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Developmental regulation and functions of the TGFβ signaling pathway in *Drosophila melanogaster*

Nádia Cristina Leal Eusébio

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Tese de Doutoramento apresentada à
Faculdade de Ciências da Universidade do Porto
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Doutoramento em Biologia
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2018
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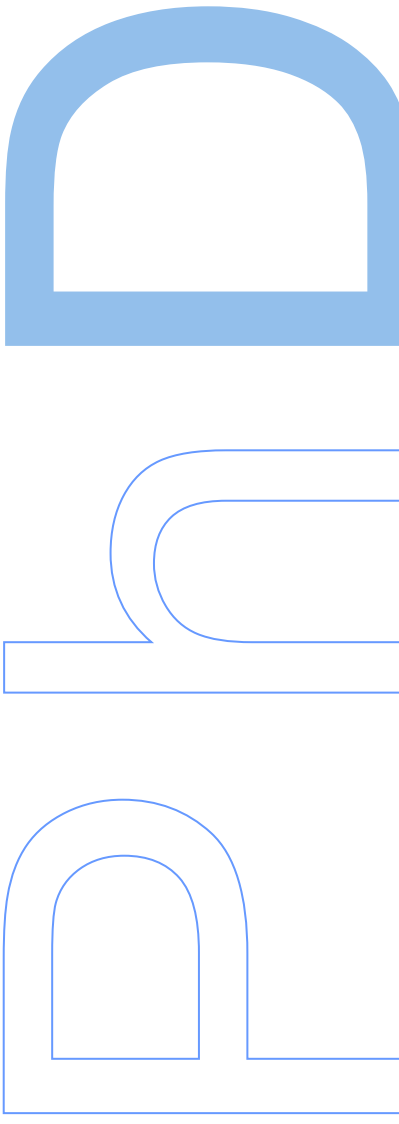

You see things; you say, 'Why?' But I dream things that never were; and I say 'Why not?'

George Bernard Shaw, Back to Methuselah

Dissertação apresentada à Faculdade de Ciências da Universidade do Porto para a obtenção do grau de Doutor em Biologia, no âmbito do programa doutoral em Biologia. Orientador – Doutor Paulo Pereira e Coorientador – Professor Doutor Fernando Tavares. Esta tese foi escrita no abrigo do número 2 do Artigo 4° do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31° do D.L. 74/2006, de 24 de Março, alterado e republicado no D.L. 230/2009, de 14 de Setembro, e D.L. 115/2013, de 7 de agosto. Esta dissertação é composta por um conjunto coerente de trabalhos de investigação, já publicados ou em preparação, sob o nome de **Eusebio N**:

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Dado que os artigos foram feitos em colaboração com outros autores, a candidata clarifica que, em todos eles participou ativamente no desenho, trabalho experimental, obtenção, análise e discussão dos dados, preparação e publicação dos artigos.

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Nádia

Resumo

A via de sinalização da família Fator de Transformação de Crescimento β (TGFβ) é conservada de forma evolutiva e ubíqua em Metazoa. Esta via está envolvida numa coleção de processos biológicos, tanto durante o desenvolvimento quanto na idade adulta. A via de sinalização da família TGFβ em *Drosophila* está dividida em dois ramos, ativados por diferentes ligandos, Ativinas e Proteínas Morfogénicas Ósseas (BMPs). Ao contrário dos vertebrados, são necessários menos fatores para modular cada ramo, simplificando o estudo mecanístico da via de sinalização. Embora alguns dos membros da família TGFβ e outros não membros desta família necessários à regulação desta via tenham sido identificados, é necessária a caracterização de novos efetores de crescimento celular, diferenciação e polaridade durante o desenvolvimento de *Drosophila*. Nesta tese, detalhamos as funções e a regulação da via de sinalização TGFβ durante o desenvolvimento de *Drosophila*.

No Capítulo 2, relatamos que tanto Punt, o recetor tipo II, quanto Smad2, R-Smad, são fortemente necessários para o crescimento de células e tecidos. A diminuição da expressão de Punt ou Smad2 nas células da glândula causam alterações na estrutura e funções nucleolares. As células da glândula com diminuição da sinalização de TGFβ/Activina acumulam transcritos intermediários de pré-rRNA que contêm regiões internas transcritas do espaçador 1 (ITS1) acompanhadas da retenção nucleolar de proteínas ribossómicas. Mostramos ainda que a sobrexpressão de Punt aumenta o crescimento celular induzido por *Drosophila* Myc (dMyc), um indutor bem caracterizado de hipertrofia nucleolar e biogênese ribossómica.

No Capítulo 3, descrevemos uma triagem genética dirigida de duplo RNAi com o objetivo de melhor conhecer genes que cooperam com a via TGFβ durante o desenvolvimento do olho. Uma investigação posterior culminou na identificação de uma forte interação genética entre a via de sinalização TGFβ, em particular entre *punt*, e *ago*, *brk*, *CtBP* e *dad*. Curiosamente, tanto Brk como Ago são reguladores negativos do crescimento do tecido e da atividade de dMyc. Mostramos ainda que o aumento da capacidade de crescimento dos tecidos, pela sobrexpressão de dMyc ou CyclinD-Cdk4, é suficiente para recuperar o crescimento e diferenciação dos fotorreceptores dependente de *punt*. Além disso, identificamos uma nova função de CtBP na inibição da ativação de Mad dependente de Dpp, a jusante ou paralelamente a *dad*, a Smad Inibitória.

No Capítulo 4, caracterizamos a localização e a função de Dad nas células da glândula salivar e nos discos imaginários do olho de *Drosophila*. Dad é uma proteína

nucleolar que regula o crescimento celular e a diferenciação através da regulação da ativação de Mad. O domínio N-terminal de *dad* é importante para uma inibição eficiente da via de sinalização de TGFβ e determina a localização subcelular desta Smad Inibitória. Particularmente, o domínio N-terminal de Dad é necessário para uma ligação eficiente desta proteína à membrana celular. Finalmente, a sobrexpressão de *punt* e *tkv* é capaz de induzir a fosforilação de Dad e bloquear a sua capacidade de inibir a sinalização de TGFβ.

No Capítulo 5, mostramos que a sobrexpressão de Punt e Tkv nas células da glândula compromete a polaridade celular e a integridade epitelial. A regulação positiva da sinalização de TGFβ induz características mesenquimais nas células epiteliais das glândulas salivares, que resultam de um aumento do stress das fibras de Actina, sinalização da Quinase Regulada pela Sinalização Extracelular (Erk) e expressão de Metaloproteínase de Matriz 1 (Mmp1). A diminuição da expressão de Bnl, um homólogo de *Drosophila* do Factor de Crescimento de Fibroblastos (FGF) humano, é suficiente para recuperar parcialmente a polaridade celular e a integridade epitelial dependentes da família TGFβ. Consequentemente, sugerimos que Bnl e TGFβ atuem em conjunto para induzir eficientemente múltiplas alterações moleculares que permitem às células epiteliais assumirem características de células mesenquimais.

Esta tese revela como diferentes fatores interagem com a sinalização da via TGFβ na regulação das suas funções durante o desenvolvimento. A regulação inadequada desta via conduz a diferentes problemas de desenvolvimento.

Palavras-chave: Via TGFβ, Punt, Tkv, Dad, CtBP, Ago, Mad, Ribosomas, Crescimento, Diferenciação, N-terminal, Polaridade, Características Mesenquimais

Abstract

The Transforming Growth Factor β (TGFβ) family signaling is evolutionary conserved and ubiquitous in Metazoa. This pathway is involved in a collection of biological processes, both during development and adulthood. The *Drosophila* TGFβ signaling pathway is divided in two branches, activated by different ligands, Activins, and Bone Morphogenetic Proteins (BMPs). Unlike in vertebrates, fewer factors are required to modulate each branch, simplifying the mechanistic study of the pathway. While some of the TGFβ family members and others not members of this family required for the regulation of this pathway have been identified, the characterization of new effectors of cell growth, differentiation and polarity during *Drosophila* development is necessary. In this thesis, we detail the developmental regulation and functions of the TGFβ signaling pathway in the *Drosophila* model system.

In Chapter 2, we report that both the type II receptor, Punt, and the R-Smad, Smad2, are strongly required for cell and tissue growth. Knocking down the expression of Punt or Smad2 in gland cells causes alterations in nucleolar structure and functions. Gland cells with decreased TGFβ/Activin signaling accumulate intermediate pre-rRNA transcripts containing Internal Transcribed Spacer 1 (ITS1) regions accompanied by the nucleolar retention of ribosomal proteins. We have further shown that overexpression of Punt enhances cell growth induced by *Drosophila* Myc (dMyc), a well-characterized inducer of nucleolar hypertrophy and ribosome biogenesis.

In Chapter 3, we describe a targeted double RNAi screen performed in order to get a deep knowledge about the genes working with TGFβ signaling during *Drosophila* eye development. Further investigation culminated with the identification of a strong genetic interaction between TGFβ signaling, in particular between *punt*, and *ago*, *brk*, *CtBP* and *dad*. Interestingly, both Brk and Ago are negative regulators of tissue growth and dMyc activity, and we show that increased tissue growth ability, by overexpression of dMyc or CyclinD-Cdk4 is sufficient to partially rescue Punt-dependent growth and photoreceptor differentiation. Furthermore, we identify a novel role of CtBP in inhibiting Dpp-dependent Mad activation by phosphorylation, downstream or in parallel of Dad, the Inhibitory Smad (I-Smad).

In Chapter 4, we characterize the localization and function of Dad in cell glands and imaginal eye discs. Dad is a nucleolar protein that regulates cell growth and differentiation through the regulation of Mad activation. The N-terminal domain of Dad is important for an efficient inhibition of TGFβ signaling and it determines the subcellular localization of this I-Smad. Particularly, the N-terminal domain of Dad is

required for an efficient binding of this protein to the cell membrane. Finally, the overexpression of *punt* and *tkv* is able to induce Dad phosphorylation, which blocks the ability of Dad to inhibit TGFβ signaling.

In Chapter 5, we show that overexpression of Punt and Tkv in gland cells compromises cell polarity and epithelial integrity. The upregulation of TGFβ signaling induces mesenchymal features in epithelial cells of salivary glands, which result from an increase of Actin stress fibers, Extracellular Signal-regulated Kinase (Erk) signaling and Matrix Metalloproteinase Protein 1 (Mmp1) expression. The downregulation of Bnl, a *Drosophila* homolog of Human Fibroblast Growth Factor (FGF), is sufficient to partially rescue TGFβ signaling-dependent cell polarity and epithelial integrity. Accordingly, we suggest that Bnl and TGFβ signaling cooperate to efficiently induce multiple molecular changes that enable epithelial cells to assume mesenchymal cell features.

This thesis reveals how different factors interact with TGFβ signaling to regulate its functions during development and how an inappropriate regulation of this pathway could lead to development problems.

Key-words: TGFβ signaling, Punt, Tkv, Dad, CtBP, Ago, Mad, Ribosomes, Growth, Differentiation, N-terminal, Polarity, Mesenchymal features

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Chapter 4

Abbreviations

µL	microlitre
µM	micromolar
Actβ	Activinβ
Ago	Archipelago
AIP4	Atrophin 1-Interacting Protein 4
Ato	Atonal
Babo	Baboon
BMP	Bone Morphogenetic Protein
Bnl	Branchless
bp	base pair
Brk	Brinker
ºC	degree Celsius
Cas-L	Crk-associated substrate lymphocyte type
Co-Smad	Common-mediator Smad
CtBP	C-terminal-binding protein
Cyc	Cyclin
Cdk	Cyclin-dependent kinase
Dac	Dachshund
Dad	Daughters against dpp
Dart1	*Drosophila* arginine methyltransferase 1
Daw	Dawdle
DNA	DeoxyriboNucleic Acid
Dome	Domeless
Dpp	Decapentaplegic
dNTP	dideoxyNucleotide TriPhosphate
dsRNA	double stranded RNA
Erk	Extracellular signal-regulated kinase
ETS	External Transcribed Spacer
Ex	Expanded
Exd	Extradenticle
Ey	Eyeless
Eya	Eyes absent
Eyg	Eyegone

FGF	Fibroblast Growth Factor
Flp	flippase
FRAP	Fluorescence Recovery After Photobleaching
Gbb	Glass Bottom Boat
Hh	Hedgehog
Hic5	H2O2-inducible clone 5
Hip14	Huntingtin interacting protein 14
Hth	Homothorax
Hop	Hopscotch
Hpo	Hippo
ITS1	Internal Transcribed Spacer 1
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	c-Jun Kinase
Lat	Latran
Mad	Mothers against dpp
MAPK	Mitogen-Activated Protein Kinase
Mav	Maverick
Med	Medea
Mer	Merlin
MF	Morphogenetic Furrow
MMK	Mitogen-activated protein Kinase
MMP	Matrix Metalloproteinase
Myo	Myoglianin
mg	milligrams
min	minute
mL	milliLitre
MPK38	Murine Protein Kinase 38
n	number
ng	nanogram
NICD	Notch Intracellular Domain
NS	Nucleostemin
Pax6	Paired Box 6
PCR	Polymerase Chain Reaction
pMad	phosphorylated Mad
PI3K	Phosphoinositide 3-kinase
Pol I	RNA polymerase I

Pol II	RNA polymerase II
Pol III	RNA polymerase III
PP1	Protein Phosphatase 1
pre-rRNA	precursor ribosomal RNA
PRMT1	Protein arginine N-methyltransferase 1
PS	Position Specific
Put	Punt
qPCR	quantitative Real-Time PCR
Ras	Raspberry
rRNA	ribossomal RNA
RI	receptor type I
RII	receptor type II
RNA	RiboNucleic Acid
RNAi	RNA of interference
ROCK	Rho-associated protein kinase
rpm	rotations per minute
RpL	Ribosomal protein (Large subunit)
RpS	Ribosomal protein (Small subunit)
R-Smad	Receptor-activated Smad
s	second
S/T	Serine/Threonine
Sav	Salvador
Sax	Saxophone
SCF	Skp/Cullin/F-box
Scr	Sex combs reduced
Scw	Screw
SIK	Salt-Inducible Kinase
SnoRNP	Small nucleolar ribonucleoproteins
So	Sine oculis
Spi	Spitz
STRAP	Serine/Threonine Kinase Receptor-Associated Protein
TAB1	Transforming Growth Factor Beta-Activated Kinase-Binding protein 1
TAK1	Transforming Growth Factor Beta-Activated Kinase 1
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor Beta
Tkv	Thickveins

TOR	Target Of Rapamycin
Toy	Twin of eyeless
TRAF 6	TNF Receptor Associated Factor 6
Tsh	Teashirt
UAS	Upstream Activating Sequence
Upd	Unpaired
USP15	Ubiquitin-Specific Peptidase 15
Vito	Viriato
Wg	Wingless
Wit	Wishful thinking
Wts	Warts
YAP65	Yes-Associated Protein 65
Yki	Yorkie

Thesis framework

Transforming Growth Factor β (TGFβ) signaling provides cells with a multiple means of driving developmental programs and controlling cell behavior, a function that is evident in the several effects of TGFβ signaling on cell proliferation, differentiation, apoptosis, morphogenesis, cellular homeostasis and regeneration. Deregulation of TGFβ signaling is associated with several illnesses including cancer, fibrosis and inflammatory and cardiovascular diseases. The work developed in this thesis aimed to contribute for the current knowledge of TGFβ signaling in basic cell properties of *Drosophila melanogaster*, including growth, differentiation and polarity.

This thesis is organized in six chapters and includes four scientific manuscripts.

The first chapter, entitled Chapter 1. General Introduction, contains a revision of the literature on key aspects necessary for a better interpretation of the different chapters that I present afterwards: (i) *Drosophila melanogaster* life cycle, (ii) genetic mechanisms underlying the development and transformation of primordial structures to adult structures, (iii) cell growth during development, and (iv) the contribution of canonical and non-canonical TGFβ signaling pathway during development.

In Chapter 2, I present a study on the consequences of knocking down the TGFβ signaling in eye imaginal discs and salivary glands during development. With this study, I prove that decreased TGFβ/Activin signaling accumulates intermediate pre-rRNA transcripts containing internal transcribed spacer 1 regions accompanied by the nucleolar retention of ribosomal proteins. Therefore, TGFβ/Activin signaling is required for ribosomal biogenesis, an important aspect of cellular growth control.

In Chapter 3, I advance in the identification and characterization of the regulators and targets of Dpp signaling required for retinal development. I carry out an in vivo eye-targeted double-RNAi screen to identify genes interacting with *punt*. Using a set of 251 genes associated with eye development, I identify four negative regulators of the Dpp pathway.

In Chapter 4, I characterize the localization, function and biochemical regulation of the Inhibitory Smad of TGFβ signaling, Dad. Dad is a nucleolar protein that controls cell growth and differentiation through the regulation of Mad activation. Furthermore, I determine that TGFβ signaling is able to inhibit Dad function through a post-translational modification.

In Chapter 5, I characterize the effects of the overexpression of TGFβ in different tissues of *Drosophila melanogaster*. During this analysis, I have observed a

deregulation of Actin filaments, as well as, alterations in cell polarity. These alterations are associated with mesenchymal cell features.

In Chapter 6. Final considerations, the results obtained within the thesis are discussed. I present the key conclusions and insights of this dissertation, as well as the implications for future research.

CHAPTER 1

General Introduction

Contents

Here I review the current *state of the art* regarding theoretical and methodological aspects required for a better interpretation of the different chapters. I briefly describe *Drosophila melanogaster* life cycle and the genetic mechanisms underlying its embryonic development. The importance of some signaling pathways responsible for cell growth are highlighted including JAK/STAT, Notch, Hippo, and Myc. Finally, I present a description of the canonical and non-canonical TGFβ signaling pathway.

The genetic mechanisms responsible for morphological development and diversification were first addressed by notable researchers such as Thomas Hunt Morgan. This extraordinary geneticist got his PhD in 1890 and was appointed associate professor at Johns Hopkins' sister school Bryn Mawr College. During this period, he studied development and regeneration on different organisms and published *The Development of the Frog's Egg* in 1897 and *Regeneration* in 1901. Later in 1904, Thomas Morgan moved to Columbia University, participating in the intense debate generated by the publication of *The Origin of Species*, by Charles Darwin. In his view, many questions related to the diversity of morphological characteristics and how they were transmitted to the next generation remained to be explained. Driven by these questions, he established the 'Fly Room' to use the common fruit fly (*Drosophila melanogaster*) to study genetic characteristics and their transmission (1911). These studies gave him the Nobel Prize in Physiology or Medicine in 1933 for the discoveries clarifying the role of chromosomes in heredity.

1. *Drosophila melanogaster*

Drosophila melanogaster is a powerful model to study mechanisms underlining cell growth and differentiation. Some characteristics that make *D. melanogaster* so attractive are its rapid life cycle, small genome size (with around 14000 genes), low chromosome number, ease of culture and manipulation. Additionally, it has been predicted that more than 70% of known human disease related genes have homologues in the genome of fruit flies (Reiter, Potocki et al. 2001).

A large range of genetic tools are available for this model, such as the possibility to generate clones of cells of a given genotype within a normal tissue environment, by mitotic recombination (Xu and Rubin 1993). The heterologous GAL4/UAS system is another important tool for targeted gene expression in a given tissue (Brand and Perrimon 1993, Dietzl, Chen et al. 2007). This system is based on the properties of the yeast GAL4 transcription factor which induces transcription of its target genes by binding to UAS cis-regulatory sites. In *Drosophila*, the two components, GAL4 and UAS, are carried in different fly lines allowing for several combinatorial possibilities (Figure 1.1.). In the progeny of the crossing between two flies with the different components, the produced Gal4 protein binds to the UAS component in the DNA, which induces the expression of it downstream target gene (Figure 1.1.). The expression of the Gal4 gene is regulated by a given promoter or enhancer that determines the stage, tissue, and pattern of expression (Caygill and Brand 2016). The Gal4-UAS system can also control RNA of interference (RNAi) to knockdown

endogenous genes in a specific type of cells. The expression of double stranded RNA (dsRNA) targeting the UAS-target gene is activated by the Gal4 driver and, consequently, the generated siRNAs induce the degradation of the target gene mRNA (Duffy 2002). This binary system offers a powerful tool to study gene function in *Drosophila* enabling the regulation over the timing, tissue specificity, and intensity of gene expression.

