*, 10.1038/s41592-025-02610-9. Advance online publication. <https://doi.org/10.1038/s41592-025-02610-9>

- Kamato, D., Burch, M. L., Piva, T. J., Rezaei, H. B., Rostam, M. A., Xu, S., Zheng, W., Little, P. J., & Osman, N. (2013). Transforming growth factor- β signalling: role and consequences of Smad linker region phosphorylation. *Cellular signalling*, 25(10), 2017–2024. <https://doi.org/10.1016/j.cellsig.2013.06.001>
- Kannaiyan, R., & Mahadevan, D. (2018). A comprehensive review of protein kinase inhibitors for cancer therapy. *Expert review of anticancer therapy*, 18(12), 1249–1270. <https://doi.org/10.1080/14737140.2018.1527688>
- Khatibi, S., Zhu, H. J., Wagner, J., Tan, C. W., Manton, J. H., & Burgess, A. W. (2017). Mathematical model of TGF- β signalling: feedback coupling is consistent with signal switching. *BMC systems biology*, 11(1), 48. <https://doi.org/10.1186/s12918-017-0421-5>
- Kholodenko, B. N., Rauch, N., Kolch, W., & Rukhlenko, O. S. (2021). A systematic analysis of signaling reactivation and drug resistance. *Cell reports*, 35(8), 109157. <https://doi.org/10.1016/j.celrep.2021.109157>
- Kim, B. G., Malek, E., Choi, S. H., Ignatz-Hoover, J. J., & Driscoll, J. J. (2021). Novel therapies emerging in oncology to target the TGF- β pathway. *Journal of hematology & oncology*, 14(1), 55. <https://doi.org/10.1186/s13045-021-01053-x>
- Kimble, J., & Nüsslein-Volhard, C. (2022). The great small organisms of developmental genetics: *Caenorhabditis elegans* and *Drosophila melanogaster*. *Developmental biology*, 485, 93–122. <https://doi.org/10.1016/j.ydbio.2022.02.013>
- Kimura, T., Imaishi, K., Hagiwara, Y., Horibe, T., Hayano, T., Takahashi, N., Urade, R., Kato, K., & Kikuchi, M. (2005). ERp57 binds competitively to protein disulfide isomerase and calreticulin. *Biochemical and biophysical research communications*, 331(1), 224–230. <https://doi.org/10.1016/j.bbrc.2005.03.147>
- Kretschmar, M., Doody, J., Timokhina, I., & Massagué, J. (1999). A mechanism of repression of TGF β / Smad signaling by oncogenic Ras. *Genes & development*, 13(7), 804–816. <https://doi.org/10.1101/gad.13.7.804>
- Krishnan V. (2023). The RUNX Family of Proteins, DNA Repair, and Cancer. *Cells*, 12(8), 1106. <https://doi.org/10.3390/cells12081106>

- Kuang, C., & Chen, Y. (2004). Tumor-derived C-terminal mutations of Smad4 with decreased DNA binding activity and enhanced intramolecular interaction. *Oncogene*, 23(5), 1021–1029. <https://doi.org/10.1038/sj.onc.1207219>
- Kulkarni, A. B., Thyagarajan, T., & Letterio, J. J. (2002). Function of cytokines within the TGF-beta superfamily as determined from transgenic and gene knockout studies in mice. *Current molecular medicine*, 2(3), 303–327. <https://doi.org/10.2174/1566524024605699>
- Lampe, M., Vassilopoulos, S., & Merrifield, C. (2016). Clathrin coated pits, plaques and adhesion. *Journal of structural biology*, 196(1), 48–56. <https://doi.org/10.1016/j.jsb.2016.07.009>
- Li, N., Yang, Y., He, K., Zhang, F., Zhao, L., Zhou, W., Yuan, J., Liang, W., & Fang, X. (2016). Single-Molecule Imaging Reveals the Activation Dynamics of Intracellular Protein Smad3 on Cell Membrane. *Scientific reports*, 6, 33469. <https://doi.org/10.1038/srep33469>
- Li, Y., Lee, H., Heo, W. D., & Zi, Z. (2021). Revisiting the Role of TGFβ Receptor Internalization for Smad Signaling: It is Not Required in Optogenetic TGFβ Signaling Systems. *Advanced biology*, 5(10), e2101008. <https://doi.org/10.1002/adbi.202101008>
- Li, Y., Luo, W., & Yang, W. (2018). Nuclear Transport and Accumulation of Smad Proteins Studied by Single-Molecule Microscopy. *Biophysical journal*, 114(9), 2243–2251. <https://doi.org/10.1016/j.bpj.2018.03.018>
- Lim, F., Khalique, H., Ventosa, M., & Baldo, A. (2013). Biosafety of gene therapy vectors derived from herpes simplex virus type 1. *Current gene therapy*, 13(6), 478–491. <https://doi.org/10.2174/156652321306140103224550>
- Lim, J., Petersen, M., Bunz, M., Simon, C., & Schindler, M. (2022). Flow cytometry based-FRET: basics, novel developments and future perspectives. *Cellular and molecular life sciences : CMLS*, 79(4), 217. <https://doi.org/10.1007/s00018-022-04232-2>
- Liu, L., Li, Q., Yang, L., Li, Q., & Du, X. (2021). SMAD4 Feedback Activates the Canonical TGF-β Family Signaling Pathways. *International journal of molecular sciences*, 22(18), 10024. <https://doi.org/10.3390/ijms221810024>