1.1. *Drosophila* life cycle

The *Drosophila* life cycle is a fast process, lasting about 9-10 days from egg deposition to an adult fly, at 25ºC. The period of life cycle decreases at high temperatures and increases at low temperatures. The female fly lays out approximately 400 eggs (embryos) in normal conditions, at 25ºC. The eggs, which are about 0.5 mm long, hatch after 12–15 hours as larvae. The resulting larvae grow for about 4-5 days passing through three larvae stages (L1, L2 and L3). During these stages, it is possible to find sac-like structures (imaginal discs) inside the larva that are the precursors of adult structures. The location of the seven major structures in the mature larva (third

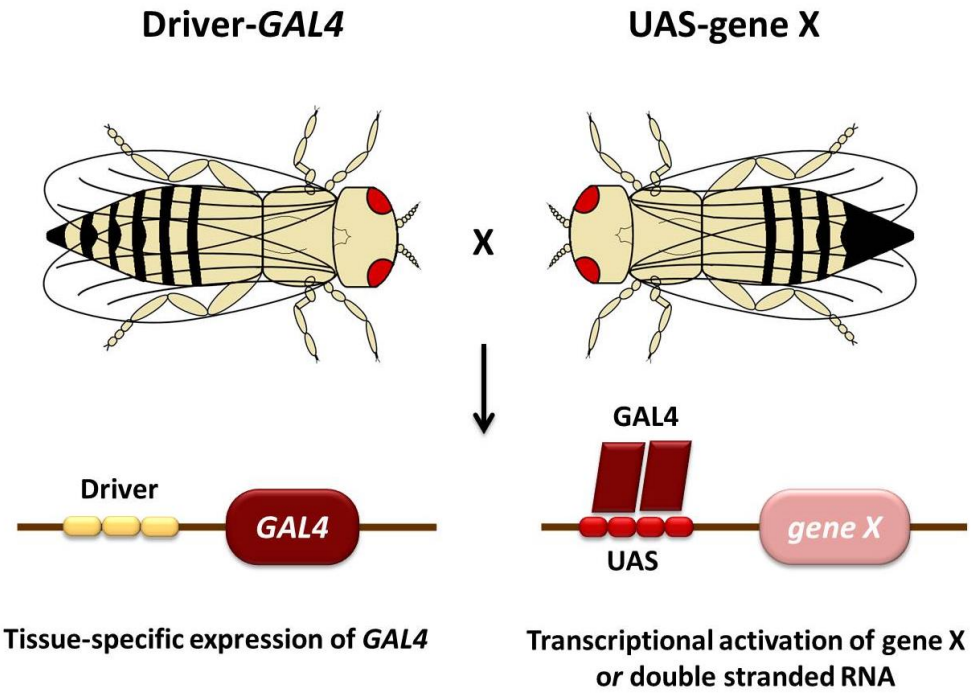

Figure 1.1. The GAL4–UAS system for open reading frame expression. The yeast Gal4-UAS system is a two components method in which one fly line carries a regulatory region that induces Gal4 expression (the driver) and a second fly line that carries the UAS upstream to the transgene. The resultant descendants from the crossing of the two *Drosophila melanogaster* lines will carry both Gal4 and UAS. Thus, the Gal4 will be transcribed and translated into a protein that will bind to the upstream activating sequence (UAS) activating the expression of an open reading frames or double stranded RNA. Adapted from Busson 2007.

instar stage) is illustrated in Figure 1.2 B. After larval stages, the larvae encapsulate forming a pupa that undergo a four-day-long metamorphosis, after which the adults hatch (Figure 1.2. A). As insects do not grow during adulthood, their final size is a reflection of the duration of the growth period as well as the growth rate during larval phases (Edgar 2006). Thus, for us, who study cell growth and differentiation, the most important developmental phase is the larval stage.

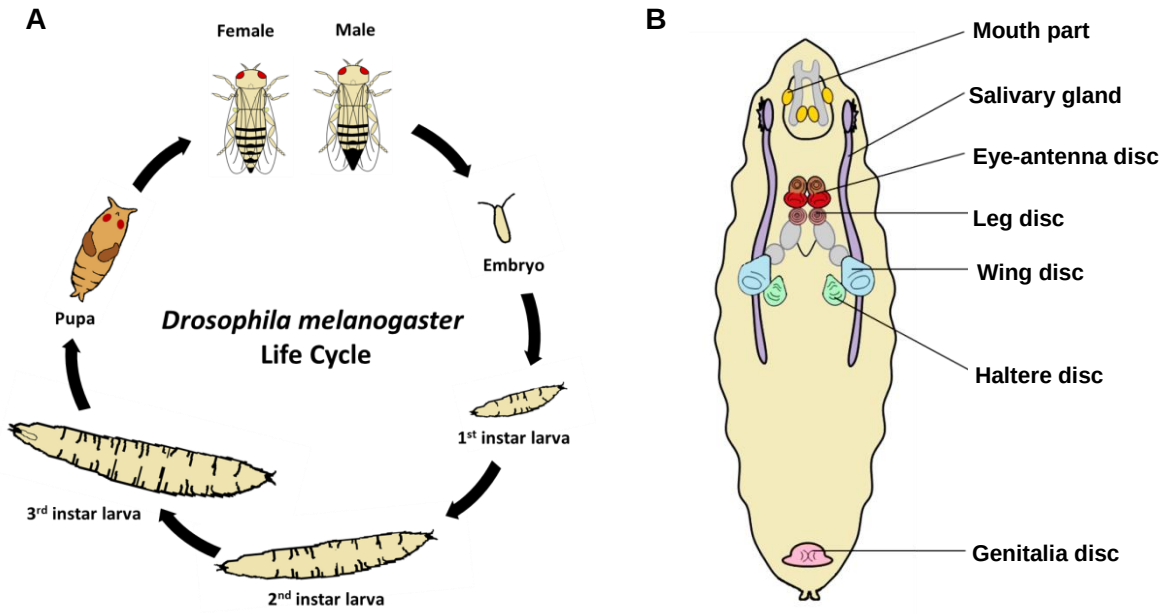

Figure 1.2. Life cycle of *Drosophila melanogaster* (A) and a rough fate map representing the seven major imaginal discs in the mature larva (B). (A) Life cycle of *Drosophila melanogaster* takes approximately 9-10 days to complete at 25ºC. After mating, eggs deposited in the food successive molts to become the first, second, and third instar larva. Then, the larvae encapsulate developing a pupa that undergoes a four-day-long metamorphosis, followed by the emergence of the adult fly. (B) The imaginal discs are the precursors of the adult structures. Adapted from Arias 2008 and Aldaz 2010.

1.2. Eye development

The *Drosophila* compound adult eye is a very organized structure that derives from the eye-antennal imaginal disc (Haynie and Bryant 1986). This imaginal disc gives rise to most adult head structures, with the exception of the proboscis (Figure 1.3.). The eye-antennal disc develops from a group of coalescent cells from the dorsal pouch of the embryo and gives origin to the eye, antenna, ocelli, head capsule and maxillary palp (Figure 1.3.) (Jurgens G and V. 1993, Younossi-Hartenstein, Tepass et al. 1993). Later during the first larval stage, this monolayer sac-like structure is composed by about 30 cells and expresses two *Pax6* genes, *eyeless* (*ey*) and *twin of eyeless* (*toy*) in a uniform expression manner, promoting cell growth and division (Quiring, Walldorf et al.

1994, Czerny, Halder et al. 1999). At this time, the eye-antenna disc territory is determined. During the second larval stage, cell proliferation continues and two different regions in the imaginal eye disc are possible to distinguish, the antennal lobe characterized by the expression of gene *cut* and the eye lobe that remains expressing *ey* (Kenyon, Ranade et al. 2003). This determination is followed by cell-shape changes forming two distinct epithelial layers, the peripodial epithelium and the disc proper (McClure and Schubiger 2005, Pallavi and Shashidhara 2005). The disc proper is characterized by columnar cells, while peripodial epithelium presents flattened squamous cells. Both epithelial layers are in dynamic communication and peripodial epithelium has a main contribution in disc proper development and regulates several signaling molecules important for eye differentiation (Atkins and Mardon 2009). During the third larval stage, the disc has grown until about 2000 cells and the peripodial epithelium cells closest to the disc proper become cuboidal leading to head capsule formation (Haynie and Bryant 1986).

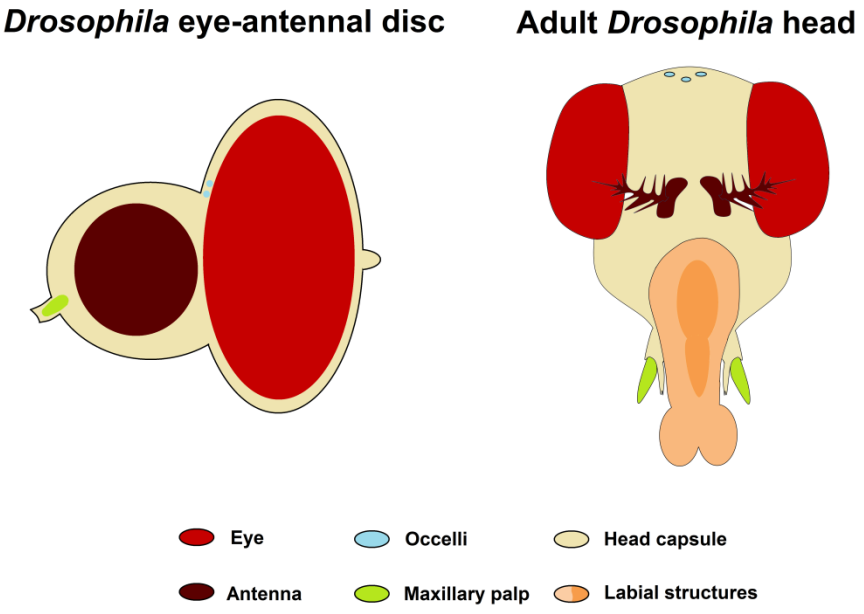

Figure 1.3. A simplified representation of the correspondent elements of eye-antennal disc and adult head of *Drosophila melanogaster*. The eye-antennal imaginal disc gives rise to the most adult head structures of flies, including the eye, the antenna, the ocelli, the maxillary palp and the head capsule. Adapted from Haynie and Bryant 1986.

1.2.1. Genetic regulation of eye specification: The Retinal Determination Genes

The retinal determination genes are a set of genes that collectively direct the specification of the eye (Pappu and Mardon 2002, Silver and Rebay 2005). Accordingly

to their role in early eye development, this regulatory framework is expressed before the initiation of retinal differentiation and anterior to the morphogenetic furrow (MF), an epithelial indentation that advances from the posterior margin to the anterior region of the eye imaginal disc. With the exception of *sine oculis* (*so*), the ectopic expression of retinal determination genes induces retinal development. The interregulation of this network is mediated by physical interaction of the conserved protein domains encoded by these genes. The core retinal determination genes are divided in two main groups: the eye determination effectors, *eyeless* (*ey*) and *twin of eyeless* (*toy*), and the early retinal determination genes, *eyes absent* (*eya*), *sine oculis* (*so*), and *dachshund* (*dac*). The eye determination effectors initiate the genetic hierarchy that controls the eye compound development, promoting the expression of early retinal determination genes that are expressed at the eye primordium and during retinal differentiation (Halder, Callaerts et al. 1998).

1.2.1.1. *Pax6* genes: the eye determination effectors

Drosophila ey and *toy* genes, arose from a gene duplication event during arthropod evolution (Czerny, Halder et al. 1999) and are located close to each other on the forth chromosome (Dahl, Koseki et al. 1997). Nevertheless, the paired domains of *ey* and *toy* present distinct DNA-binding properties and the C-terminal motifs are very distinct, suggesting that *ey* and *toy* exert distinct functions as transcription factors by regulating different sets of target genes (Punzo, Plaza et al. 2004). Pax6 regulates eye progenitor cell survival and proliferation through the activation of the non-core members of the retinal determination network, *teashirt* (*tsh*) and *eyegone* (*eyg*), thereby initiating both eye specification and proliferation (Zhu, Palliyil et al. 2017). The loss of function of both *pax6* genes induces very strong phenotypes, the downregulation of *toy* result in a headless phenotype (Kronhamn, Frei et al. 2002), while the lacking expression of *ey* only affect the development of the compound eye development (Punzo, Plaza et al. 2004). Importantly, both *ey* and *toy* are capable of initiating the eye developmental program (Halder, Callaerts et al. 1998, Czerny, Halder et al. 1999), but *toy* cannot induce the development of ectopic eyes in an *ey* deficient background (Czerny, Halder et al. 1999). Thus, *toy* acts upstream of *ey* in the retinal determination genes network (Figure 1.4 A).

The expression of *toy* and *ey* is not only present in the eye primordia but also in the embryonic brain and the central nervous system (Quiring, Walldorf et al. 1994, Czerny, Halder et al. 1999). In eye imaginal disc, during the first and second larval stage, *toy* and *ey* are expressed in the entire eye field (Halder, Callaerts et al. 1998, Czerny,

Halder et al. 1999). Later, during the later second - early third instar stages the expression of the *pax6* genes remains very active in undifferentiated cells at the anterior region of the eye imaginal disc, however, they are downregulated in differentiating cells (Czerny, Halder et al. 1999). At this point early retinal genes start to be expressed regulating the timing of differentiation of retinal cells.

1.2.1.2. Early Retinal Genes

There are three main early retinal genes: *eya*, *so* and *dac*. The expression of these genes is induced by *ey* and they are required for *ey*-induced retinal formation, which indicates that these genes work downstream of *ey* in the retinal determination gene network (Bonini, Bui et al. 1997, Shen and Mardon 1997, Halder, Callaerts et al. 1998). The combinatorial upregulation of *so* and *eya* is able to induce ectopic eye formation (Pignoni, Hu et al. 1997). The single upregulation of *eya* is also sufficient to produce ectopic eye induction (Bonini, Bui et al. 1997, Pignoni, Hu et al. 1997, Jemc and Rebay 2007). However, this eye ectopic induction is considerably less effective than *ey* in both frequency and magnitude (Bonini, Bui et al. 1997, Pignoni, Hu et al. 1997, Seimiya and Gehring 2000). These results suggest that *ey* acts upstream of *eya* during normal eye development and that *ey* also regulate other important genes that control retinal determination. Indeed, *ey* is also required for *so* expression (Cheyette, Green et al. 1994, Halder, Callaerts et al. 1998, Niimi, Seimiya et al. 1999, Ostrin, Li et al. 2006). While *ey* is necessary to induce the expression of *eya* and *so*, these target genes may also regulate the expression of each other and *ey* (Pignoni, Hu et al. 1997). More recently, *so* has been shown to induce eye formation on its own while the sort of tissues and cell types that can be transformed into retina by this gene is far less than that of ey (Weasner, Salzer et al. 2007). Ectopic eyes induced by the combinatorial expression of *eya* and *so* are only a subclass of those where *ey* alone is effective, which indicates that *ey* may regulate other target genes. In fact, upregulation of *ey* or *eya* induces expression of *dac* (Chen, Amoui et al. 1997, Pappu, Ostrin et al. 2005, Anderson, Salzer et al. 2006). The upregulation of this gene is sufficient to induce expression of *ey*, *eya* and *so*, which indicates a positive-feedback loop (Chen, Amoui et al. 1997, Pignoni, Hu et al. 1997). Additionally, the Eya-Dac complex may also play a relevant role in the eye determination as Dac is predicted to be a DNA binding protein and co-expression of *eya* and *dac* also increases the ectopic eye formation (Chen, Amoui et al. 1997). Curiously, in the developing eye Dac function is not categorically dependent of the domain that mediates interactions with Eya (Tavsanli, Ostrin et al. 2004). Moreover, numerous retinal determination family members bind to members of

the Dach family (Li, Perissi et al. 2002), which suggests that So may act as a bond for interactions between Eya and Dac within the developing eye (Kumar 2009).

The expression of early retinal genes occurs earlier than photoreceptor differentiation during the second and early third instar larval stages. *eya* and *so* are expressed in a gradient manner with highest levels in the posterior-lateral margins of the eye imaginal disc and narrowing off to the middle region (Cheyette, Green et al. 1994, Bonini, Bui et al. 1997, Halder, Callaerts et al. 1998). Moreover, *dac* is initially expressed at the anterior region of the eye disc and during the differentiation progression its expression extends to the posterior region (Mardon, Solomon et al. 1994). The expression of *dac* potentiates Hedgehog signaling pathway that ensures a fast and robust differentiation of the retina (Brás-Pereira, Potier et al. 2016). Null mutations in early retinal genes result in flies without eyes, which is caused by a failure to initiate differentiation. However, these genes are not required for photoreceptor differentiation or progression, which is attributed to other genes.

1.2.2. Genetic signaling molecules regulating eye differentiation

The differentiation of the eye imaginal disc is a continuous process, where columns of cells successively differentiate from posterior to anterior region, establishing individual photoreceptors differentiation in a defined order within each cluster. At early stages of *Drosophila* eye development, *wg* and *dpp* are expressed in dorsal/anterior (Baker 1988, Pereira, Pinho et al. 2006) and ventral/posterior regions (Heberlein, Wolff et al. 1993), respectively. Nevertheless, at early eye development stages, the eye primordium is very small and as Wg and Dpp are long range secreted molecules, cells receive both signals. Therefore, Wg is able to repress Dpp activity (Hazelett, Bourouis et al. 1998), which avoids a premature differentiation onset (Figure 1.4 B). However, during late second - early third larval stages, the proliferation regulated by Notch, Eyg and Upd (Domínguez and Celis 1998, Chao, Tsai et al. 2004) induces the growth of the disc, and *wg* and *dpp* expression ranges are separated. This separation is further stimulated by *wg* repression through the JAK/STAT signaling (Tsai and Sun 2004, Ekas, Baeg et al. 2006). Therefore, the antagonizing effect of Wg upon Dpp is unloaded (Kenyon, Ranade et al. 2003) and retinal differentiation starts with the expression of the unblocked *dpp* within the MF (Ready, Hanson et al. 1976). At this stage, Dpp induces the expression of retinal determination genes closer to the margin by *hth* repression (Bessa, Gebelein et al. 2002). These cells can now differentiate, giving rise to the first photoreceptors. With the exception of the R8 cells, these photoreceptors activate *hedgehog* (*hh*) expression (Rogers, Brennan et al. 2005). In

addition, *hh* is secreted in and behind the MF (Treisman and Heberlein 1998) and controls the expression of *dpp* in the furrow (Heberlein, Wolff et al. 1993, Heberlein, Singh et al. 1995, Greenwood and Struhl 1999). In turn, Dpp spreads and represses *hth* expression in the next cells (Lopes and Casares 2010). This mechanism is necessary to induce the progenitor cells to enter a precursor state allowing the initiation and progression of retinal differentiation (Bessa, Gebelein et al. 2002, Lopes and Casares 2010). Hh also activates the expression of *atonal* (*ato*) in the furrow. The expression of this protein is restricted to the future R8 photoreceptor and is required for the recruitment of the other neuronal and non-neuronal cells required to form the ommatidial clusters (Tomlinson 1985, Domínguez and Casares 2005). The anterior region to the MF is characterized by a cell population that proliferates via asynchronous cell divisions. However, the posterior region of the furrow presents pre-clusters of photoreceptors cells. The R8 is the first type of photoreceptor to have its fate determined, followed by the differentiation of two photoreceptor pairs: R2/5 and R3/4. The cells that are not incorporated into the pre-ommatidial clusters undergo a second mitotic wave, which is required to produce the remaining photoreceptors. With the end of the second mitotic wave, several cells immediately join the pre-ommatidial cluster and assume the fates of the final three photoreceptors (R1/6/7). These photoreceptors and accessory cells are then recruited to each ommatidial cluster by waves of expression of the ligand spitz (spi) for the EGF receptor.

The photoreceptors axons are surrounded by several types of glia. Glial migration depends on photoreceptor axons, as glia progenitors are held in their precursor regions when retinal innervation is eliminated (Dearborn and Kunes 2004). Integrins and JNK signaling regulates retinal glia migration from the brain into the eye disc (Tavares, Pereira et al. 2015, Tavares, Correia et al. 2017), a process that is tightly coordinated with the ongoing photoreceptor differentiation (Tayler and Garrity 2003, Silies, Yuva et al. 2007).

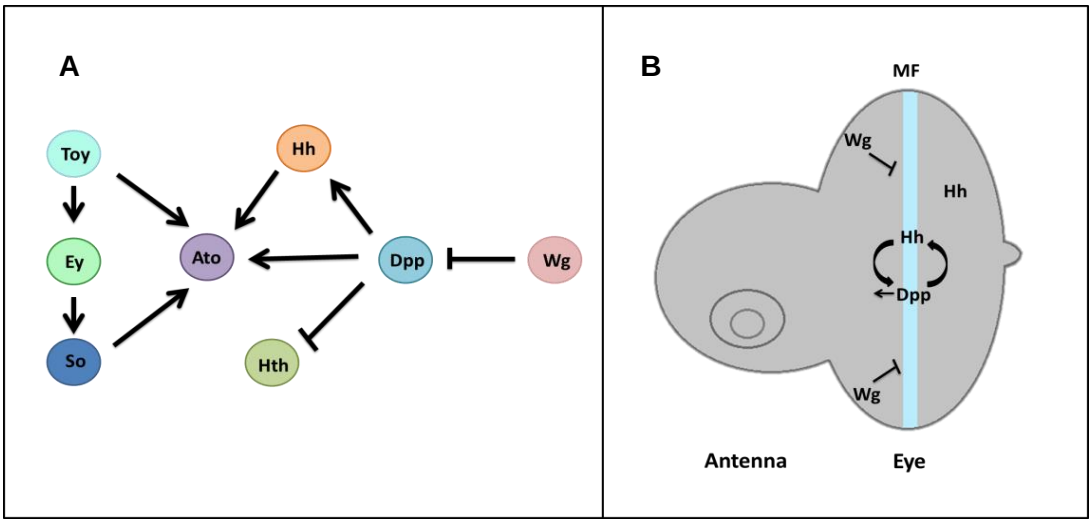

Figure 1.4. Representation of the mechanisms evolved in eye imaginal disc development. (A) A hierarchy scheme of transcription factors operating during embryonic development of the eye imaginal disc. Progression of the Morphogenetic Furrow (MF) in the eye imaginal disc is regulated by Hh and Dpp. Wg secreted at the ventral and dorsal edges regulates photoreceptor formation at the posterior region of eye imaginal disc.

1.3. Salivary gland development

Drosophila salivary glands are simple tubular organs that produce a glue fluid secretion, which is essential for larvae to undergo pupariation (Myat 2005). They are a pair of elongated tubes, composed by secretory and duct cells. Secretory cells are columnar epithelial cells which secrete high levels of protein. Duct cells are cuboidal epithelial cells surrounding an inner lumen, which are connected to the larval mouth. The salivary glands arise from two ventral ectodermal clusters of approximately 100 cells each, in the region of the presumptive posterior head, during mid-embryogenesis (Campos-Ortega and Hartenstein 1997). During mid-embryogenesis, *Drosophila* salivary cells invaginate from the ventral ectoderm of the embryo, which results in the formation of the tubular structure (Figure 1.5). Once primordial cells have been internalized and an elementary tube is established, distal cells in contact with the visceral mesoderm migrate to the posterior region, lengthening their apical membrane in the direction of the migration, to give rise to the final shape and size of the tube (Myat 2005). The specification of cell fate, to determine a correct formation of salivary glands, depends on various gene expression including the homeotic genes, s*ex combs reduced* (*scr*), *extradenticle* (exd) and *homothorax* (*hth*), as well as, a signaling pathway initiated by the product of the *decapentaplegic* (*dpp*) (Panzer, Weigel et al. 1992, Henderson and Andrew 2000). Scr is responsible to restrict the salivary glands to a distinct anterior-posterior position in the embryo. Exd and Hth are required, at

multiple levels, for salivary gland formation. They are necessary for the expression of salivary gland target genes. Additionally, Exd and Hth are essential to maintain the expression of *scr* in the ventral cells of ectoderm of parasegment 2, which will form the salivary glands (Henderson and Andrew 2000). Furthermore, in mutant embryos for Scr, Exd and Hth, no salivary glands are formed (Henderson and Andrew 2000). Dpp signaling limits which cells become committed to form salivary glands (Panzer, Weigel et al. 1992, Isaac and Andrew 1996, Henderson, Isaac et al. 1999). Relatively to the lumen size, GTPase Rho1 seems to have a crucial role in its determination by controlling the apical and cell rearrangement, through the regulation of the Actin cytoskeleton and Moesin (Hogan and Kolodziej 2002, Myat 2005, Xu, Bagumian et al. 2011).

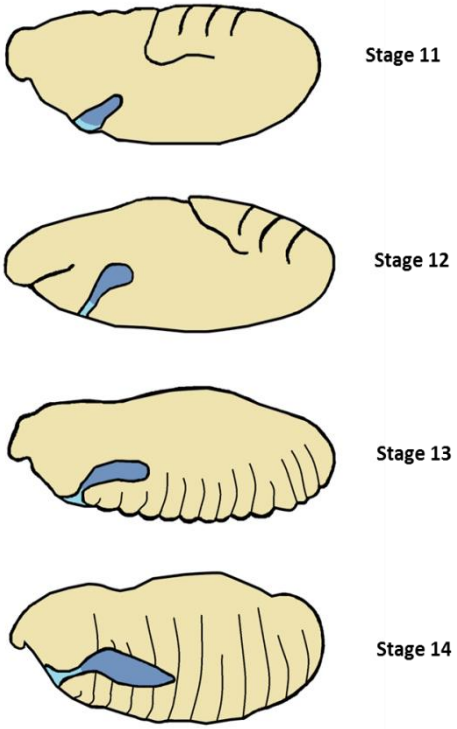

Figure 1.5. A schematic representation of salivary gland development. The salivary gland primordia forms during stage 11 (light and dark blue) on the ventral side of the embryo as two placodes. During the stage 12, the salivary gland cells invaginate into the interior of the embryo and contact the visceral mesoderm. This invagination continues until approximately the end of stage 12, when all of the salivary gland cells have been internalized. During stages 13-14 the salivary gland development continue with the formation of the duct and the glands adopt a dorsally oriented position within the embryo. Adapted from Vining 2005.

The differentiation of the salivary glands is not dependent of cell division and the increase of total size is only dependent on volume of individual cells (Figure 1.6) (Smith and Orr-Weaver 1991, Edgar and Orr-Weaver 2001). Therefore, the changes that occur during later development happen within and between pre-existing cells, which simplifies its study. Mitotically differentiated cells of salivary glands lose the capacity to divide and undergo successive cycles of endoreplication. These endoreplicative cycles are characterized by successive S phases of DNA synthesis and gap phases, without cell division. These polyploid cells are controlled by exogenous factors, such as temperature and nutritional conditions, as well as by growth-related signaling pathways. For example, the loss of a growth regulator associated with nutritional signaling, such as the *Drosophila* serine/threonine kinase target-of rapamycin (dTOR), decreases the ability of cells to

endoreplicate (Zhang, Stallock et al. 2000). Furthermore, expression of *dMyc* regulates rRNA synthesis and ribosome biogenesis which is required for essential dMyc functions, including cell growth (Grewal, Li et al. 2005, Marinho, Casares et al. 2011). Therefore, the salivary gland is an excellent experimental organ to study both cell growth and cell cycle. Additionally, their large size at the cellular level, offers an easier way to study small structures, such as the nucleolus.

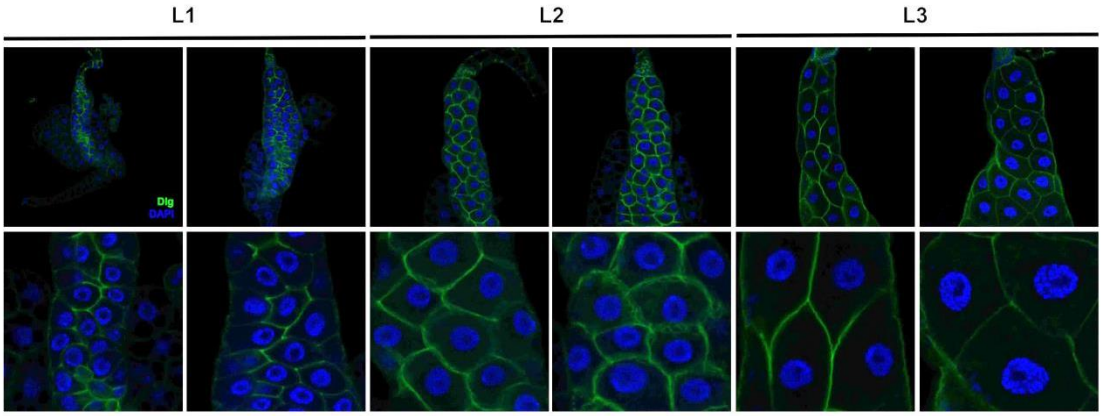

Figure 1.6. The increase of the total size of the salivary glands is only dependent of the volume of individual cells. Blue represents DNA and Green symbolizes the membranes, stained with Dlg. L1: First Larval Instar; L2: Second Larval Instar; L3: Third Larval Instar.

2. Cell growth and proliferation during animal development

Cell growth requires an extraordinary number of machineries that carry out protein synthesis, the ribosomes. Therefore, the efficient ribosome production reflects the cell capacity to grow. In eukaryotes, this process involves the coordinated function of more than 200 proteins and takes place in the nucleolus, nucleoplasm and cytoplasm (Thomson, Ferreira-Cerca et al. 2013). The activity of the three RNA polymerases (I, II and III) is necessary to synthesize the components essential for ribosome biogenesis (Figure 1.7). Ribosome biogenesis begins in the nucleolus, with the co-transcription of 18S, 5.8S and 28S rRNA genes, by RNA polymerase I (Pol I) as a single polycistronic transcript. A fourth pre-rRNA 5S is transcribed at the nucleus, by the RNA polymerase III (Pol III), and imported to the nucleolus to undergo maturation (Lafontaine and Tollervey 2001, Raska, Koberna et al. 2004, Xue and Barna 2012). The transcribed rRNA is subjected to several modifications, such as site-specific pseudouridylation and methylation, carried by small nucleolar ribonucleoproteins (snoRNP). Ribosomal proteins and accessory factors are transcribed by RNA polymerase II (Pol II) and the subsequent RNA's are translated in cytoplasm. Then, these proteins are imported into the nucleus to be assembled into the small and large ribosomal subunits. The small

pre-40S ribosomal subunit corresponds to 18S rRNA and the pre-60S large ribosomal subunit is composed of 28S, 5.8S and 5S rRNA. Finally, the pre-ribosomes are exported to the cytoplasm for protein translation. The ribosome production is correlated with cell capacity to grow and to proliferate. Therefore, the dysregulation of this machinery conduces to cellular transformations, either by overproduction or haploinsufficiency of ribosomal biogenesis (Montanaro, Treré et al. 2008, Silvera, Formenti et al. 2010).

2.1. *Drosophila* nucleolus is a multifunctional nuclear structure that controls cell growth through ribosomal biogenesis

The nucleolus is a multifunctional nuclear structure that controls cell growth through ribosomal biogenesis. Nucleoli have been shown to behave like liquid droplets, where proteins and other molecules required for ribosome biogenesis dynamically self-segregate into the three different infra-nucleolar regions dense-fibrillar component (DFC), fibrillar center (FC) and granular component (GC) (Brangwynne, Mitchison et al. 2011, Feric, Vaidya et al. 2016). These microscopically recognized regions are the sites of progressive steps of rRNA transcription, processing, and ribosome assembly. In electronic microscopy, the *Drosophila* nucleolus is less organized and the three nucleolar components are not distinguishable (Orihara-Ono, Suzuki et al. 2005). In this model, the nucleolar structural architecture is regulated by *viriato* (*vito*), the single *Drosophila* member of the Nol12/Nop25 gene family (Marinho, Casares et al. 2011). In the nucleolus, the genes encoding rDNA are present in tandem repeats, organized as clusters embedded in heterochromatin and cytologically visible as nucleolus organizers (NORs), when they are transcriptionally active. In *Drosophila*, NORs are responsible for nucleoli organization, proper pairing of X and Y during meiosis and rDNA exchange and deletions at a high rate (McStay 2016). The localization and number of NORs differ from species to species and may sometimes be autosomal, as well as, frequently associated with sex chromosomes, which is the case of *Drosophila*.

The main role of the nucleoli is to create the appropriate environment for an efficient ribosome biogenesis. However, many non-traditional functions of the nucleolus have been proposed, such as signal recognition particle assembly, small RNA modification, RNA editing, telomerase maturation, nuclear export, cell cycle and cell growth control, and stress sensor (Lo, Lee et al. 2006).

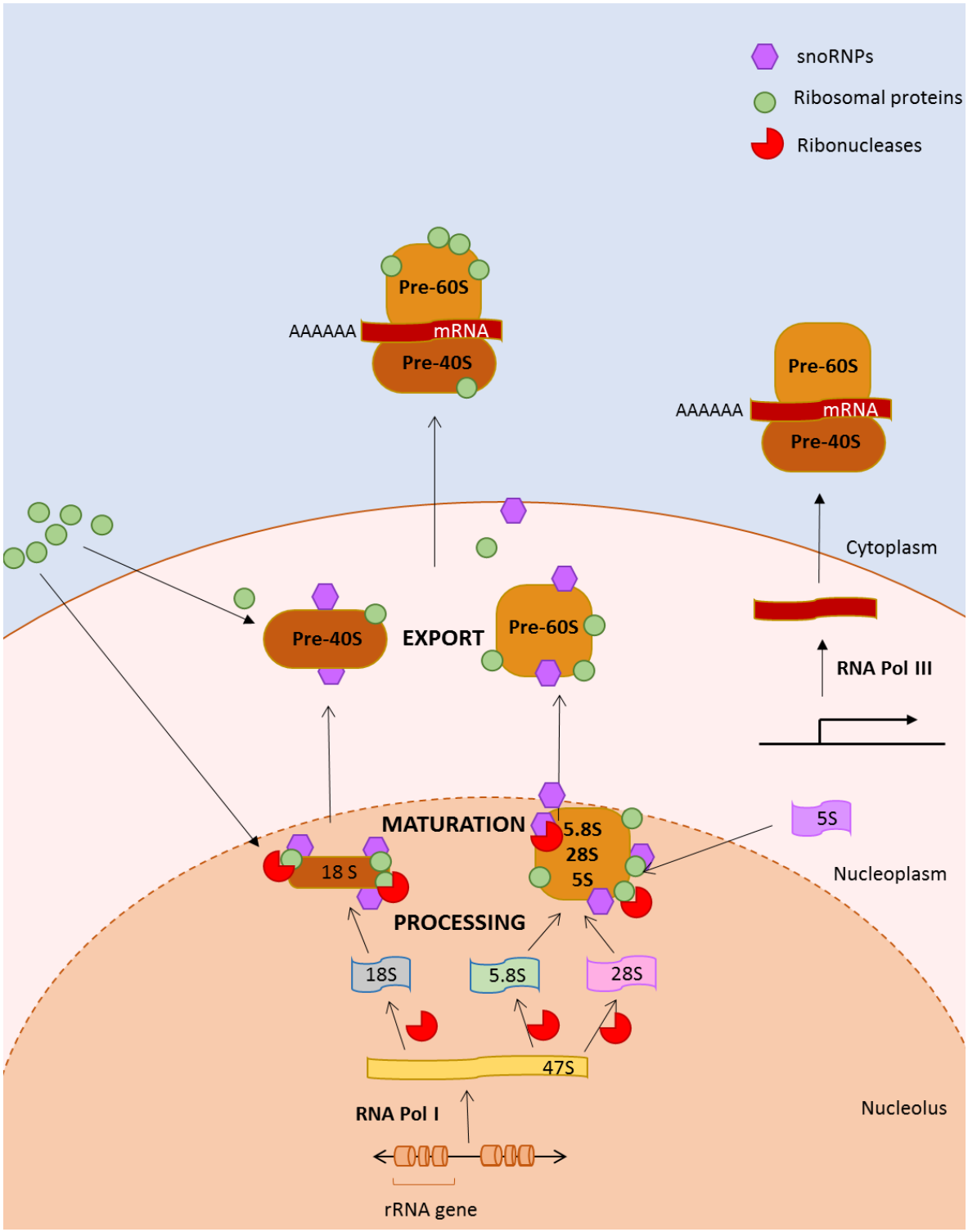

Figure 1.7. Ribosomal biogenesis. The nucleolus has a cluster of rRNA genes, which are transcribed by RNA polymerase I into the 5.8S, 28S and 18S pre-rRNAs. The 5S pre-rRNA is transcribed by RNA polymerase III at the nucleoplasm and imported to the nucleolus. Processing, maturation and assembly of pre-rRNAs to form the ribosome subunits requires ribonucleases, ribosomal proteins and small nucleolar ribonucleoproteins (snoRNPs). The mature small 40S and large 60S subunits are then translocated to the cytoplasm in a late maturation process, and assemble with mRNA to form functional ribosomes.

As a dynamic structure, the nucleolus is characterized by an interaction between resident nucleolar proteins and several foreign proteins that are in constant exchange between the nucleolus and the nucleoplasm (Raska, Koberna et al. 2004, Andersen, Lam et al. 2005). This constant trafficking results from cellular demands, reflecting the physiological state of the cell. In the nucleolus, the targeting of proteins can be mediated by intergenic spacer regions, which is present between the small and large subunits. Consequently, in response to cell stress stimuli these highly repeated DNA sequences are transcribed into long non-coding RNAs that are able to capture and retain proteins containing a specific peptidic motif, NoDS (Nucleolar Detention Sequence) (Audas, Jacob et al. 2012, Prasanth 2012). The transitory nature of the nucleolar proteome ensure the sensitivity of nucleolus to external changes, making possible the interaction between different proteins with related functions in a cellular context-dependent fashion (Boisvert, van Koningsbruggen et al. 2007).

Several proteins are described as nucleolar modulators. One of these modulators is dMyc, which has the ability to induce a coordinated nucleolar hypertrophy and to stimulate pre-rRNA transcription and ribosome biogenesis in general (Grewal, Li et al. 2005, Marinho, Casares et al. 2011). In *Drosophila*, *dMyc* overexpression indirectly increases rRNA transcriptional machinery, by increasing the production of the large Pol I subunit, RpI135, and the transcription initiation factor IA (TIF-IA) (Grewal, Li et al. 2005, Grewal, Evans et al. 2007). dMyc is also responsible to increase the Pol II-dependent transcription of ribosomal proteins such as Nop60 and Fibrillarin, as well as to stimulate Pol III transcription (Coller, Grandori et al. 2000, Guo, Malek et al. 2000, Boon, Caron et al. 2001, Menssen and Hermeking 2002). Moreover, in vertebrates, c-Myc binds to TFIIIB, a Pol III-specific general transcription factor, activating Pol III-mediated transfer RNA and 5S rRNA transcription (Gomez-Roman, Grandori et al. 2003) and to regulate the efficiency of rRNA processing (Schlosser, Hölzel et al. 2003).

2.2. The generic regulation of cell growth

Tissue growth is the result of both cellular growth and proliferation. Studies from *Drosophila* growth regulators have shown that cell division and cellular growth are distinct but inter-connected processes. Several signals control tissue growth like temperature fluctuations, nitric oxide or oxygen levels, hormones and many different signaling pathways (Day and Lawrence 2000, Koyama, Mendes et al. 2013, Nijhout, Riddiford et al. 2014). Here, we discuss some of the most important signals that regulate *Drosophila* tissue growth.