- Liu, N. N., Xi, Y., Callaghan, M. U., Fribley, A., Moore-Smith, L., Zimmerman, J. W., Pasche, B., Zeng, Q., & Li, Y. L. (2014). SMAD4 is a potential prognostic marker in human breast carcinomas. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, 35(1), 641–650. <https://doi.org/10.1007/s13277-013-1088-1>
- Liu, S., Ren, J., Hu, Y., Zhou, F., & Zhang, L. (2024). TGF β family signaling in human stem cell self-renewal and differentiation. *Cell regeneration (London, England)*, 13(1), 26. <https://doi.org/10.1186/s13619-024-00207-9>
- Lo, W. S., Roca, M., Dardiry, M., Mackie, M., Eberhardt, G., Witte, H., Hong, R., Sommer, R. J., & Lightfoot, J. W. (2022). Evolution and Diversity of TGF- β Pathways are Linked with Novel Developmental and Behavioral Traits. *Molecular biology and evolution*, 39(12), msac252. <https://doi.org/10.1093/molbev/msac252>
- López-Casillas, F., Wrana, J. L., & Massagué, J. (1993). Betaglycan presents ligand to the TGF beta signaling receptor. *Cell*, 73(7), 1435–1444. [https://doi.org/10.1016/0092-8674\(93\)90368-z](https://doi.org/10.1016/0092-8674(93)90368-z)
- Lu, J., Wu, T., Zhang, B., Liu, S., Song, W., Qiao, J., & Ruan, H. (2021). Types of nuclear localization signals and mechanisms of protein import into the nucleus. *Cell communication and signaling : CCS*, 19(1), 60. <https://doi.org/10.1186/s12964-021-00741-y>
- Luo, Q., Nieves, E., Kzhyshkowska, J., & Angeletti, R. H. (2006). Endogenous transforming growth factor-beta receptor-mediated Smad signaling complexes analyzed by mass spectrometry. *Molecular & cellular proteomics : MCP*, 5(7), 1245–1260. <https://doi.org/10.1074/mcp.M600065-MCP200>
- Makvandi, P., Chen, M., Sartorius, R., Zarrabi, A., Ashrafizadeh, M., Dabbagh Moghaddam, F., Ma, J., Mattoli, V., & Tay, F. R. (2021). Endocytosis of abiotic nanomaterials and nanobiovectors: Inhibition of membrane trafficking. *Nano today*, 40, 101279. <https://doi.org/10.1016/j.nantod.2021.101279>

- Marques, L., Costa, B., Pereira, M., Silva, A., Santos, J., Saldanha, L., Silva, I., Magalhães, P., Schmidt, S., & Vale, N. (2024). Advancing Precision Medicine: A Review of Innovative In Silico Approaches for Drug Development, Clinical Pharmacology and Personalized Healthcare. *Pharmaceutics*, 16(3), 332. <https://doi.org/10.3390/pharmaceutics16030332>
- Maruyama I. N. (2014). Mechanisms of activation of receptor tyrosine kinases: monomers or dimers. *Cells*, 3(2), 304–330. <https://doi.org/10.3390/cells3020304>
- Marvin, D. L., You, L., Bornes, L., van Dinther, M., Peters, N., Dang, H., Hakuno, S. K., Hornsvel, M., Kranenburg, O., van Rhee, J., Rohling, J. H. T., Chien, M. P., Ten Dijke, P., & Ritsma, L. (2022). Dynamic Visualization of TGF- β /SMAD3 Transcriptional Responses in Single Living Cells. *Cancers*, 14(10), 2508. <https://doi.org/10.3390/cancers14102508>
- Massagué J. (1998). TGF-beta signal transduction. *Annual review of biochemistry*, 67, 753–791. <https://doi.org/10.1146/annurev.biochem.67.1.753>
- Massagué, J., & Sheppard, D. (2023). TGF- β signaling in health and disease. *Cell*, 186(19), 4007–4037. <https://doi.org/10.1016/j.cell.2023.07.036>
- Massagué, J., & Wotton, D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. *The EMBO journal*, 19(8), 1745–1754. <https://doi.org/10.1093/emboj/19.8.1745>
- Massague, J., Czech, M. P., Iwata, K., DeLarco, J. E., & Todaro, G. J. (1982). Affinity labeling of a transforming growth factor receptor that does not interact with epidermal growth factor. *Proceedings of the National Academy of Sciences of the United States of America*, 79(22), 6822–6826. <https://doi.org/10.1073/pnas.79.22.6822>
- Matschinsky F. M. (2001). Ebb and tide of glucokinase. *International journal of experimental diabetes research*, 2(3), 168–171. <https://doi.org/10.1155/edr.2001.168>
- Mehrvarz Sarshekeh, A., Advani, S., Overman, M. J., Manyam, G., Kee, B. K., Fogelman, D. R., Dasari, A., Raghav, K., Vilar, E., Manuel, S., Shureiqi, I., Wolff, R. A., Patel, K. P., Luthra, R., Shaw, K., Eng, C., Maru, D. M., Routbort, M. J., Meric-Bernstam, F., & Kopetz, S. (2017). Association of SMAD4 mutation with patient demographics, tumor characteristics, and clinical outcomes in colorectal cancer. *PloS one*, 12(3), e0173345. <https://doi.org/10.1371/journal.pone.0173345>

- Mesaeli, N., & Phillipson, C. (2004). Impaired p53 expression, function, and nuclear localization in calreticulin-deficient cells. *Molecular biology of the cell*, 15(4), 1862–1870. <https://doi.org/10.1091/mbc.e03-04-0251>
- Míguez, D. G., Gil-Guiñón, E., Pons, S., & Martí, E. (2013). Smad2 and Smad3 cooperate and antagonize simultaneously in vertebrate neurogenesis. *Journal of cell science*, 126(Pt 23), 5335–5343. <https://doi.org/10.1242/jcs.130435>
- Míguez, D. G., Satnoianu, R. A., & Muñuzuri, A. P. (2006). Experimental steady pattern formation in reaction-diffusion-advection systems. *Physical review. E, Statistical, nonlinear, and soft matter physics*, 73(2 Pt 2), 025201. <https://doi.org/10.1103/PhysRevE.73.025201>
- Míguez, D.G. (2010), The role of asymmetric binding in ligand–receptor systems with 1:2 interaction ratio. *Biophysical Chemistry*, vol. 148, no. 1–3, pp. 74–81. <https://doi.org/10.1016/j.bpc.2010.02.012>
- Miyazawa, K., & Miyazono, K. (2017). Regulation of TGF- β Family Signaling by Inhibitory Smads. *Cold Spring Harbor perspectives in biology*, 9(3), a022095. <https://doi.org/10.1101/cshperspect.a022095>
- Miyazawa, K., Itoh, Y., Fu, H., & Miyazono, K. (2024). Receptor-activated transcription factors and beyond: multiple modes of Smad2/3-dependent transmission of TGF- β signaling. *The Journal of biological chemistry*, 300(5), 107256. <https://doi.org/10.1016/j.jbc.2024.107256>
- Miyazono, K., ten Dijke, P., & Heldin, C. H. (2000). TGF-beta signaling by Smad proteins. *Advances in immunology*, 75, 115–157. [https://doi.org/10.1016/s0065-2776\(00\)75003-6](https://doi.org/10.1016/s0065-2776(00)75003-6)
- Morikawa, M., Derynck, R., & Miyazono, K. (2016). TGF- β and the TGF- β Family: Context-Dependent Roles in Cell and Tissue Physiology. *Cold Spring Harbor perspectives in biology*, 8(5), a021873. <https://doi.org/10.1101/cshperspect.a021873>
- Morikawa, M., Koinuma, D., Miyazono, K., & Heldin, C. H. (2013). Genome-wide mechanisms of Smad binding. *Oncogene*, 32(13), 1609–1615. <https://doi.org/10.1038/onc.2012.191>