2.2.1. JAK/STAT pathway

The Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway is one of the key pathways required for proper tissue growth in *Drosophila melanogaster.* While the activation of JAK/STAT pathway signaling in mammals is activated by several interleukins, interferons and growth factors (Subramaniam, Torres et al. 2001, Langer, Cutrone et al. 2004), only three interleukin-6 (IL-6)-like cytokines—Unpaired (Upd), also named Outstretched, Upd2, and Upd3, have been identified in invertebrates (Harrison, McCoon et al. 1998, Agaisse, Petersen et al. 2003, Gilbert, Weaver et al. 2005, Hombría, Brown et al. 2005). The canonical JAK/STAT pathway initiates with the binding of a ligand to the dimeric receptor Domeless (Dome) (Figure 1.8). This binding induces the activation of the JAK Hopscotch (Hop), leading to tyrosine phosphorylation of Dome. Once phosphorylated, receptor/JAK complex phosphorylates STAT92E dimer producing an active STAT92E dimer. The active STAT92E dimer is then translocated to the nucleus where it controls the expression of several target genes. There is a second transmembrane receptor called Latran (Lat) or Eye Transformer (ET), which forms heterodimers with Dome receptor and inhibits JAK-STAT signaling (Kallio, Myllymäki et al. 2010, Makki, Meister et al. 2010). One of the most important functions of JAK-STAT signaling is the regulation of growth and the competitive status of proliferating cells (Mukherjee, Hombría et al. 2005, Rodrigues, Zoranovic et al. 2012). In larval blood cells, a constitutive active *Drosophila* HOP version induces the overproliferation and premature differentiation of these cells, which then form melanotic tumours (Hanratty and Dearolf 1993, Harrison, Binari et al. 1995, Luo, Hanratty et al. 1995). Another tissue in which this occurs is the developing eye imaginal disc. JAK-STAT signaling promotes proliferation of cells in the eye field, which leads to an increase of the number of mitotic cells within the first mitotic wave (Bach, Vincent et al. 2003, Tsai and Sun 2004). As consequence, more cells are available to be differentiated into ommatidia, leading to an overgrowth of adult eye (Bach, Vincent et al. 2003, Mukherjee, Schäfer et al. 2006). Moreover, loss-of-function mutations of *upd* or *hop* cause small eye phenotype, indicating that JAK/STAT pathway is required for normal cell proliferation (Perrimon and Mahowald 1986, Tsai and Sun 2004, Mukherjee, Hombría et al. 2005, Mukherjee, Schäfer et al. 2006). Similarly effects were observed in wing discs mutant for hop^{M13} (Mukherjee, Hombría et al. 2005). One interesting finding is the dual function of JAK/STAT pathway during wing larval development. During early larval development, this pathway promotes proliferation, nevertheless by later larval stages STAT92E is stimulated by a non-canonical signaling playing an important role as antiproliferative factor (Mukherjee, Hombría et al. 2005).

Relatively to cell size, JAK/STAT pathway does not appear to have a significant contribution on its regulation (Mukherjee, Hombría et al. 2005).

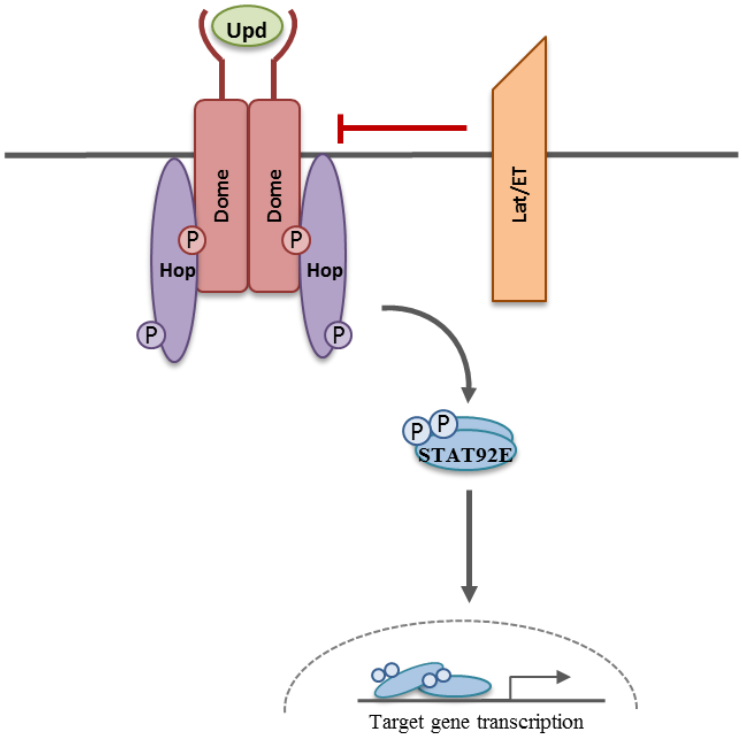

Figure 1.8. Diagram representing the JAK/STAT signaling pathway in *Drosophila*. This schematic diagram represents the core components of JAK/STAT pathway. Arrows represent activation and bar-ended line represents repression. For more details, see text. Adapted from Zoranovic 2013.

2.2.2. Notch signaling

Notch signaling is an evolutionarily conserved pathway that controls cell fate during developmental through local cell interactions. The canonical Notch signaling initiates with the interaction between the transmembrane proteins ligands, Serrate and Delta, and the Notch receptor in the neighbor cell (Figure 1.9). This receptor/ligand interaction leads to proteolytic cleavages of Notch by ADAM metalloproteases and γ-secretase in the membrane-tethered intracellular domain. After γ-secretase cleaves Notch, the Notch intracellular domain (NICD) is free to translocate to the nucleus. There, NICD forms a complex with the Supressor of Hairless protein (Su(H)), Mastermind protein (MAM) and Histone acetyltransferases (Hac), inducing transcriptional activation of Notch target genes (Kopan and Ilagan 2009). In the *Drosophila* eye disc, the activation of Notch along the dorsoventral boundary results in eye disc growth, while the downregulation of Notch signaling results in smaller or absent eyes (Cho and Choi 1998, Domínguez and Celis 1998, Papayannopoulos, Tomlinson et al. 1998, Chao, Tsai et al. 2004, Reynolds-Kenneally and Mlodzik 2005, Yao, Phin et al. 2008). In the

early wing imaginal disc, the activation of Notch promotes tissue growth (de Celis and García-Bellido 1994), while loss of Notch signaling during second larval stage leads to a failure in the induction of wing fate (Couso and Arias 1994). The regulation of tissue growth by Notch seems to result from proliferation and cell growth. The activation of Notch signaling in wing discs is associated to high mitotic activity, however this cell proliferation is not a direct consequence of Notch overexpression (Go, Eastman et al. 1998). Instead, it results from a synergy of Notch with several other proteins, including Vestigial and Wingless. Moreover, in stem cells, Notch signaling plays a very important role in cell growth (Song and Lu 2011). Furthermore, the activation of Notch signaling has been implicated in tumor development (Thompson, Mathieu et al. 2005, Vaccari and Bilder 2005). Hyperactivation of Notch is observed in numerous types of cancers including breast cancer (Brzozowa-Zasada, Piecuch et al. 2017) and T-cell acute lymphoblastic leukemia in humans (Tosello and Ferrando 2013).

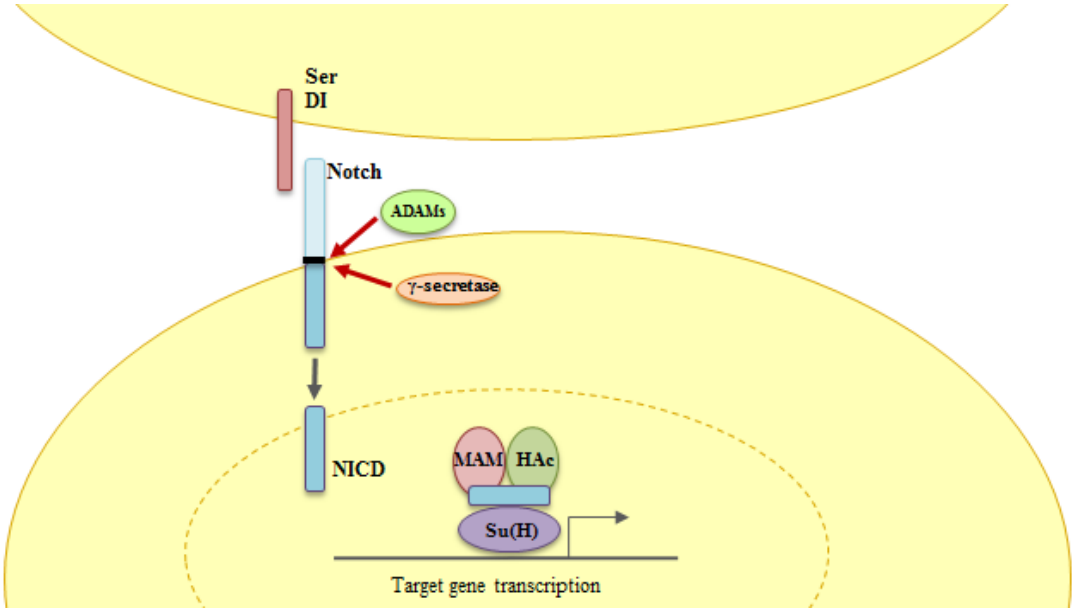

Figure 1.9. Diagram representing the Notch pathway in *Drosophila*. This diagram represents the core components of Notch pathway. Dark-grey arrows represent activation and red arrows represent cleavage. For more details, see text. Adapted from Kopan 2009.

2.2.3. Hippo pathway

The Hippo (Hpo) pathway is known for its great contribution in the growth control during *Drosophila* development. The canonical Hippo pathway initiates with the phosphorylation and activation of Wts by Hpo-Sav complex (Wu, Huang et al. 2003, Wei, Shimizu et al. 2007) (Figure 1.10). Together, Wts, Hpo and Sav recruit Mats (Lai, Wei et al. 2005) and phosphorylate the transcriptional coactivator Yorkie (Yki) at

Ser111, Ser168 and Ser250 residues, being Ser168 residue the most critical site (Huang, Wu et al. 2005, Dong, Feldmann et al. 2007, Oh and Irvine 2008, Zhang, Ren et al. 2008, Ren, Wang et al. 2010). After Yki phosphorylation by Wts, this phospho-protein binds to 14-3-3 proteins, which negatively regulate Yki function by keeping it localized in the cytoplasm (Dong, Feldmann et al. 2007, Oh and Irvine 2008, Zhang, Ren et al. 2008, Ren, Zhang et al. 2010). This cytoplasmic location results from nuclear export and cytoplasmic anchoring (Ren, Zhang et al. 2010). The inhibition of Yki upstream kinases leads to the entrance of Yki into the nucleus and drives the transcription of its targets genes. The most relevant functions of Yki are cell growth and proliferation, as well as the inhibition of cell death (Huang, Wu et al. 2005). Several Yki targets are already described, including CycE that controls the transition of G1 to S phase during cell cycle (Wu, Huang et al. 2003), Diap-1 that controls cell death (Tapon, Harvey et al. 2002, Wu, Huang et al. 2003), the microRNA bantam which regulates cell survival and proliferation (Nolo, Morrison et al. 2006, Thompson and Cohen 2006) and also the proto-oncogene dMyc (Neto, Aguilar-Hidalgo et al. 2016).

Contrasting with other signaling pathways, Hippo pathway is regulated by multiple and ever increasing number of upstream inputs that either act directly on Yki or control the core kinase cassette (Staley and Irvine 2012, Irvine and Harvey 2015). Some of these regulators are the atypical Cadherin Fat linked with the related Cadherin Dachsous; the membrane-associated proteins from the Ex-Mer-Kibra complex; cell-cell adhesion and junctional proteins, such as Ajuba, E-cadherin and Echinoid; cell polarity regulators,

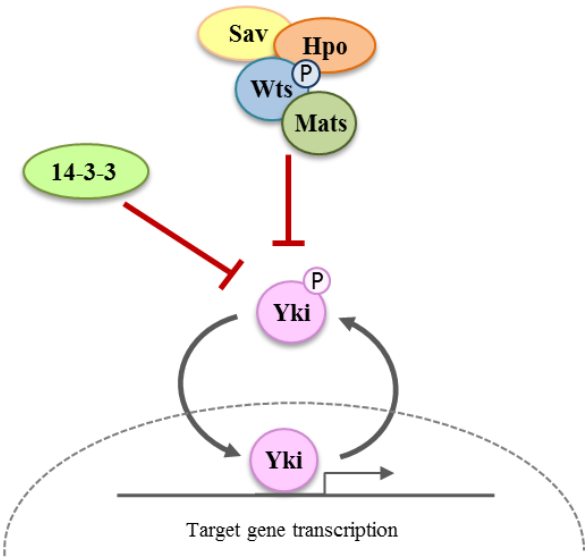

Figure 1.10. Diagram representing the Hippo pathway in *Drosophila*. This diagram represents the core components of Hippo pathway. Arrows represent activation and bar-ended lines represent repression. For more details, see text. Adapted from Zhao 2011.

including Crumbs, Discs large, Lethal giant larvae and Scribble; and F-actin (Staley and Irvine 2012, Irvine and Harvey 2015). These regulators notably associate the Hippo pathway with numerous growth regulatory signals, which suggests this pathway as an emergent regulator of growth control.

2.2.4. dMyc

Mammalian Myc is a multifunctional, nuclear phosphoprotein involved in cell cycle progression, apoptosis and cellular transformation (Ruf, Rhyne et al. 2001). In vertebrates, it is well known that Myc family comprises three types of transcriptional activators (c-, N-, L-Myc), which are expressed in distinct patterns during embryogenesis (Zimmerman, Yancopoulos et al. 1986, Zimmerman and Alt 1990). One of the partners involved in almost all c-Myc biological functions is MAX protein. This protein, when dimerized with c-Myc, acts as transcriptional activator (Tansey 2014). Like c-Myc protein, Mnt, Mga and Mxd1-4 use Max as a cofactor for DNA binding at E-box sequences, but instead of activating transcription they appear to function as transcriptional repressors. In *Drosophila*, this network is much more simple, comprising a single transcriptional activator (*dMyc*), a single repressor (*dMnt*), and their common partner (*dMax*) (Gallant 2013) (Figure 1.11). All three proteins show sequence and biochemical similarities to their vertebrate orthologous. Additionally, *Drosophila* and vertebrate Myc proteins can functionally substitute for each other. dMyc can cooperate with the active version of oncogenic Ras [V12] to transform rat fibroblasts (Schreiber-Agus et al., 1997). Inversely, a human translation variant of c-Myc with a truncated N-terminus rescues the development of flies carrying a lethal Myc allele (Benassayag,

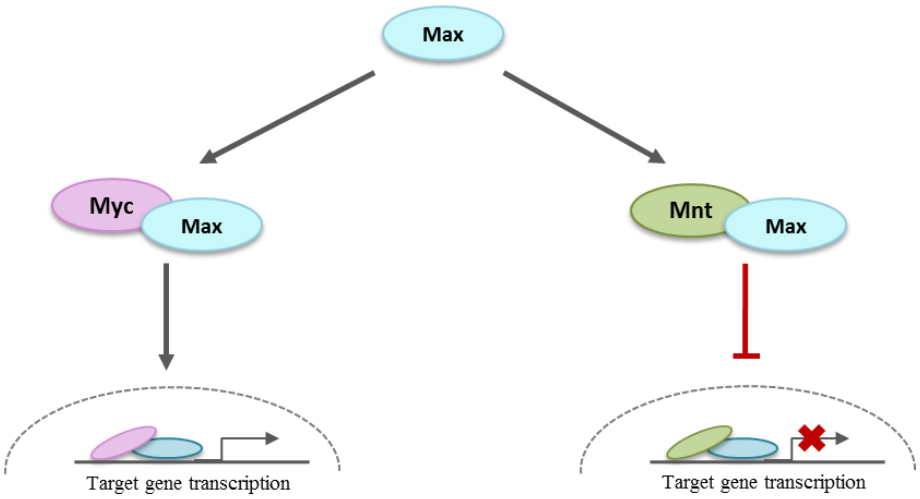

Figure 1.11. Diagram representing Myc/Max/Mnt network and its impact on gene expression. Arrows represent activation and bar-ended lines represent repression. For more details, see text. Adapted from Chanu 2014.

Montero et al. 2005).

One of the most important functions of *dMyc* is the regulation of cell growth and animal size (de la Cova and Johnston 2006). The study of this gene in *Drosophila* has contributed to the understanding of c-Myc function. In 1935, a *Drosophila* Myc mutant was identified as a spontaneous mutation that conduced to a smaller body size (Bridges 1935), whereas flies overexpressing *dMyc* are bigger than controls (Johnston, Prober et al. 1999). Furthermore, it was shown that flies carrying null mutants for *dMyc* die very early in development, at the beginning of the second instar (Pierce, Yost et al. 2004). In the case of hypomorphic mutants, the consequences during development are not so drastic, but a developmental delay is observed in larval phases. A very strong effect is reported in the larval endoreplicating tissues, such as fat body cells and salivary glands. Endoreplicative tissues are indispensable for larval development and are characterized by their cells that grow without dividing, reaching polyploidy and increasing in size (Edgar and Orr-Weaver 2001). In these tissues, *dMyc* mutants exhibit small nucleus that fail to reach normal DNA content (Pierce, Yost et al. 2004). In contrast, cells with *dMyc* overexpressed revealed huge nucleus and nucleolus, with higher ploidy (Pierce, Yost et al. 2004, Grewal, Li et al. 2005, Marinho, Casares et al. 2011). In vertebrates, Myc is also involved in growth regulation: *c-Myc* null mice die among 10 days of gestation and presented smaller size together with a variety of developmental defects and pathologic anomalies (Davis, Wims et al. 1993).

In a previous study conducted in our lab, a novel gene was identified in a screen for genes required for tissue growth in *Drosophila*, *viriato* (*vito*) (Marinho, Martins et al. 2013). This gene is expressed in the anterior region of eye imaginal discs, where cells are proliferating, and codifies for a nucleolar protein. Vito mutants exhibit a developmental delay, and its depletion in the eye imaginal disc results in reduction of its size. Furthermore, *vito* regulates the levels of Fibrillarin at the nucleolus and maintain proper nucleolar structure. In salivary glands, it was shown that Vito is necessary for the dMyc-induced growth, stimulating nucleolar biogenesis and mass accumulation during development. Additionally, null *dMyc* mutants display decreased levels of Vito, through regulation of vito mRNA levels. These results suggest that gene as a mediator of nucleolar response of dMyc-induced growth (Marinho, Casares et al. 2011).

Another protein that is very important for dMyc regulation is *brinker* (*bkr*). This gene is a transcription factor that negatively regulates Dpp target genes (Bray 1999). The *bkr* function was demonstrated by an elegant experiment where *brk* mutant induces a strong increase of the dorsal epidermis and a reduction of the ventral epidermis, which

is a characteristic of a defect in Dpp signaling. Thus, Brk negatively affects either the distribution of Dpp, by interfering with the reception of the Dpp signal or blocking the activation of Dpp target genes. Moreover, clones expressing activated type I receptor of TGFβ signaling, Thickveins (Tkv^{Q235D}), in which Brk expression is inhibited present a robust elevation of dMyc protein (Doumpas, Ruiz-Romero et al. 2013). In contrast, transient induction of Brk expression caused a clear reduction in dMyc protein levels and *dMyc* mRNA. Thus, Dpp signaling inhibits Brk, thereby inducing expression of dMyc leading to tissue growth.

3. TGFβ signaling pathway

The Transforming Growth Factor β (TGFβ) superfamily is a large family of structurally related cell regulatory proteins, which are highly conserved along metazoans (Padgett, St. Johnston et al. 1987, Huminiecki, Goldovsky et al. 2009). TGFβ signaling pathway is involved in several cellular processes in both the adult organism and the developing embryo, including cell proliferation, differentiation, survival/or apoptosis, among other functions. The canonical TGFβ signaling pathway can be divided into two main branches, Bone Morphogenetic Proteins (BMP) and Activin, activated by specific ligands. The first ligand of *Drosophila* TGFβ signaling pathway was discovered in 1987 and named Decapentaplegic (Dpp) (Padgett, St. Johnston et al. 1987) (Figure 1.12). After this, seven other ligand family members were identified in this organism: Activinβ (Actβ), Dawdle (Daw), Maverick (Mav) and Myoglianin (Myo) belonging to TGFβ/Activin family (Parker, Stathakis et al. 2004), and Dpp, Screw (Scw) and Glass bottom boat (Gbb) belonging to BMP family. The canonical TGFβ signaling pathway initiates the signaling activity with the binding of a ligand to type II receptor activation, Punt (Put) or Wishful thinking (Wit), common to both branches. Once phosphorylated, type II receptor binds to type I receptor, Tkv and Saxophone (Sax) in BMP branch and Baboon (Babo) in Activin branch. Then, type I receptor phosphorylates its specific R-Smad, Mad in the Dpp branch and Smad2 (or Smox) in the Activin branch. R-Smads activation promotes its homodimerization and induces the formation of a trimeric complex with the common co-Smad Medea. This complex is then translocated to the nucleus where they control the expression of several target genes (Dijke and Hill 2004, Rahimi and Leof 2007).