- Moses, H. L., Roberts, A. B., & Derynck, R. (2016). The Discovery and Early Days of TGF- β : A Historical Perspective. *Cold Spring Harbor perspectives in biology*, 8(7), a021865. <https://doi.org/10.1101/cshperspect.a021865>
- Moses, H., & Barcellos-Hoff, M. H. (2011). TGF-beta biology in mammary development and breast cancer. *Cold Spring Harbor perspectives in biology*, 3(1), a003277. <https://doi.org/10.1101/cshperspect.a003277>
- Nair, A., Chauhan, P., Saha, B., & Kubatzky, K. F. (2019). Conceptual Evolution of Cell Signaling. *International journal of molecular sciences*, 20(13), 3292. <https://doi.org/10.3390/ijms20133292>
- Naito, J., Kaji, H., Sowa, H., Hendy, G. N., Sugimoto, T., & Chihara, K. (2005). Menin suppresses osteoblast differentiation by antagonizing the AP-1 factor, JunD. *The Journal of biological chemistry*, 280(6), 4785–4791. <https://doi.org/10.1074/jbc.M408143200>
- Nakagoshi, H., Hoshi, M., Nabeshima, Y., & Matsuzaki, F. (1998). A novel homeobox gene mediates the Dpp signal to establish functional specificity within target cells. *Genes & development*, 12(17), 2724–2734. <https://doi.org/10.1101/gad.12.17.2724>
- Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., & ten Dijke, P. (1997). TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *The EMBO journal*, 16(17), 5353–5362. <https://doi.org/10.1093/emboj/16.17.5353>
- Nam K. H. (2023). The Conformational Change of the L3 Loop Affects the Structural Changes in the Substrate Binding Pocket Entrance of β -Glucosidase. *Molecules (Basel, Switzerland)*, 28(23), 7807. <https://doi.org/10.3390/molecules28237807>
- Newfeld, S. J., & O'Connor, M. B. (2022). New aspects of TGF- β superfamily signaling in development and disease (2022 FASEB meeting review). *Faculty reviews*, 11, 36. <https://doi.org/10.12703/r/11-36>
- Nickel, J., Ten Dijke, P., & Mueller, T. D. (2018). TGF- β family co-receptor function and signaling. *Acta biochimica et biophysica Sinica*, 50(1), 12–36. <https://doi.org/10.1093/abbs/gmx126>

- Nicolás, F. J., De Bosscher, K., Schmierer, B., & Hill, C. S. (2004). Analysis of Smad nucleocytoplasmic shuttling in living cells. *Journal of cell science*, 117(Pt 18), 4113–4125. <https://doi.org/10.1242/jcs.01289>
- Nofrini, V., Di Giacomo, D., & Mecucci, C. (2016). Nucleoporin genes in human diseases. *European journal of human genetics : EJHG*, 24(10), 1388–1395. <https://doi.org/10.1038/ejhg.2016.25>
- Oliver, J. D., Roderick, H. L., Llewellyn, D. H., & High, S. (1999). ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. *Molecular biology of the cell*, 10(8), 2573–2582. <https://doi.org/10.1091/mbc.10.8.2573>
- Oshimori, N., & Fuchs, E. (2012). The harmonies played by TGF- β in stem cell biology. *Cell stem cell*, 11(6), 751–764. <https://doi.org/10.1016/j.stem.2012.11.001>
- Otsuka, S., & Ellenberg, J. (2018). Mechanisms of nuclear pore complex assembly - two different ways of building one molecular machine. *FEBS letters*, 592(4), 475–488. <https://doi.org/10.1002/1873-3468.12905>
- Paci, G., Caria, J., & Lemke, E. A. (2021). Cargo transport through the nuclear pore complex at a glance. *Journal of cell science*, 134(2), jcs247874. <https://doi.org/10.1242/jcs.247874>
- Pardali, K., & Moustakas, A. (2007). Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. *Biochimica et biophysica acta*, 1775(1), 21–62. <https://doi.org/10.1016/j.bbcan.2006.06.004>
- Pierreux, C. E., Nicolás, F. J., & Hill, C. S. (2000). Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. *Molecular and cellular biology*, 20(23), 9041–9054. <https://doi.org/10.1128/MCB.20.23.9041-9054.2000>
- Pomeraniec, L., Hector-Greene, M., Ehrlich, M., Blobe, G. C., & Henis, Y. I. (2015). Regulation of TGF- β receptor hetero-oligomerization and signaling by endoglin. *Molecular biology of the cell*, 26(17), 3117–3127. <https://doi.org/10.1091/mbc.E15-02-0069>
- Randall, R. A., Howell, M., Page, C. S., Daly, A., Bates, P. A., & Hill, C. S. (2004). Recognition of phosphorylated-Smad2-containing complexes by a novel Smad interaction motif. *Molecular and cellular biology*, 24(3), 1106–1121. <https://doi.org/10.1128/MCB.24.3.1106-1121.2004>

- Renati, P. (2023). Reframing Some Methods in Life Sciences: Relationship and Causation in Living Matter. *Cut. Edge Res. Biol.* 8, 127–194. <https://doi.org/10.9734/bpi/cerb/v8/19273D>
- Ross, S., Cheung, E., Petrakis, T. G., Howell, M., Kraus, W. L., & Hill, C. S. (2006). Smads orchestrate specific histone modifications and chromatin remodeling to activate transcription. *The EMBO journal*, 25(19), 4490–4502. <https://doi.org/10.1038/sj.emboj.7601332>
- Ruiz, L., Kaczmarek, Z., Gomes, T., Aragon, E., Torner, C., Freier, R., Baginski, B., Martin-Malpartida, P., de Martin Garrido, N., Marquez, J. A., Cordeiro, T. N., Pluta, R., & Macias, M. J. (2021). Unveiling the dimer/monomer propensities of Smad MH1-DNA complexes. *Computational and structural biotechnology journal*, 19, 632–646. <https://doi.org/10.1016/j.csbj.2020.12.044>
- Ruiz-Herrero, T., Estrada, J., Guantes, R., & Miguez, D. G. (2013). A tunable coarse-grained model for ligand-receptor interaction. *PLoS computational biology*, 9(11), e1003274. <https://doi.org/10.1371/journal.pcbi.1003274>
- Runa, F., Ortiz-Soto, G., de Barros, N. R., & Kelber, J. A. (2024). Targeting SMAD-Dependent Signaling: Considerations in Epithelial and Mesenchymal Solid Tumors. *Pharmaceuticals (Basel, Switzerland)*, 17(3), 326. <https://doi.org/10.3390/ph17030326>
- Sabbah, D. A., Hajjo, R., Bardaweel, S. K., & Zhong, H. A. (2024). Targeting the PI3K/AKT signaling pathway in anticancer research: a recent update on inhibitor design and clinical trials (2020-2023). *Expert opinion on therapeutic patents*, 34(3), 141–158. <https://doi.org/10.1080/13543776.2024.2338100>
- Sanz, A.L. & Míguez, D.G. (2014). Dual R-Smads interplay in the regulation of vertebrate neurogenesis, *Neurogenesis*, 1(1), e29529. <https://doi.org/10.4161/neur.29529>
- Savage, C., Das, P., Finelli, A. L., Townsend, S. R., Sun, C. Y., Baird, S. E., & Padgett, R. W. (1996). Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. *Proceedings of the National Academy of Sciences of the United States of America*, 93(2), 790–794. <https://doi.org/10.1073/pnas.93.2.790>