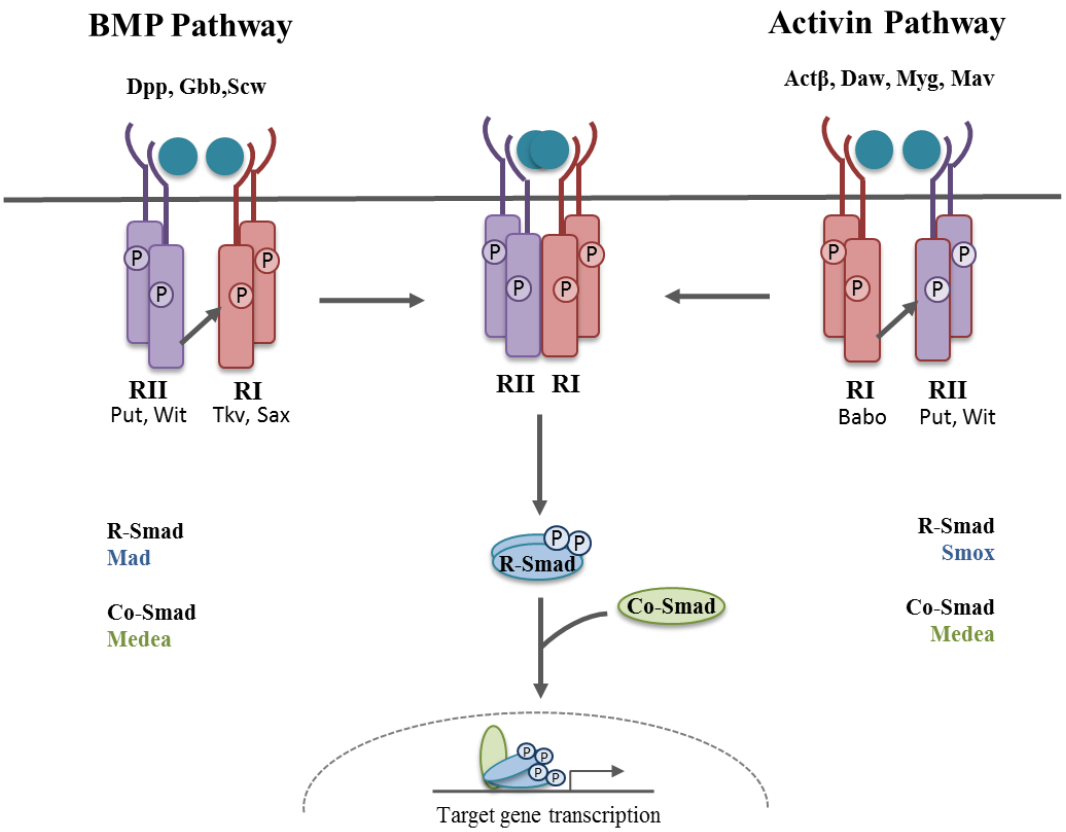

Figure 1.12. Transforming Growth Factor β signaling pathway. In *Drosophila melanogaster*, TGBβ signaling is divided into two branches, BMP and Activin. Decapentaplegic (Dpp), Glass Bottom Boat (Gbb) and Screw (Scw) are the BMP pathway ligands, whereas Activin β (Actβ), Dawdle (Daw), Myoglanin (Myo) and Maverick (Mav) are the Activin branch ligands. After binding of the dimeric ligands to the heteromeric receptor complex, the two activated R-Smads (Mad in BMP branch or Smox in Activin branch) will constitute a trimeric complex with the common Co-Smad Medea that translocates to the nucleus, and activates the transcription of target genes. Adapted from Hamaratoglu 2014.

3.1. BMP branch

From the two TGFβ signaling branches, BMP/Dpp branch is the most exhaustively studied. Dpp, the homolog of vertebrate BMP-2 and BMP-4, is involved in several processes of development including the establishment of the dorsal fate during embryogenesis; maintenance of the germ line; the formation of dorsal-ventral axis formation in the early embryo; patterning of the germ layers (ectoderm, mesoderm, and endoderm); tracheal morphogenesis; patterning of the appendages; and growth control (Ferguson and Anderson 1992, Affolter, Marty et al. 2001, Parker, Stathakis et al. 2004, Affolter and Basler 2007). One of the most fascinating properties of Dpp is its capacity to function as a morphogen, inducing target genes in a concentration-dependent manner. This Dpp function has been deeply dissected in the dorsal-ventral axis during embryonic development and during wing patterning (Podos and Ferguson 1999, Strigini and Cohen 1999, Raftery and Sutherland 2003). In cells nearby the

anterior-posterior axis, *dpp* is expressed in an extracellular gradient across the wing disc that signals directly to distant cells, regulating the expression domains of several genes (Basler and Struhl 1994, Lecuit, Brook et al. 1996, Nellen, Burke et al. 1996, Entchev, Schwabedissen et al. 2000, Teleman and Cohen 2000). The dynamic of the Dpp gradient is similar to that of the Wg gradient (Strigini and Cohen 2000), since both ligands are unstable and spread rapidly across the wing disc. One of the genes that are regulated by Dpp is *brk*, which has its transcription downregulated by Dpp. Therefore the expression pattern of *brk* is a mirror of Dpp gradient (Campbell and Tomlinson 1999, Jaźwińska, Kirov et al. 1999, Affolter, Pyrowolakis et al. 2008). Thus, Dpp has a morphogen action in the surrounding tissue by modelling cellular behavior in a positional-dependent manner. Besides its role on patterning, Dpp is also important for size control (Restrepo, Zartman et al. 2014). Flies with low levels of *dpp* in the wing disc, exhibit a severe size reduction in this tissue (Zecca, Basler et al. 1995, Tsuneizumi, Nakayama et al. 1997), whereas ubiquitous expression of *dpp* results in overgrowth of the wing disc (Nellen, Burke et al. 1996). In particular, Dpp expression in the wing A/P boundary is responsible for this tissue growth (Matsuda and Affolter 2017, Barrio and Milán 2017, Bosch, Ziukaite et al. 2017).

3.2. Activin/TGFβ branch

The Activin/TGFβ signaling branch has been less studied when compared with BMP/Dpp branch. This branch is involved in cell growth control during imaginal discs development. Mutants for the type I receptor Baboon (Babo) display smaller imaginal discs, while the ubiquitous expression of a constitutively active version of Babo results in wing imaginal discs overgrowth (Zecca, Basler et al. 1995, Brummel, Abdollah et al. 1999). Smad2, the mammalian R-Smad2 and Smad3 orthologous, is important for patterning in the wing disc (Sander, Eivers et al. 2010). RNAi for *smad2* induces abnormal wing discs and venation defects in the adult wing. Importantly, null mutants of Smad2 produce overgrown wing discs that resemble the phenotypes generated by the gain of function of BMP subfamily signaling (Peterson and O'Connor 2013). Notably, larvae without *babo* and *smad2* genes are not capable to undergo pupariation and die in late larval or during early pupal stages. This downregulation of Activin branch increases Cyclin A levels, with subsequent delay in M phase exiting during cell cycle (Zhu, Boone et al. 2008). Studies in the brain revealed that signaling activation is also important for neuronal remodeling during methamorphosis, when larval neuronal projections are replaced by adult projections. Defects of *babo/smad2* conduce to a decrease in the number of brain precursor cells, due to its low levels of proliferation

(Brummel, Abdollah et al. 1999, Zhu, Boone et al. 2008). Furthermore, mutations in *babo* and *smad2* block remodeling in different neurons of the *Drosophila* brain (Zheng, Zugates et al. 2006). This process also requires the type II receptors, Punt and Wit, common to both branches of the TGFβ signaling pathway. In the wing disc, the ubiquitous expression of *babo* in the expression domain of a Dpp target gene, *vestigial* (*vg*), results in blistered and crumpled adult wings. This phenotype is inhibited when *mad* expression is depleted or when *smad2* is overexpressed, which suggests that the R-Smads compete for Babo phosphorylation. The depletion of *smad2* conduces to an increase of pMad levels in the *babo*-expressing domains, while co-depletion of *babo* and *smad2* does not change the normal pMad expression pattern. Thus, Mad is phosphorylated by Babo in a Smad2 level-dependent manner (Peterson, Jensen et al. 2012). In a recent work developed in our lab, the nucleolar regulator Vito/Nol12 was identified as a protein that interacts with several members of the BMP and Activin branch (including Put, Tkv, Baboon, and Smad2) (Marinho, Martins et al. 2013). This nucleolar protein acts downstream of Dpp, having a role in growth and differentiation of the eye disc.

3.3. Structure of I-Smads

The TGFβ signaling pathway is regulated through several negative and positive regulators (Miyazawa and Miyazono 2016). Interestingly, one of these negative regulators is a structurally related member of the signaling pathway, I-Smad (Miyazawa and Miyazono 2016), which is also induced by this signaling (Tsuneizumi, Nakayama et al. 1997). Thus, I-Smads promote a negative feedback regulation. Mammalian I-Smads have highly conserved Mad homology 2 (MH2) domains with others Smads but lack the phosphorylation site in R-Smads required for their activation by the type I receptors (Figure 1.13). Moreover, the N-domains (MH1 domain and linker regions) are very divergent. The N-domains of Smad 6 and Smad 7 are conserved in only 36.7% of the sequence. In mammals, the N domains of I-Smads have been described as very important for its functions. The N-domain of Smad7 is important for an efficient inhibition of TGFβ signaling and it determines the subcellular localization of these I-Smads (Itoh, Landström et al. 1998, Hanyu, Ishidou et al. 2001). In Smad6, the MH1-linker region also plays an important role in the inhibition of BMP signaling (Lin, Liang et al. 2003). Remarkably, MH2 domain is essential for the inhibition of BMP and TGFβ signaling by both Smad 6 and Smad 7 (Hanyu, Ishidou et al. 2001). The MH2 domain of Smad7 is necessary for competition with R-Smads for receptor activation (Hayashi, Abdollah et al. 1997, Nakao, Afrakhte et al. 1997). Moreover, the MH2 domain of

Smad7 has high affinity for DNA binding and is required for nuclear localization (Zhang, Fei et al. 2007, Shi, Chen et al. 2008). MH2 domain also has an important role in interaction with type I receptors (Mochizuki, Miyazaki et al. 2004).

In *Drosophila*, Dad is the orthologous of Smad6 and Smad7. Dad is a 568 aa Smad protein that, similarly to the other members of the inhibitory group, has a MH1 domain, a linker domain and a MH2 domain (Figure 1.14). The MH2 domain of Dad is conserved in Smad6 and Smad7 sequences, in 41.1% and 36.7%, respectively. However, the N-domain (MH1 domain and linker regions) is conserved only in 26.1% of the sequence with both Smad6 and Smad7. Despite the low conservation rate of this domain, Dad N-terminal is rich in proline content (19 prolines residues), which might be important for several transient intermolecular interactions such as signal transduction, cell-cell communication and cytoskeletal organization (Williamson 1994, Ball, Kühne et al. 2005).

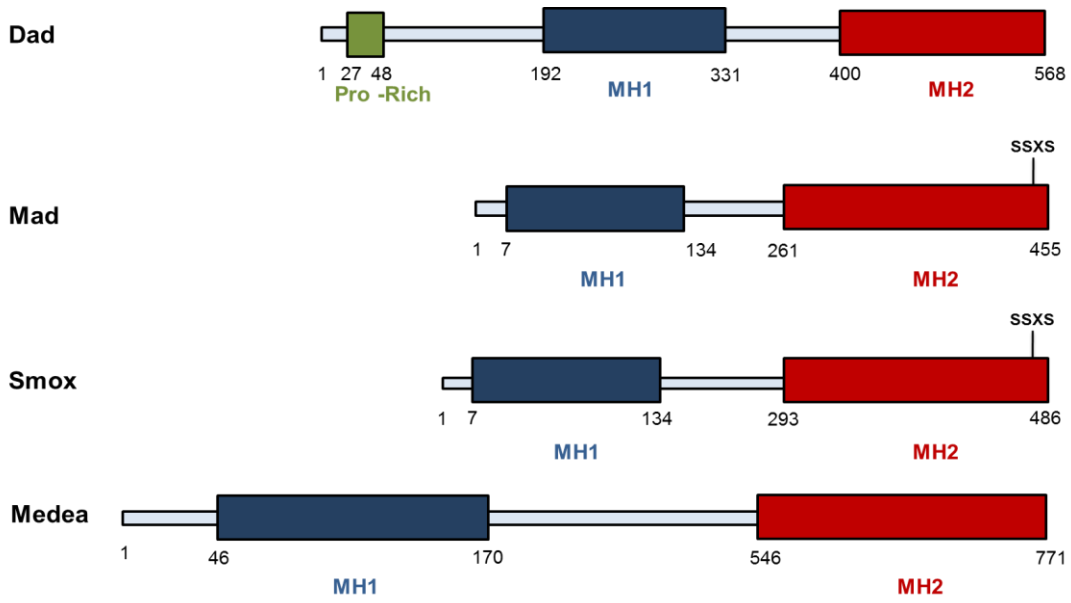

Figure 1.13. Structural organization of the three classes of Smad proteins. The overall structure of Dad, Mad, Smox and Medea. Receptor-mediated phosphorylation of the C-terminal sequence SSXS in the MH2 domain is conserved between R-Smads (Mad and Smox).

3.3.1. Mechanisms of TGFβ inhibition thought I-Smad

In *Drosophila*, it is known that Dad can negatively regulate TGFβ signaling preventing Mad activation by competing with R-Smads for receptors and, thereby, inhibits Tkv-induced Mad phosphorylation. It may also compete for Co-Smad interactions, blocking hetero-oligomerization and nuclear translocation of Mad (Tsuneizumi, Nakayama et al.

1997, Kamiya, Miyazono et al. 2008). Moreover, in vertebrates, the mechanisms of TGFβ inhibition through I-Smad are better studied and multiple mechanisms are already characterized (Miyazawa and Miyazono 2016) (Figure 1.15.). Smad6 and Smad7 differ in their inhibitory preferences on the TGFβ signaling pathway. Smad7 inhibits TGF-β signaling pathway by both BMPs and TGFβ branches while Smad6 preferentially inhibits BMP branch (Miyazawa and Miyazono 2016). Smad7 stably associates with type I receptor, thus inhibiting the recruitment and subsequently phosphorylation of R-Smads and R-Smad/Co-Smad complex formation (Figure 1.15. A) (Hayashi, Abdollah et al. 1997, Nakao, Afrakhte et al. 1997, Souchelnytskyi, Nakayama et al. 1998). Smad6 may also interfere with the formation of an R-Smad/Co-Smad complex interacting with the phosphorylated R-Smad and preventing BMP-induced transcription (Hata, Lagna et al. 1998). It has also been reported that Smad7 is able to form a complex with type I receptor and BAMBI (BMP and Activin membrane-bound inhibitor) in interfering with the R-Smads recruitment (Figure 1.15. B) (Yan, Lin et al. 2009). Several studies have shown that Smad7 has an important role as an adaptor for recruitment of WW domains of type E3 ubiquitin ligases responsible for type I receptor degradation (Miyazawa and Miyazono 2016). Both Smad 7 and Smad 6 have a Pro-Tyr motif that is necessary for the Trp-Trp domains recognition of Smurf (Smad

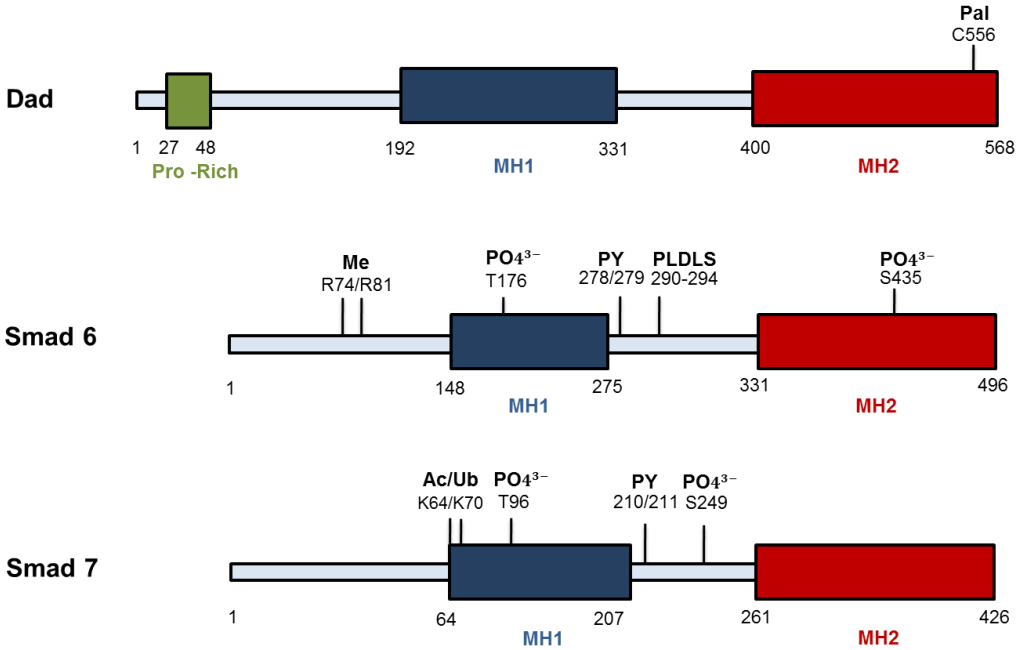

Figure 1.14. Structures of I-Smad proteins. Schematic representation of the structures of Dad, Smad6 and Smad7 and their post-translational modifications. Pal, Palmitoylation site; PY, Pro-Tyr; PLDLS; PO_4^{3-}, Phosphorylation; Ac/Ub, acetylation and ubiquitylation site; C, Cysteine; S, Serine; R, Arginine; K, Lysine; T, Threonine.

ubiquitin regulatory factor) E3 ubiquitin ligases (Zhu, Kavsak et al. 1999, Zhang, Chang et al. 2001). Thus, I-Smads bind to Smurfs and facilitates their association with type I receptors, competing with the R-Smads for receptors (Kavsak, Rasmussen et al. 2000, Ebisawa, Fukuchi et al. 2001, Murakami, Watabe et al. 2003). Additionally, Smad7 may also recruit the E2-conjugating enzyme UbcH7 and assist Smurf2 on the type I receptor degradation (Figure 1.15. C) (Ogunjimi, Briant et al. 2005). The Smad7/Smurf2-mediated type I receptor degradation is enhanced by HSP90 (heat-shock protein 90) (Wrighton, Lin et al. 2008) and Salt-inducible kinase (SIK) (Kowanetz, Lönn et al. 2008, Lönn, Vanlandewijck et al. 2012). Furthermore, Smad7/Smurf2 complex in contact with type I receptor may also recruits ubiquitin-specific peptidase 15 (USP15) which interferes with the degradation of type I receptors (Figure 1.15. C) (Eichhorn, Rodón et al. 2012). Moreover, the Smad7 MH2 domain interacts with Tollip C2 domain and this complex associates with type I receptor, facilitating its translocation to endosomes (Figure 1.15. D) (Zhu, Wang et al. 2012). Smad7 may also interact with a regulatory subunit of the protein phosphatase 1 (PP1), GADD34, that recruits the catalytic subunit of PP1 (PP1c) and this complex facilitates dephosphorylation of the type I receptor (Figure 1.15. E) (Shi et al. 2004). The regulation of TGFβ signaling pathway is also done at the nuclear level with Smad6, which recruits the transcriptional corepressor CtBP through its PLDLS motif and subsequently represses BMP-induced transcription (Figure 1.15. F) (Lin, Liang et al. 2003). This motif is not conserved in Smad7, thus CtBP can only interact with Smad6. Many other proteins are recruited by I-Smads to regulate TGFβ signaling pathway, including Crk-associated substrate lymphocyte type (Cas-L) (Inamoto, Iwata et al. 2006), atrophin 1-interacting protein 4 (AIP4) (Lallemand, Seo et al. 2005), Yes-associated protein 65 (YAP65) (Ferrigno, Lallemand et al. 2002), serine/threonine kinase receptor-associated protein (STRAP) (Datta and Moses 2000) and H2O2-inducible clone 5 (Hic5) (Wang, Song et al. 2008).