- Schmierer, B., & Hill, C. S. (2005). Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads. *Molecular and cellular biology*, 25(22), 9845–9858. <https://doi.org/10.1128/MCB.25.22.9845-9858.2005>
- Schmierer, B., Tournier, A. L., Bates, P. A., & Hill, C. S. (2008). Mathematical modeling identifies Smad nucleocytoplasmic shuttling as a dynamic signal-interpreting system. *Proceedings of the National Academy of Sciences of the United States of America*, 105(18), 6608–6613. <https://doi.org/10.1073/pnas.0710134105>
- Schummer, J. (2006). The Philosophy of Chemistry. In *Philosophy of Chemistry: Synthesis of a New Discipline*; Baird, D., Scerri, E., McIntyre, L., Eds.; Springer: Dordrecht, The Netherlands, pp. 19–39. https://link.springer.com/chapter/10.1007/1-4020-3261-7_2
- Scott, M. S., Calafell, S. J., Thomas, D. Y., & Hallett, M. T. (2005). Refining protein subcellular localization. *PLoS computational biology*, 1(6), e66. <https://doi.org/10.1371/journal.pcbi.0010066>
- Seoane, J., & Gomis, R. R. (2017). TGF- β Family Signaling in Tumor Suppression and Cancer Progression. *Cold Spring Harbor perspectives in biology*, 9(12), a022277. <https://doi.org/10.1101/cshperspect.a022277>
- Sharmin, N., Nganwuchu, C. C., & Nasim, M. T. (2021). Targeting the TGF- β signaling pathway for resolution of pulmonary arterial hypertension. *Trends in pharmacological sciences*, 42(7), 510–513. <https://doi.org/10.1016/j.tips.2021.04.002>
- Shi, X., Yang, J., Deng, S., Xu, H., Wu, D., Zeng, Q., Wang, S., Hu, T., Wu, F., & Zhou, H. (2022). TGF- β signaling in the tumor metabolic microenvironment and targeted therapies. *Journal of hematology & oncology*, 15(1), 135. <https://doi.org/10.1186/s13045-022-01349-6>
- Silnitsky, S., Rubin, S. J. S., Zerihun, M., & Qvit, N. (2023). An Update on Protein Kinases as Therapeutic Targets-Part I: Protein Kinase C Activation and Its Role in Cancer and Cardiovascular Diseases. *International journal of molecular sciences*, 24(24), 17600. <https://doi.org/10.3390/ijms242417600>

- Sisto, M., Ribatti, D., & Lisi, S. (2021). SMADS-Mediate Molecular Mechanisms in Sjögren's Syndrome. *International journal of molecular sciences*, 22(6), 3203. <https://doi.org/10.3390/ijms22063203>
- Smith, E. R., & Hewitson, T. D. (2020). TGF- β 1 is a regulator of the pyruvate dehydrogenase complex in fibroblasts. *Scientific reports*, 10(1), 17914. <https://doi.org/10.1038/s41598-020-74919-8>
- Song, R., Tikoo, S., Jain, R., Pinello, N., Au, A. Y. M., Nagarajah, R., Porse, B., Rasko, J. E. J., & J-L Wong, J. (2022). Dynamic intron retention modulates gene expression in the monocytic differentiation pathway. *Immunology*, 165(2), 274–286. <https://doi.org/10.1111/imm.13435>
- Sorre, B., Warmflash, A., Brivanlou, A. H., & Siggia, E. D. (2014). Encoding of temporal signals by the TGF- β pathway and implications for embryonic patterning. *Developmental cell*, 30(3), 334–342. <https://doi.org/10.1016/j.devcel.2014.05.022>
- Steelman, L. S., Chappell, W. H., Abrams, S. L., Kempf, R. C., Long, J., Laidler, P., Mijatovic, S., Maksimovic-Ivanic, D., Stivala, F., Mazzarino, M. C., Donia, M., Fagone, P., Malaponte, G., Nicoletti, F., Libra, M., Milella, M., Tafuri, A., Bonati, A., Bäsecke, J., Cocco, L., ... McCubrey, J. A. (2011). Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging*, 3(3), 192–222. <https://doi.org/10.18632/aging.100296>
- Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., & Wahl, G. M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *The EMBO journal*, 18(6), 1660–1672. <https://doi.org/10.1093/emboj/18.6.1660>
- Su, J., Song, Y., Zhu, Z., Huang, X., Fan, J., Qiao, J., & Mao, F. (2024). Cell-cell communication: new insights and clinical implications. *Signal transduction and targeted therapy*, 9(1), 196. <https://doi.org/10.1038/s41392-024-01888-z>