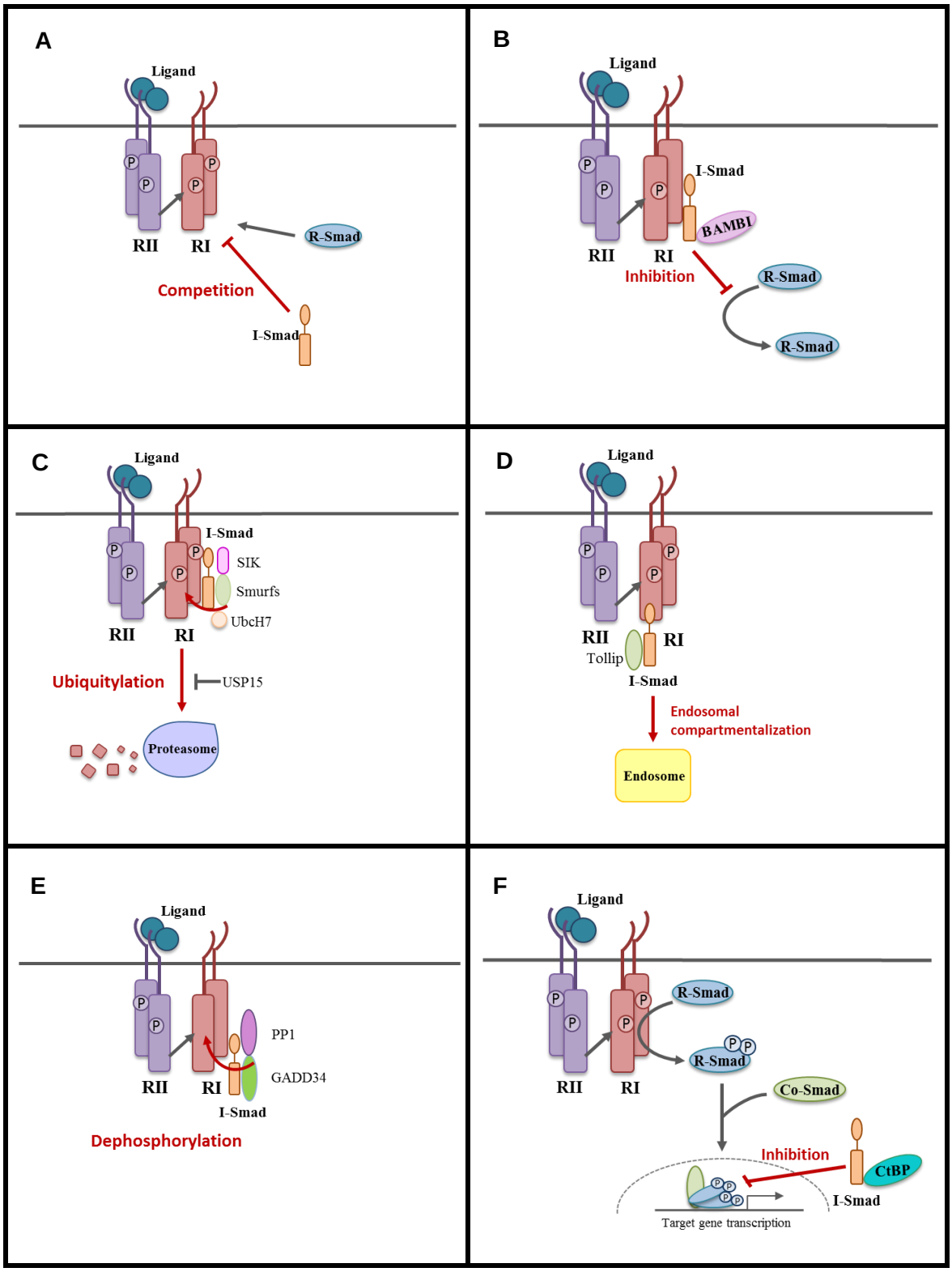

Figure 1.15. Inhibition of TGFβ Family Signaling through I-Smads. (A) I-Smads inhibit TGFβ signaling by interacting with type I receptor and also competing with R-Smads for receptor. (B) I-Smads are able to form a complex with type I receptor and BAMBI interfering with the R-Smads recruitment (C) Smurfs and other E3 ubiquitin ligases interact with I-Smads facilitating receptor degradation. (D) Tollip associates with I-Smad and ubiquitylated type I receptor to drives the endosomal localization of receptors. (E) GADD34–PP1c complex dephosphorylates type I receptors through association

with I-Smads. (F) I-Smads recruit the transcriptional corepressor CtBP and subsequently represses BMP-induced transcription. Arrows represent activation and bar-ended lines represent repression. Adapted from Miyazawa 2016.

3.3.2. Mechanisms for I-Smad regulation

I-Smads undergoes several post-translational modifications (Figure 1.14). Smad7 stability is regulated by ubiquitination-acetylation mechanisms. Ubiquitination and degradation of Smad7 is regulated by multiple factors including Smurf1, Itch, Jab1 and Arkadia (Grönroos, Hellman et al. 2002, Koinuma, Shinozaki et al. 2003, Kim, Lee et al. 2004, Park, Jung et al. 2015). In contrast, the acetylation enhances the stability of Smad7. Smad7 is acetylated at Lys64 and Lys70, the same sites that Smurf ubiquitinates, which inhibits Smurf1-mediated ubiquitination (Grönroos, Hellman et al. 2002). Additionally, HDAC1 (Simonsson, Heldin et al. 2005) and SIRT1 (Kume, Haneda et al. 2007) proteins deacetylate Smad7 promoting its ubiquitination and degradation (Simonsson, Heldin et al. 2005). Phosphorylation is another mechanism that contributes for I-Smad regulation. Smad6 is phosphorylated at Ser435 by protein kinase X, and thereby induces a change in Smad6-DNA binding activity or protein partner complex formation playing an important role in the regulation of Smad6 nuclear function (Glesne and Huberman 2006). Murine protein serine/threonine kinase 38 (MPK38) phosphorylates Smad7 at Thr96 and Smad6 at Thr176, enhancing the inhibition of TGFβ signaling pathway (Seong, Jung et al. 2010). Nevertheless, the phosphorylation of Ser249 of Smad7 by an unknown kinase does not interfere with Smad7 inhibitory role in TGFβ signaling pathway but disturbs Smad7-dependent transcription activation (Pulaski, Landström et al. 2001). Methylation is also a very important process in the regulation of I-Smad activity (Xu, Wang et al. 2013). The arginine methylation of Smad6 by PRMT1, the orthologous of *Drosophila* arginine methyltransferase 1 (Dart1), initiates BMP signaling through Smads. When BMP ligand binds to the receptor complex, PRMT1 methylates Smad6 and this last is dissociated from the type I receptor, derepressing BMP-induced Smad activation by phosphorylation. The methylation of I-Smad seems to be conserved across species, because Dart1 methylates Dad and regulates BMP signaling in *Drosophila* wing development (Xu, Wang et al. 2013). Another post-translational modification that regulates I-Smad activity is palmitoylation. *Drosophila* I-Smad, Dad, is palmitoylated by palmitoyltransferase dHIP14 and this modification is critical for membrane-function of Dad. Thus, Dad palmitoylation is important for the inhibitory function of I-Smads in TGFβ signaling pathway activity (Li, Li et al. 2017).

3.4. Non-Smad pathways of the TGFβ signaling and their roles in epithelial–mesenchymal transition

In *Drosophila,* the first report of non-canonical TGFβ signaling involved signals that regulate the neuromuscular junction (Easton, Cho et al. 2005). The proper development of the neuromuscular junction involves the presynaptic BMP signaling. In addition to the role of canonical BMP signaling, the type II receptor Wit also stimulates the kinase LIMK1 in a Mad-independent way (Easton, Cho et al. 2005). LIMK1 regulates development in the nervous system by phosphorylating Twinstar which inhibits polymerization of the Actin cytoskeleton (Ohashi, Nagata et al. 2000, Ng and Luo 2004). Other signaling components that have been shown to mediate non-canonical signaling in vertebrates are conserved in *Drosophila.* In vertebrates, several signaling pathways are activated and regulated by activated TGFβ receptors (Figure 1.16). These non-Smad pathways include the mitogen-activated protein kinase (MAPK) pathways, including the extracellular signal regulated kinases (Erks), c-Jun amino terminal kinase (JNK) and p38 MAPK, phosphatidylinositol-3 kinase (PI3K) and Akt, and Rho family GTPases (Zhang 2017). These non-canonical TGFβ signaling may regulate epithelial–mesenchymal transition (EMT) through their functions on distinct mechanisms, such as cell growth, cytoskeleton organization, migration and invasion (Derynck and Zhang 2003). The canonical TGFβ signaling pathway can also induces EMT through the expression of diverse EMT-inducing transcription factors, including Snail/Slug, Twist and ZEB1/2 (Xu, Lamouille et al. 2009, Moustakas and Heldin 2012). In this section, I will focus on non-canonical TGFβ induced-EMT.

3.4.1. Erk MAP kinase activation in TGFβ induced-EMT

TGFβ signaling stimulates Erk MAP kinase signaling through a non-Smad dependent pathway (Figure 1.16 A). In mammals, the activation of Erk MAP kinase signaling by TGFβ family initiates with the binding of a TGFβ ligand to a type II receptor, which catalyzes the phosphorylation of type I receptor. The phosphorylated receptor I directly phosphorylates Shc on serine and tyrosine to induce its binding to Grb2/Sos. The Shc/Grb2/Sos complex initiates Ras activation upstream from the kinase cascade, which in turn activates Raf. Then, Raf phosphorylates and activates MEK1/2 and ERK1/2 (Morrison and Cutler 1997, Kolch 2000, Lee, Pardoux et al. 2007). Activated ERK may translocate to the nucleus regulating the activity of several transcription factors and suppressors (Zehorai, Yao et al. 2010). Increased Erk MAP kinase signaling enhances TGFβ-induced EMT, causing disassembly of adherens junctions and promoting cell motility (Zavadil et al. 2001; Xie et al. 2004). As Erk MAP kinase

signaling activates several transcription factors, this interface with TGFβ signaling may result in altered gene expression. Therefore, induction of Erk MAP kinase signaling amplifies TGFβ-induced responses, enabling the downregulation of E-cadherin, upregulation of N-cadherin and matrix metalloproteinase (MMP) expression (Grände, Franzen et al. 2002, Uttamsingh, Bao et al. 2007). In fact, this regulation may be a consequence of the increased expression of Snail2 which downregulates E-cadherin expression in mammals (Schmidt, Gi et al. 2005). Additionally, the activation of Erk MAP kinase signaling can also phosphorylate and promote the nuclear accumulation of Smads, consequently amplifying TGFβ response or inducing secondary responses (Lehmann, Janda et al. 2000). In *Drosophila*, oncogenic Ras (RasV12) cooperate with mutants that disturb cell polarity to drive metastatic behavior, such as basement membrane degradation, loss of E-cadherin expression, migration, invasion and secondary tumor formation (Pagliarini and Xu 2003). Erk/rolled signaling is also important for actin dynamics, in particular Erk signaling is involved in Actin cable formation (Wang, Tsarouhas et al. 2009). Moreover, Rolled mutations alter EGF-R signaling deregulating dorsoventral polarity of the egg shell and the embryo (Brunner, Oellers et al. 1994). However, in *Drosophila* the induction of Erk signaling by TGFβ family is not described.

3.4.2. JNK and p38 MAPK activation in TGFβ induced-EMT

JNK MAPK (Santibañez 2006, Alcorn, Guala et al. 2008) and p38 MAPK (Bakin, Rinehart et al. 2002, Yu, Hébert et al. 2002) pathways are also important mediators of TGFβ induced-EMT. In mammals, the activation of both p38 MAPK and JNK MAPK signaling initiates with the interaction of TGFβ receptors with polyubiquitinated TRAF6 (Figure 1.16 B). Polyubiquitinated TRAF6 recruits TAK1 and activates JNK MAPK and p38 MAPK pathways through MKK4 and MKK3/6, respectively. Active JNK/p38 MAPK may regulate several transcription factors important for TGFβ induced-EMT. Downregulation of JNK/p38 MAPK inhibits alterations in cell shape and reorganization of Actin cytoskeleton linked to EMT (Bakin, Rinehart et al. 2002, Santibañez 2006). Similarly, *Drosophila* JNK has also an important role in the control of Actin stress fibers, cell shape and migration (Martín-Blanco, Pastor-Pareja et al. 2000, Reed, Wilk et al. 2001, Kaltschmidt, Lawrence et al. 2002). Moreover, in human prostate cancer cells (PC-3U), the cleavage of the intracellular domain of TGFβ type I receptor induced by TRAF6 (Mu, Sundar et al. 2011) activates the transcription of p300 stimulating invasion through the expression of *Snail*, *MMP2* and *p300* genes (Sundar, Gudey et al. 2015). In human breast cancer, ubiquitin-conjugating enzyme Ubc13

regulates cell metastasis through activation of p38 MAPK pathway (Wu, Zhang et al. 2014). Moreover, the upregulation of p38 is also involved in TGFβ-mediated metastasis by TAK1-NF-κB-MMP9 pathway (Safina, Ren et al. 2007) and controls cell invasion by up regulation of MMP2 (Xu, Chen et al. 2006, Mu, Sundar et al. 2011). In the other hand, the blockage of p38 MAPK activity, using specific inhibitors, decreases metastasis kinetics (Wu, Zhang et al. 2014). In human gastric cancer cells, TGFβ also promotes invasion and metastasis increasing fascin1 expression via JNK MAPK signal pathway (Fu, Hu et al. 2009). In addition, JNK can also regulate TGFβ signaling through a feedback loop (Ventura, Kennedy et al. 2004). The downregulation of JNK in fibroblasts increases the autocrine signaling of TGFβ and this mechanism has a role in several biological responses to TGFβ, such as invasion and migration (Massagué 1999). While the activation of JNK/p38 MAPK is done by TGFβ receptors through a non-Smad way, the Inhibitory Smads (Smad6 and Smad7) of TGFβ signaling also regulate JNK/p38 MAPK. Smad6 inhibits TGFβ-induced activation of JNK/p38 MAPK pathways through the recruitment of the deubiquitylase A20 to TRAF6, which blocks polyubiquitylation of TRAF6 (Jung, Lee et al. 2013). Smad6 also inhibit BMP-induced p38 MAPK activation, maybe by an interface with TAK1 or the TAK1-binding protein, TAB1 (Kimura, Matsuo et al. 2000, Yanagisawa, Nakashima et al. 2001). Nevertheless, Smad7 may enhance JNK/ p38 MAPK cascade activity, facilitating TGFβ–induced JNK/ p38 MAPK activation and apoptosis (Mazars, Lallemand et al. 2001, Edlund, Bu et al. 2003). Thus, Smad6 and Smad7 seem to play opposite functions in the regulation of TGFβ-induced activation of JNK/p38 MAPK. In *Drosophila*, JNK signaling pathway is an important mediator of tumor invasion and cell apoptosis (Behrens, Sibilia et al. 1999, Huang, Rajfur et al. 2003, Uhlirova, Jasper et al. 2005, Igaki, Pagliarini et al. 2006, Jiang, Scott et al. 2011). JNK functions in part by modulating expression of MMP1 which is necessary for degradation of the basement membrane, and is therefore necessary for metastatic potential of *Drosophila* tumors (Jasper, Benes et al. 2001, Deryugina and Quigley 2006, Uhlirova and Bohmann 2006, Beaucher, Hersperger et al. 2007). Conversely, JNK signaling dynamically direct Actin remodeling proteins to orchestrate the cytoskeletal changes required for cell migration (Rudrapatna, Bangi et al. 2013). Moreover, p38 MAPK signaling also modulates Actin filaments and regulates Hippo signaling which controls cell growth during development, and its dysregulation contributes to tumorigenesis (Huang, Li et al. 2016). Nevertheless, the stimulation of JNK/ p38 MAPK signaling by TGFβ family is not described in *Drosophila* models.

3.4.3. Phosphatidylinositol-3 kinase and Akt activation in TGFβ induced-EMT

TGFβ signaling can also induce Phosphatidylinositol-3-kinase (PI3K)/Akt pathway which plays an important role in EMT. In mammals, the activation of PI3K/Akt pathway initiates with the conversion of PIP2 (phosphatidylinositol 4,5-bisphosphate) into PIP3 by PI3K (Figure 1.16 C). Then, mTOR2 (mTOR, mSin1, mLST8 and Rictor) and PDK1 phosphorylate Akt at Ser473 and Thr308, respectively (Easton, Cho et al. 2005, Tschopp, Yang et al. 2005, Wright, Maroulakou et al. 2008). In turn, activated Akt induces mTORC1/S6 kinase 1 pathway, which increases protein synthesis and cell size. Similarly, in *Drosophila,* the upregulation of TOR also increases ribosome biogenesis and protein synthesis (Sanchez, Teixeira et al. 2016) stimulating also cell growth (Zhang, Stallock et al. 2000). However, stimulation of PI3K/Akt pathway by TGFβ family is not described in *Drosophila* models. In mammals, TGFβ family can also activate mTORC2, regulate cytoskeletal reorganization, RhoA activation and cell migration (Lamouille, Connolly et al. 2012). At a late stage of EMT, mTORC2 enhances Akt activation, acting as a positive feedback loop in the PI3K/Akt pathway (Lamouille, Connolly et al. 2012). Downregulation of PI3K/Akt pathway inhibits downregulation of E-Cadherin, α-smooth muscle Actin expression and other morphological, transcriptional, and migratory activities induced by TGFβ (Bakin, Tomlinson et al. 2000, Kattla, Carew et al. 2008). Furthermore, an inhibitor of mTOR activity, Rapamycin, decreases cell migration and invasion kinetics that are linked to TGFβ-induced EMT, however does not recue the phenotypic changes associated to TGFβ induced-EMT (Lamouille and Derynck 2007). The activation of Akt phosphorylates Twist1 which induces TGFβ receptor signaling, PI3K/Akt pathway activity and consequently EMT (Xue, Restuccia et al. 2012). Akt also induces the transcription of Snail, which is an important factor for EMT (Zhou, Deng et al. 2004, Julien, Puig et al. 2007). In human prostate cancer, PI3K-AKT signaling promotes cell migration through TRAF6-mediated ubiquitylation of p85α (Hamidi, Song et al. 2017) and this PI3K/Akt upregulation is associated with a poor prognosis (Wegiel, Bjartell et al. 2008). In fibroblast, PI3K is important for proliferation and morphological changes induced by TGFβ (Wilkes, Mitchell et al. 2005).