- Sun, G., Hu, Z., Min, Z., Yan, X., Guan, Z., Su, H., Fu, Y., Ma, X., Chen, Y. G., Zhang, M. Q., Tao, Q., & Wu, W. (2015). Small C-terminal Domain Phosphatase 3 Dephosphorylates the Linker Sites of Receptor-regulated Smads (R-Smads) to Ensure Transforming Growth Factor β (TGF β)-mediated Germ Layer Induction in *Xenopus* Embryos. *The Journal of biological chemistry*, 290(28), 17239–17249. <https://doi.org/10.1074/jbc.M115.655605>
- Tan, X., Tong, L., Li, L., Xu, J., Xie, S., Ji, L., Fu, J., Liu, Q., Shen, S., Liu, Y., Xiao, Y., Gao, F., Moses, R. E., Bardeesy, N., Wang, Y., Zhang, J., Tang, L., Li, L., Wong, K. K., Song, D., ... Li, X. (2021). Loss of Smad4 promotes aggressive lung cancer metastasis by de-repression of PAK3 via miRNA regulation. *Nature communications*, 12(1), 4853. <https://doi.org/10.1038/s41467-021-24898-9>
- Tie, Y., Tang, F., Peng, D., Zhang, Y., & Shi, H. (2022). TGF-beta signal transduction: biology, function and therapy for diseases. *Molecular biomedicine*, 3(1), 45. <https://doi.org/10.1186/s43556-022-00109-9>
- Timney, B. L., Raveh, B., Mironska, R., Trivedi, J. M., Kim, S. J., Russel, D., Wente, S. R., Sali, A., & Rout, M. P. (2016). Simple rules for passive diffusion through the nuclear pore complex. *The Journal of cell biology*, 215(1), 57–76. <https://doi.org/10.1083/jcb.201601004>
- Tuncer, E., Calçada, R. R., Zingg, D., Varum, S., Cheng, P., Freiburger, S. N., Deng, C. X., Kleiter, I., Levesque, M. P., Dummer, R., & Sommer, L. (2019). SMAD signaling promotes melanoma metastasis independently of phenotype switching. *The Journal of clinical investigation*, 129(7), 2702–2716. <https://doi.org/10.1172/JCI94295>
- Tzavlaki, K., & Moustakas, A. (2020). TGF- β Signaling. *Biomolecules*, 10(3), 487. <https://doi.org/10.3390/biom10030487>
- Upadhyay, A., Moss-Taylor, L., Kim, M. J., Ghosh, A. C., & O'Connor, M. B. (2017). TGF- β Family Signaling in *Drosophila*. *Cold Spring Harbor perspectives in biology*, 9(9), a022152. <https://doi.org/10.1101/cshperspect.a022152>
- Valkenburg, K. C., De Marzo, A. M., & Williams, B. O. (2017). Deletion of tumor suppressors adenomatous polyposis coli and Smad4 in murine luminal epithelial cells causes invasive prostate cancer and loss of androgen receptor expression. *Oncotarget*, 8(46), 80265–80277. <https://doi.org/10.18632/oncotarget.17919>

- Van Munster, E. B., Kremers, G. J., Adjobo-Hermans, M. J., & Gadella, T. W., Jr (2005). Fluorescence resonance energy transfer (FRET) measurement by gradual acceptor photobleaching. *Journal of microscopy*, 218(Pt 3), 253–262. <https://doi.org/10.1111/j.1365-2818.2005.01483.x>
- Verma, A. K., Noumani, A., Yadav, A. K., & Solanki, P. R. (2023). FRET Based Biosensor: Principle Applications Recent Advances and Challenges. *Diagnostics (Basel, Switzerland)*, 13(8), 1375. <https://doi.org/10.3390/diagnostics13081375>
- Verrecchia, F., Mauviel, A., & Farge, D. (2006). Transforming growth factor-beta signaling through the Smad proteins: role in systemic sclerosis. *Autoimmunity reviews*, 5(8), 563–569. <https://doi.org/10.1016/j.autrev.2006.06.001>
- Visan, A. I., & Negut, I. (2024). Integrating Artificial Intelligence for Drug Discovery in the Context of Revolutionizing Drug Delivery. *Life (Basel, Switzerland)*, 14(2), 233. <https://doi.org/10.3390/life14020233>
- Vora, L. K., Gholap, A. D., Jetha, K., Thakur, R. R. S., Solanki, H. K., & Chavda, V. P. (2023). Artificial Intelligence in Pharmaceutical Technology and Drug Delivery Design. *Pharmaceutics*, 15(7), 1916. <https://doi.org/10.3390/pharmaceutics15071916>
- Wan, R., Feng, J., & Tang, L. (2021). Consequences of Mutations and Abnormal Expression of SMAD4 in Tumors and T Cells. *OncoTargets and therapy*, 14, 2531–2540. <https://doi.org/10.2147/OTT.S297855>
- Wang, Q., Xiong, F., Wu, G., Wang, D., Liu, W., Chen, J., Qi, Y., Wang, B., & Chen, Y. (2023). SMAD Proteins in TGF- β Signalling Pathway in Cancer: Regulatory Mechanisms and Clinical Applications. *Diagnostics (Basel, Switzerland)*, 13(17), 2769. <https://doi.org/10.3390/diagnostics13172769>
- Warmflash, A., Zhang, Q., Sorre, B., Vonica, A., Siggia, E. D., & Brivanlou, A. H. (2012). Dynamics of TGF- β signaling reveal adaptive and pulsatile behaviors reflected in the nuclear localization of transcription factor Smad4. *Proceedings of the National Academy of Sciences of the United States of America*, 109(28), E1947–E1956. <https://doi.org/10.1073/pnas.1207607109>

- Weiss, L. (1943). A simple demonstration of the law of conservation of mass, *Journal of Chemical Education*, 20(12) 579. <https://doi.org/10.1021/ed020p579>
- Winkler, M. E., O'Connor, L., Winget, M., & Fendly, B. (1989). Epidermal growth factor and transforming growth factor alpha bind differently to the epidermal growth factor receptor. *Biochemistry*, 28(15), 6373–6378. <https://doi.org/10.1021/bi00441a033>
- Wu, J. W., Fairman, R., Penry, J., & Shi, Y. (2001). Formation of a stable heterodimer between Smad2 and Smad4. *The Journal of biological chemistry*, 276(23), 20688–20694. <https://doi.org/10.1074/jbc.M100174200>
- Xie, L., Vo-Ransdell, C., Abel, B., Willoughby, C., Jang, S., & Sowa, G. (2011). Caveolin-2 is a negative regulator of anti-proliferative function and signaling of transforming growth factor- β in endothelial cells. *American journal of physiology. Cell physiology*, 301(5), C1161–C1174. <https://doi.org/10.1152/ajpcell.00486.2010>
- Xu, F., Liu, C., Zhou, D., & Zhang, L. (2016). TGF- β /SMAD Pathway and Its Regulation in Hepatic Fibrosis. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 64(3), 157–167. <https://doi.org/10.1369/0022155415627681>
- Yang, F., Chen, Y., Shen, T., Guo, D., Dakhova, O., Ittmann, M. M., Creighton, C. J., Zhang, Y., Dang, T. D., & Rowley, D. R. (2014). Stromal TGF- β signaling induces AR activation in prostate cancer. *Oncotarget*, 5(21), 10854–10869. <https://doi.org/10.18632/oncotarget.2536>
- Yang, Y., Guo, L., Chen, L., Gong, B., Jia, D., & Sun, Q. (2023). Nuclear transport proteins: structure, function, and disease relevance. *Signal transduction and targeted therapy*, 8(1), 425. <https://doi.org/10.1038/s41392-023-01649-4>
- Yi, M., Li, T., Niu, M., Zhang, H., Wu, Y., Wu, K., & Dai, Z. (2024). Targeting cytokine and chemokine signaling pathways for cancer therapy. *Signal transduction and targeted therapy*, 9(1), 176. <https://doi.org/10.1038/s41392-024-01868-3>
- Zhang, S., Fei, T., Zhang, L., Zhang, R., Chen, F., Ning, Y., Han, Y., Feng, X. H., Meng, A., & Chen, Y. G. (2007). Smad7 antagonizes transforming growth factor beta signaling in the nucleus by interfering with functional Smad-DNA complex formation. *Molecular and cellular biology*, 27(12), 4488–4499. <https://doi.org/10.1128/MCB.01636-06>