3.4.4. Rho family GTPases activation in TGFβ induced-EMT

Rho GTPases have been described as important factors in tumorigenesis, including in EMT, migration, invasion and metastasis (Ungefroren, Witte et al. 2017). A major regulator of RhoA activity, at the tight junctions of epithelial cells, is TGFβ (Ungefroren, Witte et al. 2017). In mammals, RhoA activity initiates with the binding of TGFβ type II

receptor to Par6 and TGFβ type I receptor complex (Figure 1.16 D). Then, TGFβ type II receptor phosphorylates Par6 at Ser345 (Ozdamar, Bose et al. 2005), which recruits the Smurf1, enhancing RhoA ubiquitination by this E3 ubiquitin ligase and degradation. The activation of RhoA-GTP in response to TGFβ also stimulates ROCK, which activate LIM kinase that blocks the Actin-depolymerizing factor Cofilin (Arber, Barbayannis et al. 1998, Yang, Higuchi et al. 1998). Thus, TGFβ seems to regulate RhoA in two different modes: 1) at early stages of TGFβ induced-EMT, induces a rapid activation of RhoA and 2) at later stages downregulates the levels of RhoA protein by proteasome degradation (Ozdamar, Bose et al. 2005). The activation of ROCK modulates Actin stress fibers-formation (Pellegrin and Mellor 2007). Nevertheless, the downregulation of LIM kinase inhibits the reorganization of TGFβ-induced Actin in fibroblasts (Vardouli, Moustakas et al. 2005). Additionally, downregulation of ROCK/ Rho activity in mammals leads to inhibition of the reorganization of TGFβ-induced Actin and the expression of α-smooth muscle Actin associated to EMT, without perturbing Smad activation (Bhowmick, Ghiassi et al. 2001, Cho and Yoo 2007). In *Drosophila* embryonic epidermis, RhoA also regulates the cytoskeleton and cell-cell adhesion, (Bloor and Kiehart 2002). Moreover, RhoA is also necessary for normal EMT during embryonic chick heart development and downregulation of TGFβ lead to a strong reduction of RhoA mRNA (Tavares, Mercado-Pimentel et al. 2006).

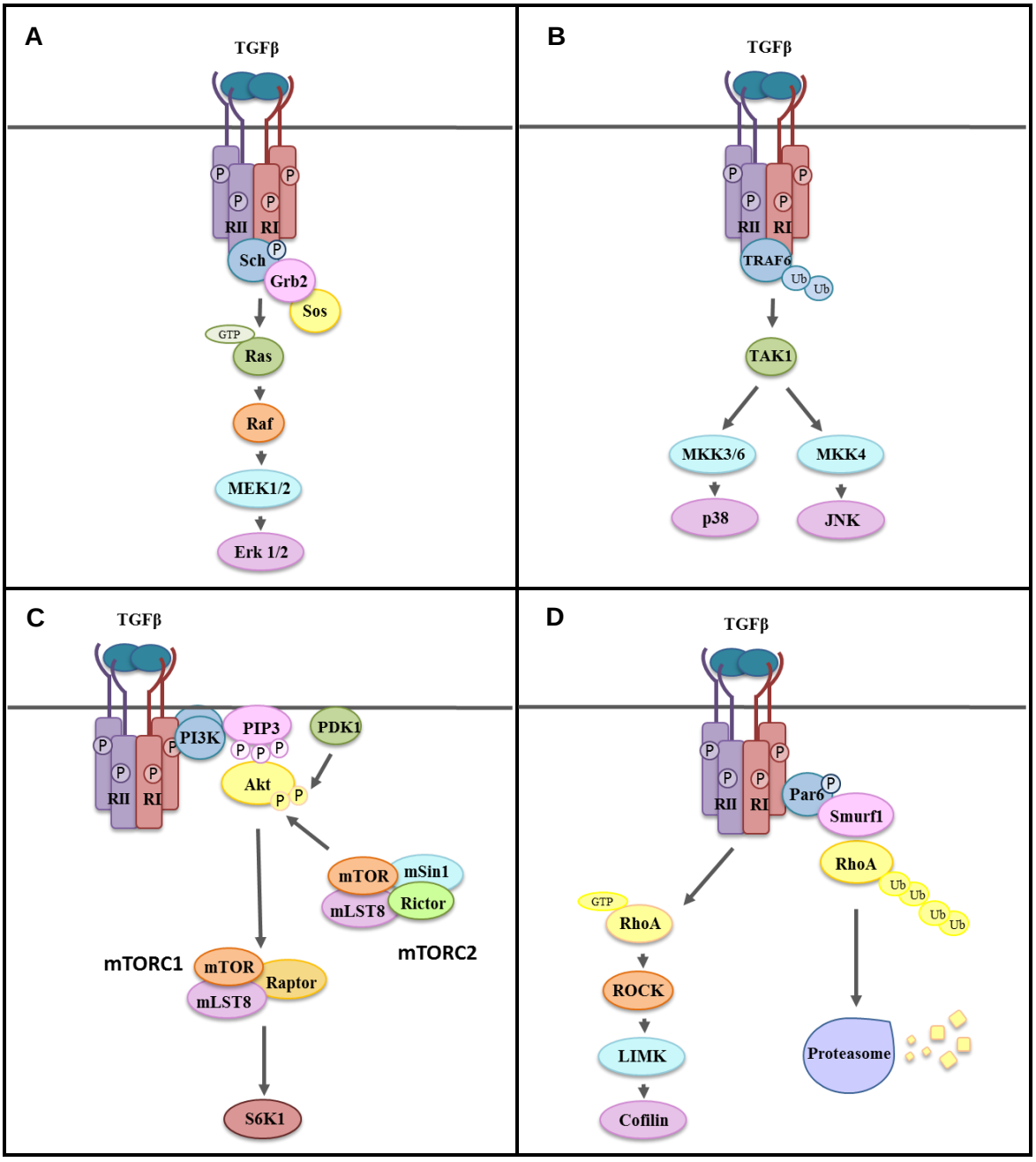

Figure 1.16.TGFβ-induced activation of non-Smad pathways in mammalian models. (A) Erk MAP kinase, (B) JNK and p38 MAPK, (C) PI3K/Akt and (D) Rho GTPases activation through TGFβ signaling. Arrows represent activation. For more details, see text. Adapted from Zhang 2017.

4. References

Affolter, M. and K. Basler (2007). "The Decapentaplegic morphogen gradient: from pattern formation to growth regulation." Nat Rev Genet **8**(9): 663-674.

Affolter, M., T. Marty, M. Vigano and A. Jaźwińska (2001). "NEW EMBO MEMBER'S REVIEW: Nuclear interpretation of Dpp signaling in *Drosophila*." The EMBO Journal **20**(13): 3298-3305.

Affolter, M., G. Pyrowolakis, A. Weiss and K. Basler (2008). "Signal-Induced Repression: The Exception or the Rule in Developmental Signaling?" Developmental Cell **15**(1): 11-22.

Agaisse, H., U.-M. Petersen, M. Boutros, B. Mathey-Prevot and N. Perrimon (2003). "Signaling Role of Hemocytes in *Drosophila* JAK/STAT-Dependent Response to Septic Injury." Developmental Cell **5**(3): 441-450.

Alcorn, J. F., A. S. Guala, J. v. d. Velden, B. McElhinney, C. G. Irvin, R. J. Davis and Y. M. W. Janssen-Heininger (2008). "Jun N-terminal kinase 1 regulates epithelial-to-mesenchymal transition induced by TGF-β1." Journal of cell science **121**(Pt 7): 1036-1045.

Aldaz, S. and L. M. Escudero (2010). "Imaginal discs." Current Biology **20**(10): R429-R431.

Andersen, J. S., Y. W. Lam, A. K. L. Leung, S.-E. Ong, C. E. Lyon, A. I. Lamond and M. Mann (2005). "Nucleolar proteome dynamics." Nature **433**(7021): 77-83.

Anderson, J., C. L. Salzer and J. P. Kumar (2006). "Regulation of the retinal determination gene *dachshund* in the embryonic head and developing eye of *Drosophila*." Developmental biology **297**(2): 536-549.

Arber, S., F. A. Barbayannis, H. Hanser, C. Schneider, C. A. Stanyon, O. Bernard and P. Caroni (1998). "Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase." Nature **393**: 805.

Arias, A. M. (2008). *Drosophila melanogaster* and the Development of Biology in the 20th Century. *Drosophila*: Methods and Protocols. C. Dahmann. Totowa, NJ, Humana Press**:** 1-25.

Atkins, M. and G. Mardon (2009). "Signaling in the Third Dimension: The Peripodial Epithelium in Eye Disc Development." Developmental dynamics : an official publication of the American Association of Anatomists **238**(9): 2139-2148.

Audas, Timothy E., Mathieu D. Jacob and S. Lee (2012). "Immobilization of Proteins in the Nucleolus by Ribosomal Intergenic Spacer Noncoding RNA." Molecular Cell **45**(2): 147-157.

Bach, E. A., S. Vincent, M. P. Zeidler and N. Perrimon (2003). "A Sensitized Genetic Screen to Identify Novel Regulators and Components of the *Drosophila* Janus Kinase/Signal Transducer and Activator of Transcription Pathway." Genetics **165**(3): 1149-1166.

Baker, N. E. (1988). "Embryonic and imaginal requirements for wingless, a segment-polarity gene in *Drosophila*." Developmental Biology **125**(1): 96-108.

Bakin, A. V., C. Rinehart, A. K. Tomlinson and C. L. Arteaga (2002). "p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration." Journal of Cell Science **115**(15): 3193-3206.

Bakin, A. V., A. K. Tomlinson, N. A. Bhowmick, H. L. Moses and C. L. Arteaga (2000). "Phosphatidylinositol 3-Kinase Function Is Required for Transforming Growth Factor β-mediated Epithelial to Mesenchymal Transition and Cell Migration." Journal of Biological Chemistry **275**(47): 36803-36810.

Ball, L. J., R. Kühne, J. Schneider-Mergener and H. Oschkinat (2005). "Recognition of Proline-Rich Motifs by Protein–Protein-Interaction Domains." Angewandte Chemie International Edition **44**(19): 2852-2869.

Barrio, L. and M. Milán (2017). "Boundary Dpp promotes growth of medial and lateral regions of the *Drosophila* wing." eLife **6**: e22013.

Basler, K. and G. Struhl (1994). "Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein." Nature **368**(6468): 208-214.

Beaucher, M., E. Hersperger, A. Page-McCaw and A. Shearn (2007). "Metastatic ability of *Drosophila* tumors depends on MMP activity." Developmental Biology **303**(2): 625-634.

Behrens, A., M. Sibilia and E. F. Wagner (1999). "Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation." Nature Genetics **21**: 326.

Benassayag, C., L. Montero, N. Colombié, P. Gallant, D. Cribbs and D. Morello (2005). "Human c-Myc Isoforms Differentially Regulate Cell Growth and Apoptosis in *Drosophila* melanogaster." Molecular and Cellular Biology **25**(22): 9897-9909.

Bessa, J., B. Gebelein, F. Pichaud, F. Casares and R. S. Mann (2002). "Combinatorial control of *Drosophila* eye development by Eyeless, Homothorax, and Teashirt." Genes & Development **16**(18): 2415-2427.

Bhowmick, N. A., M. Ghiassi, A. Bakin, M. Aakre, C. A. Lundquist, M. E. Engel, C. L. Arteaga and H. L. Moses (2001). "Transforming Growth Factor-β1 Mediates Epithelial to Mesenchymal Transdifferentiation through a RhoA-dependent Mechanism." Molecular Biology of the Cell **12**(1): 27-36.

Bloor, J. W. and D. P. Kiehart (2002). "*Drosophila* RhoA regulates the cytoskeleton and cell-cell adhesion in the developing epidermis." Development **129**(13): 3173-3183.

Boisvert, F.-M., S. van Koningsbruggen, J. Navascues and A. I. Lamond (2007). "The multifunctional nucleolus." Nat Rev Mol Cell Biol **8**(7): 574-585.

Bonini, N. M., Q. T. Bui, G. L. Gray-Board and J. M. Warrick (1997). "The Drosophila eyes absent gene directs ectopic eye formation in a pathway conserved between flies and vertebrates." Development **124**(23): 4819-4826.

Boon, K., H. N. Caron, R. van Asperen, L. Valentijn, M. C. Hermus, P. van Sluis, I. Roobeek, I. Weis, P. A. Voûte, M. Schwab and R. Versteeg (2001). N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis.

Bosch, P. S., R. Ziukaite, C. Alexandre, K. Basler and J.-P. Vincent (2017). "Dpp controls growth and patterning in *Drosophila* wing precursors through distinct modes of action." eLife **6**: e22546.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development **118**(2): 401-415.

Brangwynne, C. P., T. J. Mitchison and A. A. Hyman (2011). "Active liquid-like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes." Proceedings of the National Academy of Sciences of the United States of America **108**(11): 4334-4339.

Brás-Pereira, C., D. Potier, J. Jacobs, S. Aerts, F. Casares and F. Janody (2016). "*dachshund* Potentiates Hedgehog Signaling during *Drosophila* Retinogenesis." PLOS Genetics **12**(7): e1006204.

Bray, S. (1999). "DPP on the brinker." Trends Genet **15**(4): 140.

Bridges, C. B. (1935). "Salivary chromosome maps with a key to the banding of the chromosomes of *Drosophila melanogaster*." Journal of Heredity **26**: 60-64.

Brummel, T., S. Abdollah, T. E. Haerry, M. J. Shimell, J. Merriam, L. Raftery, J. L. Wrana and M. B. O'Connor (1999). "The *Drosophila* Activin receptor Baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development." Genes & Development **13**(1): 98-111.

Brunner, D., N. Oellers, J. Szabad, W. H. Biggs, III, S. L. Zipursky and E. Hafen (1994). "A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signaling pathways." Cell **76**(5): 875-888.

Brzozowa-Zasada, M., A. Piecuch, M. Michalski, O. Segiet, J. Kurek, M. Harabin-Słowińska and R. Wojnicz (2017). "Notch and its oncogenic activity in human malignancies." European Surgery **49**(5): 199-209.

Busson, D. and A.-M. Pret (2007). GAL4/UAS Targeted Gene Expression for Studying *Drosophila* Hedgehog Signaling. Hedgehog Signaling Protocols. J. I. Horabin. Totowa, NJ, Humana Press**:** 161-201.

Campbell, G. and A. Tomlinson (1999). "Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*." Cell **96**(4): 553-562.

Campos-Ortega, J. A. and V. Hartenstein (1997). The embryonic development of *Drosophila* melanogaster. 2nd ed.

Caygill, E. E. and A. H. Brand (2016). The GAL4 System: A Versatile System for the Manipulation and Analysis of Gene Expression. Drosophila: Methods and Protocols. C. Dahmann. New York, NY, Springer New York**:** 33-52.

Chanu, S. I. and S. Sarkar (2014). "Myc: Master Regulator of Global Genomic Expression." Austin J Genet Genomic Res **1**(2): 5.

Chao, J.-L., Y.-C. Tsai, S.-J. Chiu and Y. H. Sun (2004). "Localized Notch signal acts through *eyg* and *upd* to promote global growth in *Drosophila* eye." Development **131**(16): 3839-3847.

Chen, R., M. Amoui, Z. Zhang and G. Mardon (1997). "Dachshund and Eyes Absent Proteins Form a Complex and Function Synergistically to Induce Ectopic Eye Development in Drosophila." Cell **91**(7): 893-903.

Cheyette, B. N. R., P. J. Green, K. Martin, H. Garren, V. Hartenstein and S. L. Zipursky (1994). "The Drosophila *sine oculis* locus encodes a homeodomain-containing protein required for the development of the entire visual system." Neuron **12**(5): 977-996.

Cho, H. J. and J. Yoo (2007). "Rho activation is required for transforming growth factor-β-induced epithelial-mesenchymal transition in lens epithelial cells." Cell Biology International **31**(10): 1225-1230.

Cho, K.-O. and K.-W. Choi (1998). "Fringe is essential for mirror symmetry and morphogenesis in the *Drosophila* eye." Nature **396**: 272.

Coller, H. A., C. Grandori, P. Tamayo, T. Colbert, E. S. Lander, R. N. Eisenman and T. R. Golub (2000). "Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion." Proceedings of the National Academy of Sciences **97**(7): 3260-3265.

Couso, J. P. and A. M. Arias (1994). "*Notch* is required for *wingless* signaling in the epidermis of Drosophila." Cell **79**(2): 259-272.

Czerny, T., G. Halder, U. Kloter, A. Souabni, W. J. Gehring and M. Busslinger (1999). "*twin of eyeless*, a Second *Pax-6* Gene of *Drosophila*, Acts Upstream of *eyeless* in the Control of Eye Development." Molecular Cell **3**(3): 297-307.

Dahl, E., H. Koseki and R. Balling (1997). "Pax genes and organogenesis." BioEssays **19**(9): 755-765.

Datta, P. K. and H. L. Moses (2000). "STRAP and Smad7 Synergize in the Inhibition of Transforming Growth Factor β Signaling." Molecular and Cellular Biology **20**(9): 3157-3167.

Davis, A. C., M. Wims, G. D. Spotts, S. R. Hann and A. Bradley (1993). "A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice." Genes & Development **7**(4): 671-682.

Day, S. J. and P. A. Lawrence (2000). "Measuring dimensions: the regulation of size and shape." Development **127**(14): 2977.

de Celis, J. and A. García-Bellido (1994). "Roles of the Notch gene in *Drosophila* wing morphogenesis." Mechanisms of Development **46**(2): 109-122.

de la Cova, C. and L. A. Johnston (2006). "Myc in model organisms: A view from the flyroom." Seminars in Cancer Biology **16**(4): 303-312.

Dearborn, R. and S. Kunes (2004). "An axon scaffold induced by retinal axons directs glia to destinations in the *Drosophila* optic lobe." Development **131**(10): 2291.

Derynck, R. and Y. E. Zhang (2003). "Smad-dependent and Smad-independent pathways in TGF-β family signalling." Nature **425**: 577.

Deryugina, E. I. and J. P. Quigley (2006). "Matrix metalloproteinases and tumor metastasis." Cancer and Metastasis Reviews **25**(1): 9-34.

Dietzl, G., D. Chen, F. Schnorrer, K.-C. Su, Y. Barinova, M. Fellner, B. Gasser, K. Kinsey, S. Oppel, S. Scheiblauer, A. Couto, V. Marra, K. Keleman and B. J. Dickson (2007). "A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*." Nature **448**(7150): 151-156.

Dijke, P. t. and C. S. Hill (2004). "New insights into TGF-β–Smad signalling." Trends in Biochemical Sciences **29**(5): 265-273.

Domínguez, M. and F. Casares (2005). "Organ specification–growth control connection: New in-sights from the *Drosophila* eye–antennal disc." Developmental Dynamics **232**(3): 673-684.

Domínguez, M. and J. F. d. Celis (1998). "A dorsal/ventral boundary established by Notch controls growth and polarity in the *Drosophila* eye." Nature **396**: 276.

Dong, J., G. Feldmann, J. Huang, S. Wu, N. Zhang, S. A. Comerford, M. F. Gayyed, R. A. Anders, A. Maitra and D. Pan (2007). "Elucidation of a Universal Size-Control Mechanism in *Drosophila* and Mammals." Cell **130**(6): 1120-1133.

Doumpas, N., M. Ruiz-Romero, E. Blanco, B. Edgar, M. Corominas and A. A. Teleman (2013). Brk regulates wing disc growth in part via repression of Myc expression.

Duffy, J. B. (2002). "GAL4 system in *Drosophila*: A fly geneticist's swiss army knife." genesis **34**(1-2): 1-15.

Easton, R. M., H. Cho, K. Roovers, D. W. Shineman, M. Mizrahi, M. S. Forman, V. M. Y. Lee, M. Szabolcs, R. de Jong, T. Oltersdorf, T. Ludwig, A. Efstratiadis and M. J. Birnbaum (2005). "Role for Akt3/Protein Kinase Bγ in Attainment of Normal Brain Size." Molecular and Cellular Biology **25**(5): 1869-1878.

Ebisawa, T., M. Fukuchi, G. Murakami, T. Chiba, K. Tanaka, T. Imamura and K. Miyazono (2001). "Smurf1 Interacts with Transforming Growth Factor-β Type I Receptor through Smad7 and Induces Receptor Degradation." Journal of Biological Chemistry **276**(16): 12477-12480.

Edgar, B. A. (2006). "How flies get their size: genetics meets physiology." Nat Rev Genet **7**(12): 907-916.

Edgar, B. A. and T. L. Orr-Weaver (2001). "Endoreplication Cell Cycles: More for Less." Cell **105**(3): 297-306.