- Zhao, M., Mishra, L., & Deng, C. X. (2018). The role of TGF- β /SMAD4 signaling in cancer. *International journal of biological sciences*, 14(2), 111–123. <https://doi.org/10.7150/ijbs.23230>
- Zhong, Q., Xiao, X., Qiu, Y., Xu, Z., Chen, C., Chong, B., Zhao, X., Hai, S., Li, S., An, Z., & Dai, L. (2023). Protein posttranslational modifications in health and diseases: Functions, regulatory mechanisms, and therapeutic implications. *MedComm*, 4(3), e261. <https://doi.org/10.1002/mco2.261>
- Zi, Z., Feng, Z., Chapnick, D. A., Dahl, M., Deng, D., Klipp, E., Moustakas, A., & Liu, X. (2011). Quantitative analysis of transient and sustained transforming growth factor- β signaling dynamics. *Molecular systems biology*, 7, 492. <https://doi.org/10.1038/msb.2011.22>

VIII. APPENDICES

VIII- APPENDICES

1. Supplementary Material

1.1. Step-by-Step Derivation of the Differential Equations of the System Using Mass Action Law

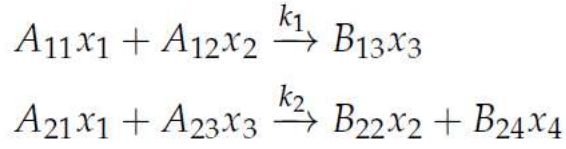
To illustrate the process of using the mass action to derive differential equations, let us first consider a general system in which several n species $x_1, x_2, \dots, x_n$ interact with each other by means of a set of m chemical reactions, with rate constants $k_1, k_2, \dots, k_m$. The concentration of the species x_j is expressed as $[x_j]$. We start with the definition of a column vector that contains the concentrations of all species at a given time point. This vector is labeled as the state vector:

$$[\vec{X}] = \begin{pmatrix} [x_1] \\ [x_2] \\ \vdots \\ [x_n] \end{pmatrix}$$

Next, we define a square matrix K of dimension $(m \times m)$ that contains all the rate constants in its diagonal as follows:

$$K = \begin{pmatrix} k_1 & 0 & \dots & 0 \\ 0 & k_2 & \ddots & \vdots \\ \vdots & \ddots & \ddots & 0 \\ 0 & \dots & 0 & k_m \end{pmatrix}$$

Next, we define two $m \times n$ matrices, A and B , that contain the stoichiometric coefficients of the reactants and products, respectively. In these matrices, the value of the A_{ij} element is the stoichiometric coefficient of species j as a reactant in reaction i , while the value of the B_{ij} element is the stoichiometric coefficient of species j as a product in reaction i (in the subindices notation, i and j denote rows and columns, respectively). If any of these coefficients do not appear in the reactions, it is set to zero. For example, in the system



the species concentration vector and the rate constants matrices are

$$[\vec{X}] = \begin{pmatrix} [x_1] \\ [x_2] \\ [x_3] \\ [x_4] \end{pmatrix} \quad K = \begin{pmatrix} k_1 & 0 \\ 0 & k_2 \end{pmatrix}$$

and, since x_3 and x_4 do not appear as reactants in reaction 1, we set $A_{13} = A_{14} = 0$.

In a similar manner, since x_2 and x_4 do not appear as reactants in reaction 2, we set $A_{22} = A_{24} = 0$.

The same rationale is applied to construct matrix B , giving the following stoichiometric matrices:

$$A = \begin{pmatrix} A_{11} & A_{12} & 0 & 0 \\ A_{21} & 0 & A_{23} & 0 \end{pmatrix} \quad B = \begin{pmatrix} 0 & 0 & B_{13} & 0 \\ 0 & B_{22} & 0 & B_{24} \end{pmatrix}$$

Finally, we define a column vector $[\vec{X}]^A$ of m rows that contains in each row the product of the concentrations of all the species concentrations raised to the power of the corresponding A matrix coefficient. This way, the i -th row of this vector can be calculated as

$$([\vec{X}]^A)_i = \prod_{j=1}^n [x_j]^{(A_{ij})} \quad (S1)$$

As an example, this vector for the previous reaction can be calculated as

$$[\vec{X}]^A = \begin{pmatrix} [x_1]^{A_{11}} [x_2]^{A_{12}} [x_3]^0 [x_4]^0 \\ [x_1]^{A_{21}} [x_2]^0 [x_3]^{A_{23}} [x_4]^0 \end{pmatrix} = \begin{pmatrix} [x_1]^{A_{11}} [x_2]^{A_{12}} \\ [x_1]^{A_{21}} [x_3]^{A_{23}} \end{pmatrix}$$

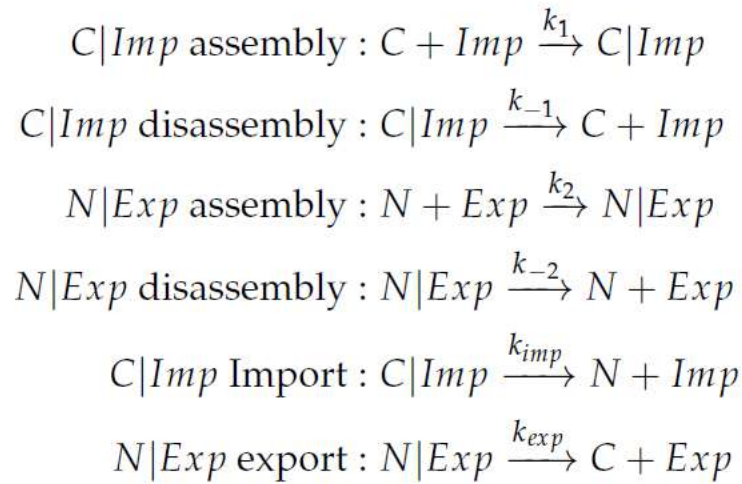
With all these definitions, the mass action law states that the temporal evolution of the concentration of the species is given by

$$\frac{d[\vec{X}]}{dt} = (B - A)^t \cdot K \cdot [\vec{X}]^A \quad (S2)$$

where the elements of the $(B - A)^t$, with dimensions $n \times m$, are given by

$$[(B - A)^t]_{ij} = B_{ji} - A_{ji} \quad (S3)$$

Now, let us apply the previous process to our system of nuclear translocation. To do so, we first need to separate each reaction as an individual process in such a way that each reversible interaction is equivalent to two irreversible interactions. This way, the system of interactions for import and export can be decomposed as the following six coupled processes:



The next step is to apply the mass action law to obtain six ordinary differential equations (ODEs, one for each species in the system) that show the temporary evolution of the concentration of the species once solved. We have 6 reactions and 6 species; therefore, we first define the state vector as

$$[\vec{X}] = \begin{pmatrix} [C] \\ [Imp] \\ [C|Imp] \\ [N] \\ [Exp] \\ [N|Exp] \end{pmatrix}$$

containing the concentration of species $[\vec{X}]$ and a diagonal matrix K containing the rates of the reactions (in the order shown above):

$$K = \begin{pmatrix} k_1 & 0 & 0 & 0 & 0 & 0 \\ 0 & k_{-1} & 0 & 0 & 0 & 0 \\ 0 & 0 & k_2 & 0 & 0 & 0 \\ 0 & 0 & 0 & k_{-2} & 0 & 0 \\ 0 & 0 & 0 & 0 & k_{imp} & 0 \\ 0 & 0 & 0 & 0 & 0 & k_{exp} \end{pmatrix}$$

Considering the order of the reactions in K and the order of the species as they appear in $[\vec{X}]$, we can define matrices A and B as

$$A = \begin{pmatrix} 1 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 \end{pmatrix}$$

$$B = \begin{pmatrix} 0 & 0 & 1 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 1 & 1 & 0 \\ 0 & 1 & 0 & 1 & 0 & 0 \\ 1 & 0 & 0 & 0 & 1 & 0 \end{pmatrix}$$

Now, we compute the $\vec{[X]}^A$ vector for these systems as

$$[\vec{X}]^A = \begin{pmatrix} [C][Imp] \\ [C|Imp] \\ [N][Exp] \\ [N|Exp] \\ [C|Imp] \\ [N|Exp] \end{pmatrix}$$

Next, we calculate the transposed matrix of $(B - A)$, which is denoted as $(B - A)^t$:

$$(B - A)^t = \begin{pmatrix} -1 & 1 & 0 & 0 & 0 & 1 \\ -1 & 1 & 0 & 0 & 1 & 0 \\ 1 & -1 & 0 & 0 & -1 & 0 \\ 0 & 0 & -1 & 1 & 1 & 0 \\ 0 & 0 & -1 & 1 & 0 & 1 \\ 0 & 0 & 1 & -1 & 0 & -1 \end{pmatrix}$$

Finally, to obtain the ODE system, we use the mass action law in Equation (S2), yielding

$$\begin{aligned}
 \frac{d[C]}{dt} &= -k_1[C][Imp] + k_{-1}[C|Imp] + k_{exp}[N|Exp] \\
 \frac{d[Imp]}{dt} &= -k_1[C][Imp] + k_{-1}[C|Imp] + k_{imp}[C|Imp] \\
 \frac{d[C|Imp]}{dt} &= k_1[C][Imp] - k_{-1}[C|Imp] - k_{imp}[C|Imp] \\
 \frac{d[N]}{dt} &= -k_2[N][Exp] + k_{-2}[N|Exp] + k_{imp}[C|Imp] \\
 \frac{d[Exp]}{dt} &= -k_2[N][Exp] + k_{-2}[N|Exp] + k_{exp}[N|Exp] \\
 \frac{d[N|Exp]}{dt} &= k_2[N][Exp] - k_{-2}[N|Exp] - k_{exp}[N|Exp]
 \end{aligned}$$

1.2. Step-by-Step Simplifying the ODEs Using the Mass Conservation Law

Let us define a row matrix C of length n . This vector contains n constants $C_1, C_2, \dots, C_n$ that give the relationships between the concentrations of the species at any time. To obtain those relationships, we have to use the following linear system:

$$C \cdot (B - A)^t = \vec{0} \quad (S4)$$

We obtain a set of n linear equations with n unknown variables. Using these equations, we must find a nontrivial relationship between all C_j coefficients. For example, if the system we obtain is

$$C_1 - C_2 = 0 \quad (S5)$$

$$C_2 - C_1 = 0 \quad (S6)$$

$$C_3 + C_4 = 0 \quad (S7)$$

We have only one relationship between the coefficients of x_1 and x_2 : $C_1 = C_2$. Also, there is a relation between the coefficients of x_3 and x_4 : $C_3 = -C_4$. Given these values, the conservation law states that for a closed system, the following relation between the amount of the reactants is always true:

$$\sum_{j=1}^n C_j \cdot [x_j] = \text{constant} \quad (\text{S8})$$

This way, if we substitute the relationships obtained using Equation (S4) for the values of coefficients C_j , Equation (S8) can be used to simplify the system of equations that describes a given system of interactions. To illustrate this with the previous example,

$$C_1[x_1] + C_2[x_2] + C_3[x_3] + C_4[x_4] = \text{constant}$$

Using the relations calculated above, it becomes

$$C_1([x_1] + [x_2]) + C_3([x_3] - [x_4]) = \text{constant}$$

This is true for all values of C_1 and C_3 . So, if we set $C_1 = 1$ and $C_3 = 0$, we obtain the following relationship:

$$[x_1] + [x_2] = \text{constant} \quad (\text{S9})$$

While, if we set $C_1 = 0$ and $C_3 = 1$, we obtain the following relationship:

$$[x_3] - [x_4] = \text{constant} \quad (\text{S10})$$

Now, it is important to realize that if this relation between the amounts of the species involved in Equations (S9) and (S10) is true at all times, it means that it is also true at the initial time point ($t = 0$). This is useful because we normally know the initial conditions of the system. This way, we can

$$\begin{aligned} [x_1] + [x_2] &= [x_1]_{t=0} + [x_2]_{t=0} \\ [x_3] - [x_4] &= [x_3]_{t=0} - [x_4]_{t=0} \end{aligned}$$

which can be used to calculate one of the variables as a function of the other, plus or minus some constant values that are known.