Edlund, S., S. Bu, N. Schuster, P. Aspenström, R. Heuchel, N.-E. Heldin, P. ten Dijke, C.-H. Heldin and M. Landström (2003). "Transforming Growth Factor-β1 (TGF-β)–induced Apoptosis of Prostate Cancer Cells Involves Smad7-dependent Activation of p38 by TGF-β-activated Kinase 1 and Mitogen-activated Protein Kinase Kinase 3." Molecular Biology of the Cell **14**(2): 529-544.

Eichhorn, P. J. A., L. Rodón, A. Gonzàlez-Juncà, A. Dirac, M. Gili, E. Martínez-Sáez, C. Aura, I. Barba, V. Peg, A. Prat, I. Cuartas, J. Jimenez, D. García-Dorado, J. Sahuquillo, R. Bernards, J. Baselga and J. Seoane (2012). "USP15 stabilizes TGF-β receptor I and promotes oncogenesis through the activation of TGF-β signaling in glioblastoma." Nature Medicine **18**: 429.

Ekas, L. A., G.-H. Baeg, M. S. Flaherty, A. Ayala-Camargo and E. A. Bach (2006). "JAK/STAT signaling promotes regional specification by negatively regulating *wingless* expression in *Drosophila*." Development **133**(23): 4721-4729.

Entchev, E. V., A. Schwabedissen and M. González-Gaitán (2000). "Gradient Formation of the TGF-β Homolog Dpp." Cell **103**(6): 981-992.

Ferguson, E. L. and K. V. Anderson (1992). "Localized enhancement and repression of the activity of the TGF-β family member, *decapentaplegic*, is necessary for dorsal-ventral pattern formation in the *Drosophila* embryo." Development **114**(3): 583-597.

Feric, M., N. Vaidya, T. S. Harmon, D. M. Mitrea, L. Zhu, T. M. Richardson, R. W. Kriwacki, R. V. Pappu and C. P. Brangwynne (2016). "Coexisting liquid phases underlie nucleolar sub-compartments." Cell **165**(7): 1686-1697.

Ferrigno, O., F. Lallemand, F. Verrecchia, S. L'Hoste, J. Camonis, A. Atfi and A. Mauviel (2002). "Yes-associated protein (YAP65) interacts with Smad7 and potentiates its inhibitory activity against TGF-β/Smad signaling." Oncogene **21**: 4879.

Fu, H., Z. Hu, J. Wen, K. Wang and Y. Liu (2009). "TGF-beta promotes invasion and metastasis of gastric cancer cells by increasing fascin1 expression via ERK and JNK signal pathways." Acta Biochim Biophys Sin (Shanghai) **41(8)**: 648-656.

Gallant, P. (2013). "Myc Function in *Drosophila*." Cold Spring Harbor Perspectives in Medicine **3**(10).

Gilbert, M. M., B. K. Weaver, J. P. Gergen and N. C. Reich (2005). "A novel functional activator of the *Drosophila* JAK/STAT pathway, unpaired2, is revealed by an in vivo reporter of pathway activation." Mechanisms of Development **122**(7): 939-948.

Glesne, D. and E. Huberman (2006). "Smad6 is a protein kinase X phosphorylation substrate and is required for HL-60 cell differentiation." Oncogene **25**: 4086.

Go, M. J., D. S. Eastman and S. Artavanis-Tsakonas (1998). "Cell proliferation control by Notch signaling in *Drosophila* development." Development **125**(11): 2031-2040.

Gomez-Roman, N., C. Grandori, R. N. Eisenman and R. J. White (2003). "Direct activation of RNA polymerase III transcription by c-Myc." Nature **421**(6920): 290-294.

Grände, M., Å. Franzen, J.-O. Karlsson, L. E. Ericson, N.-E. Heldin and M. Nilsson (2002). "Transforming growth factor-β and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes." Journal of Cell Science **115**(22): 4227-4236.

Greenwood, S. and G. Struhl (1999). "Progression of the morphogenetic furrow in the *Drosophila* eye: the roles of Hedgehog, Decapentaplegic and the Raf pathway." Development **126**(24): 5795-5808.

Grewal, S. S., J. R. Evans and B. A. Edgar (2007). "*Drosophila* TIF-IA is required for ribosome synthesis and cell growth and is regulated by the TOR pathway." The Journal of Cell Biology **179**(6): 1105-1113.

Grewal, S. S., L. Li, A. Orian, R. N. Eisenman and B. A. Edgar (2005). "Myc-dependent regulation of ribosomal RNA synthesis during *Drosophila* development." Nat Cell Biol **7**(3): 295-302.

Grönroos, E., U. Hellman, C.-H. Heldin and J. Ericsson (2002). "Control of Smad7 Stability by Competition between Acetylation and Ubiquitination." Molecular Cell **10**(3): 483-493.

Guo, Q. M., R. L. Malek, S. Kim, C. Chiao, M. He, M. Ruffy, K. Sanka, N. H. Lee, C. V. Dang and E. T. Liu (2000). "Identification of c-Myc Responsive Genes Using Rat cDNA Microarray." Cancer Research **60**(21): 5922-5928.

Halder, G., P. Callaerts, S. Flister, U. Walldorf, U. Kloter and W. J. Gehring (1998). "Eyeless initiates the expression of both sine oculis and eyes absent during *Drosophila* compound eye development." Development **125**(12): 2181-2191.

Hamaratoglu, F., M. Affolter and G. Pyrowolakis (2014). "Dpp/BMP signaling in flies: From molecules to biology." Seminars in Cell & Developmental Biology 32: 128-136.

Hamidi, A., J. Song, N. Thakur, S. Itoh, A. Marcusson, A. Bergh, C.-H. Heldin and M. Landström (2017). "TGF-β promotes PI3K-AKT signaling and prostate cancer cell migration through the TRAF6-mediated ubiquitylation of p85α." Science Signaling **10**(486).

Hanratty, W. P. and C. R. Dearolf (1993). "The *Drosophila* Tumorous lethal hematopoietic oncogene is a dominant mutation in the hopscotch locus." Molecular and General Genetics MGG **238**(1): 33-37.

Hanyu, A., Y. Ishidou, T. Ebisawa, T. Shimanuki, T. Imamura and K. Miyazono (2001). "The N domain of Smad7 is essential for specific inhibition of transforming growth factor-β signaling." The Journal of Cell Biology **155**(6): 1017-1028.

Harrison, D. A., R. Binari, T. S. Nahreini, M. Gilman and N. Perrimon (1995). "Activation of a *Drosophila* Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects." The EMBO Journal **14**(12): 2857-2865.

Harrison, D. A., P. E. McCoon, R. Binari, M. Gilman and N. Perrimon (1998). "*Drosophila* unpaired encodes a secreted protein that activates the JAK signaling pathway." Genes & Development **12**(20): 3252-3263.

Hata, A., G. Lagna, J. Massagué and A. Hemmati-Brivanlou (1998). "Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor." Genes & Development **12**(2): 186-197.

Hayashi, H., S. Abdollah, Y. Qiu, J. Cai, Y.-Y. Xu, B. W. Grinnell, M. A. Richardson, J. N. Topper, M. A. Gimbrone, Jr., J. L. Wrana and D. Falb (1997). "The MAD-Related

Protein Smad7 Associates with the TGFβ Receptor and Functions as an Antagonist of TGFβ Signaling." Cell **89**(7): 1165-1173.

Haynie, J. L. and P. J. Bryant (1986). "Development of the eye-antenna imaginal disc and morphogenesis of the adult head in *Drosophila melanogaster*." Journal of Experimental Zoology **237**(3): 293-308.

Hazelett, D. J., M. Bourouis, U. Walldorf and J. E. Treisman (1998). "decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc." Development **125**(18): 3741-3751.

Heberlein, U., C. M. Singh, A. Y. Luk and T. J. Donohoe (1995). "Growth and differentiation in the *Drosophila* eye coordinated by *hedgehog*." Nature **373**(6516): 709-711.

Heberlein, U., T. Wolff and G. M. Rubin (1993). "The TGFβ homolog *dpp* and the segment polarity gene *hedgehog* are required for propagation of a morphogenetic wave in the *Drosophila* retina." Cell **75**(5): 913-926.

Henderson, K. D. and D. J. Andrew (2000). "Regulation and Function of Scr, exd, and hth in the *Drosophila* Salivary Gland." Developmental Biology **217**(2): 362-374.

Henderson, K. D., D. D. Isaac and D. J. Andrew (1999). "Cell Fate Specification in the *Drosophila* Salivary Gland: The Integration of Homeotic Gene Function with the DPP Signaling Cascade." Developmental Biology **205**(1): 10-21.

Hogan, B. L. M. and P. A. Kolodziej (2002). "Molecular Mechanisms Of Tubulogenesis." Nat Rev Genet **3**(7): 513-523.

Hombría, J. C.-G., S. Brown, S. Häder and M. P. Zeidler (2005). "Characterisation of Upd2, a *Drosophila* JAK/STAT pathway ligand." Developmental Biology **288**(2): 420-433.

Huang, C., Z. Rajfur, C. Borchers, M. D. Schaller and K. Jacobson (2003). "JNK phosphorylates paxillin and regulates cell migration." Nature **424**: 219.

Huang, D., X. Li, L. Sun, P. Huang, H. Ying, H. Wang, J. Wu and H. Song (2016). "Regulation of Hippo signalling by p38 signalling." Journal of Molecular Cell Biology **8**(4): 328-337.

Huang, J., S. Wu, J. Barrera, K. Matthews and D. Pan (2005). "The Hippo Signaling Pathway Coordinately Regulates Cell Proliferation and Apoptosis by Inactivating Yorkie, the *Drosophila* Homolog of YAP." Cell **122**(3): 421-434.

Huminiecki, L., L. Goldovsky, S. Freilich, A. Moustakas, C. Ouzounis and C.-H. Heldin (2009). "Emergence, development and diversification of the TGF-β signalling pathway within the animal kingdom." BMC Evolutionary Biology **9**(1): 28.

Igaki, T., R. A. Pagliarini and T. Xu (2006). "Loss of Cell Polarity Drives Tumor Growth and Invasion through JNK Activation in *Drosophila*." Current Biology **16**(11): 1139-1146.

Inamoto, S., S. Iwata, T. Inamoto, S. Nomura, T. Sasaki, Y. Urasaki, O. Hosono, H. Kawasaki, H. Tanaka, N. H. Dang and C. Morimoto (2006). "Crk-associated substrate lymphocyte type regulates transforming growth factor-β signaling by inhibiting Smad6 and Smad7." Oncogene **26**: 893.

Irvine, K. D. and K. F. Harvey (2015). "Control of Organ Growth by Patterning and Hippo Signalling in *Drosophila*." Cold Spring Harbor perspectives in biology **7**(6): a019224.

Isaac, D. D. and D. J. Andrew (1996). "Tubulogenesis in *Drosophila*: a requirement for the trachealess gene product." Genes & Development **10**(1): 103-117.

Itoh, S., M. Landström, A. Hermansson, F. Itoh, C.-H. Heldin, N.-E. Heldin and P. ten Dijke (1998). "Transforming Growth Factor β1 Induces Nuclear Export of Inhibitory Smad7." Journal of Biological Chemistry **273**(44): 29195-29201.

Jasper, H., V. Benes, C. Schwager, S. Sauer, S. Clauder-Münster, W. Ansorge and D. Bohmann (2001). "The Genomic Response of the *Drosophila* Embryo to JNK Signaling." Developmental Cell **1**(4): 579-586.

Jaźwińska, A., N. Kirov, E. Wieschaus, S. Roth and C. Rushlow (1999). "The *Drosophila* Gene *brinker* Reveals a Novel Mechanism of Dpp Target Gene Regulation." Cell **96**(4): 563-573.

Jemc, J. and I. Rebay (2007). "Identification of Transcriptional Targets of the Dual Function Transcription Factor/Phosphatase Eyes Absent." Developmental biology **310**(2): 416-429.

Jiang, Y., K. L. Scott, S.-J. Kwak, R. Chen and G. Mardon (2011). "Sds22/PP1 Links Epithelial Integrity and Tumor Suppression via Regulation of Myosin II and JNK Signaling." Oncogene **30**(29): 3248-3260.

Johnston, L. A., D. A. Prober, B. A. Edgar, R. N. Eisenman and P. Gallant (1999). "*Drosophila myc* Regulates Cellular Growth during Development." Cell **98**(6): 779-790.

Julien, S., I. Puig, E. Caretti, J. Bonaventure, L. Nelles, F. van Roy, C. Dargemont, A. G. de Herreros, A. Bellacosa and L. Larue (2007). "Activation of NF-κB by Akt upregulates Snail expression and induces epithelium mesenchyme transition." Oncogene **26**: 7445.

Jung, S. M., J.-H. Lee, J. Park, Y. S. Oh, S. K. Lee, J. S. Park, Y. S. Lee, J. H. Kim, J. Y. Lee, Y.-S. Bae, S.-H. Koo, S.-J. Kim and S. H. Park (2013). "Smad6 inhibits non-

canonical TGF-β1 signalling by recruiting the deubiquitinase A20 to TRAF6." Nature Communications **4**: 2562.

Jurgens G and H. V. (1993). "The terminal regions of the body pattern." Cold Spring Harbor, NY **Cold Spring Harbor Laboratory Press.** : 687-746.

Kallio, J., H. Myllymäki, J. Grönholm, M. Armstrong, L.-M. Vanha-aho, L. Mäkinen, O. Silvennoinen, S. Valanne and M. Rämet (2010). "Eye transformer is a negative regulator of *Drosophila* JAK/STAT signaling." The FASEB Journal **24**(11): 4467-4479.

Kaltschmidt, J. A., N. Lawrence, V. Morel, T. Balayo, B. G. Fernández, A. Pelissier, A. Jacinto and A. Martinez Arias (2002). "Planar polarity and actin dynamics in the epidermis of *Drosophila*." Nature Cell Biology **4**: 937.

Kamiya, Y., K. Miyazono and K. Miyazawa (2008). "Specificity of the inhibitory effects of Dad on TGF-β family type I receptors, Thickveins, Saxophone, and Baboon in *Drosophila*." FEBS Letters **582**(17): 2496-2500.

Kattla, J. J., R. M. Carew, M. Heljić, C. Godson and D. P. Brazil (2008). "Protein kinase B/Akt activity is involved in renal TGF-β1-driven epithelial-mesenchymal transition in vitro and in vivo." American Journal of Physiology-Renal Physiology **295**(1): F215-F225.

Kavsak, P., R. K. Rasmussen, C. G. Causing, S. Bonni, H. Zhu, G. H. Thomsen and J. L. Wrana (2000). "Smad7 Binds to Smurf2 to Form an E3 Ubiquitin Ligase that Targets the TGFβ Receptor for Degradation." Molecular Cell **6**(6): 1365-1375.

Kenyon, K. L., S. S. Ranade, J. Curtiss, M. Mlodzik and F. Pignoni (2003). "Coordinating Proliferation and Tissue Specification to Promote Regional Identity in the *Drosophila* Head." Developmental Cell **5**(3): 403-414.

Kim, B.-C., H.-J. Lee, S. H. Park, S. R. Lee, T. S. Karpova, J. G. McNally, A. Felici, D. K. Lee and S.-J. Kim (2004). "Jab1/CSN5, a Component of the COP9 Signalosome, Regulates Transforming Growth Factor β Signaling by Binding to Smad7 and Promoting Its Degradation." Molecular and Cellular Biology **24**(6): 2251-2262.

Kimura, N., R. Matsuo, H. Shibuya, K. Nakashima and T. Taga (2000). "BMP2-induced Apoptosis Is Mediated by Activation of the TAK1-p38 Kinase Pathway That Is Negatively Regulated by Smad6." Journal of Biological Chemistry **275**(23): 17647-17652.

Koinuma, D., M. Shinozaki, A. Komuro, K. Goto, M. Saitoh, A. Hanyu, M. Ebina, T. Nukiwa, K. Miyazawa, T. Imamura and K. Miyazono (2003). "Arkadia amplifies TGF-β superfamily signalling through degradation of Smad7." The EMBO Journal **22**(24): 6458-6470.

Kolch, W. (2000). "Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions." Biochemical Journal **351**(Pt 2): 289-305.

Kopan, R. and M. X. G. Ilagan (2009). "The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism." Cell **137**(2): 216-233.

Kowanetz, M., P. Lönn, M. Vanlandewijck, K. Kowanetz, C.-H. Heldin and A. Moustakas (2008). "TGFβ induces SIK to negatively regulate type I receptor kinase signaling." The Journal of Cell Biology **182**(4): 655-662.

Koyama, T., C. C. Mendes and C. K. Mirth (2013). "Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects." Frontiers in Physiology **4**: 263.

Kronhamn, J., E. Frei, M. Daube, R. Jiao, Y. Shi, M. Noll and Å. Rasmuson-Lestander (2002). "Headless flies produced by mutations in the paralogous *Pax6* genes *eyeless* and *twin of eyeless*." Development **129**(4): 1015-1026.

Kumar, J. P. (2009). "The Molecular Circuitry Governing Retinal Determination." Biochimica et biophysica acta **1789**(4): 306-314.

Kume, S., M. Haneda, K. Kanasaki, T. Sugimoto, S.-i. Araki, K. Isshiki, M. Isono, T. Uzu, L. Guarente, A. Kashiwagi and D. Koya (2007). "SIRT1 Inhibits Transforming Growth Factor β-Induced Apoptosis in Glomerular Mesangial Cells via Smad7 Deacetylation." Journal of Biological Chemistry **282**(1): 151-158.

Lafontaine, D. L. J. and D. Tollervey (2001). "The function and synthesis of ribosomes." Nat Rev Mol Cell Biol **2**(7): 514-520.

Lai, Z.-C., X. Wei, T. Shimizu, E. Ramos, M. Rohrbaugh, N. Nikolaidis, L.-L. Ho and Y. Li (2005). "Control of Cell Proliferation and Apoptosis by Mob as Tumor Suppressor, Mats." Cell **120**(5): 675-685.

Lallemand, F., S. R. Seo, N. Ferrand, M. Pessah, S. L'Hoste, G. Rawadi, S. Roman-Roman, J. Camonis and A. Atfi (2005). "AIP4 Restricts Transforming Growth Factor-β Signaling through a Ubiquitination-independent Mechanism." Journal of Biological Chemistry **280**(30): 27645-27653.

Lamouille, S., E. Connolly, J. W. Smyth, R. J. Akhurst and R. Derynck (2012). "TGF-β-induced activation of mTOR complex 2 drives epithelial–mesenchymal transition and cell invasion." Journal of Cell Science **125**(5): 1259-1273.

Lamouille, S. and R. Derynck (2007). "Cell size and invasion in TGF-β–induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway." The Journal of Cell Biology **178**(3): 437-451.

Langer, J. A., E. C. Cutrone and S. Kotenko (2004). "The Class II cytokine receptor (CRF2) family: overview and patterns of receptor–ligand interactions." Cytokine & Growth Factor Reviews **15**(1): 33-48.

Lecuit, T., W. J. Brook, M. Ng, M. Calleja, H. Sun and S. M. Cohen (1996). "Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing." Nature **381**(6581): 387-393.

Lee, M. K., C. Pardoux, M. C. Hall, P. S. Lee, D. Warburton, J. Qing, S. M. Smith and R. Derynck (2007). "TGF-β activates Erk MAP kinase signalling through direct phosphorylation of ShcA." The EMBO Journal **26**(17): 3957-3967.

Lehmann, K., E. Janda, C. E. Pierreux, M. Rytömaa, A. Schulze, M. McMahon, C. S. Hill, H. Beug and J. Downward (2000). "Raf induces TGFβ production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells." Genes & Development **14**(20): 2610-2622.

Li, W., W. Li, L. Zou, S. Ji, C. Li, K. Liu, G. Zhang, Q. Sun, F. Xiao and D. Chen (2017). "Membrane targeting of inhibitory Smads through palmitoylation controls TGF-β/BMP signaling." Proceedings of the National Academy of Sciences **114**(50): 13206–13211.

Li, X., V. Perissi, F. Liu, D. W. Rose and M. G. Rosenfeld (2002). "Tissue-Specific Regulation of Retinal and Pituitary Precursor Cell Proliferation." Science **297**(5584): 1180-1183.