$$\begin{aligned} [x_2] &= [x_1]_{t=0} + [x_2]_{t=0} - [x_1] \\ [x_4] &= [x_3] - [x_3]_{t=0} - [x_4]_{t=0} \end{aligned}$$

To apply this approach to the six differential equations of the protein translocation model, we start again by defining a row vector C with six constants ($C_1, \dots, C_6$: one per species) and calculate expression (S4) for this particular system:

$$C \cdot (B - A)^t = \vec{0}$$

$$(C_1 \ C_2 \ C_3 \ C_4 \ C_5 \ C_6) \begin{pmatrix} -1 & 1 & 0 & 0 & 0 & 1 \\ -1 & 1 & 0 & 0 & 1 & 0 \\ 1 & -1 & 0 & 0 & -1 & 0 \\ 0 & 0 & -1 & 1 & 1 & 0 \\ 0 & 0 & -1 & 1 & 0 & 1 \\ 0 & 0 & 1 & -1 & 0 & -1 \end{pmatrix} = \vec{0}$$

This relation gives us a linear system of equations. Removing the independent equations and simplifying, we reach the following equivalents:

$$\begin{aligned} C_1 + C_2 &= C_3 \\ C_4 + C_5 &= C_6 \\ C_1 &= C_4 \end{aligned}$$

The mass conservation law states that the following expression has a constant value over time:

$$C_1[C] + C_2[Imp] + C_3[C|Imp] + \dots \\ + C_4[N] + C_5[Exp] + C_6[N|Exp] = constant$$

which, after applying the relations between the coefficients above, gives us

$$C_1[C] + C_2[Imp] + (C_1 + C_2)[C|Imp] + \dots \\ + C_1[N] + C_5[Exp] + (C_1 + C_5)[N|Exp] = constant$$

So, we have three independent constants (C_1 , C_2 , and C_5), and the previous equation is true for any of their values. This way, following the same rationale as in the example, we set each one to 1, while the others are set to 0. This way, we obtain the three constant relations as

$$C_1([C] + [C|Imp] + [N] + [N|Exp]) = constant \\ C_2([Imp] + [C|Imp]) = constant \\ C_5([Exp] + [N|Exp]) = constant$$

In particular, these relations are true at all time points, therefore they are true at the initial time point. This way, if we set $C_1 = C_2 = C_5 = 1$ and the constant values as the value at $t = 0$ (initial conditions) we obtain

$$[C] + [C|Imp] + [N] + [N|Exp] = [C](0) + [C|Imp](0) \\ + [N](0) + [N|Exp](0) \\ [Imp] + [C|Imp] = [Imp](0) + [C|Imp](0) \\ [Exp] + [N|Exp] = [Exp](0) + [N|Exp](0)$$

The first relation corresponds to the fact that total amount of Smad in all possible configurations (nuclear, cytoplasmic, alone, or in complex) has to remain constant. The second and third relations corresponds to the fact that the total amount of *Imp* and *Exp* proteins has to remain constant. One additional simplification can be introduced, taking into account that, at the initial time point, there are no complexes, since we start with all proteins as monomers and monitor their interaction. This way, we can assume $[C|Imp](0) = [N|Exp](0) = 0$, so the relations are

$$\begin{aligned} [C] + [C|Imp] + [N] + [N|Exp] &= [C](0) + [N](0) \\ [Imp] + [C|Imp] &= [Imp](0) + [C|Imp](0) \\ [Exp] + [N|Exp] &= [Exp](0) + [N|Exp](0) \end{aligned}$$

The next step is calculating the concentrations of some of the reactants as a function of the others. In this case, since we often focus on the amounts of R-Smads and not on the amounts of *Imp* and *Exp* proteins, it makes sense that we organize the relations in such a way that we eliminate the variables $[Imp]$ and $[Exp]$ as follows:

$$\begin{aligned} [Imp] &= [Imp](0) - [C|Imp] \\ [Exp] &= [Exp](0) - [N|Exp] \end{aligned}$$

Finally, we substitute these relations into the system of ODEs:

$$\begin{aligned} \frac{d[C]}{dt} &= -k_1[C]([Imp](0) - [C|Imp]) + \dots \\ &\quad + k_{-1}[C|Imp] + k_{exp}[N|Exp] \\ \frac{d[C|Imp]}{dt} &= k_1[C]([Imp](0) - [C|Imp]) - \dots \\ &\quad - k_{-1}[C|Imp] - k_{imp}[C|Imp] \\ \frac{d[N]}{dt} &= -k_2[N]([Exp](0) - [N|Exp]) + \dots \\ &\quad + k_{-2}[N|Exp] + k_{imp}[C|Imp] \\ \frac{d[N|Exp]}{dt} &= k_2[N]([Exp](0) - [N|Exp]) - \dots \\ &\quad - k_{-2}[N|Exp] - k_{exp}[N|Exp] \end{aligned}$$

Institutional Repository Record

This doctoral thesis is archived and publicly available through the Institutional Repository of the Universidad Autónoma de Madrid (UAM).



The screenshot shows a web browser window displaying the Institutional Repository of the Universidad Autónoma de Madrid (UAM). The page title is "Interplay between complex formation and intracellular compartmentalization in the regulation of the TGF- β Pathway signaling dynamics". The page includes the UAM logo, the "Biblos-e" logo, and a search bar. The breadcrumb trail is "Main > 2 - Docencia > doctoral studies > Interplay between co...". The record details include the URI "https://hdl.handle.net/10486/771780", the date "2026-03-16", the author "Naser Fayad, Ahmed", and the director "Miguel Gómez, David". A thumbnail image of the thesis cover is shown. The file "fayad_ahmed_naser_fayad.pdf" (8.98 MB) is listed under the "Files" section. A small bar at the bottom shows "Visitas al ítem" and "Descargas de archivos".

Interplay between complex formation and intracellular compartmentalization in the regulation of the TGF- β Pathway signaling dynamics

URI
https://hdl.handle.net/10486/771780

the date
2026-03-16

Authors
Naser Fayad, Ahmed

Directors
Miguel Gómez, David

Files
fayad_ahmed_naser_fayad.pdf (8.98 MB)

Repository URL:

<https://repositorio.uam.es/entities/publication/b32fac3e-85c8-405b-863a-46fce49d54df>

QR code:



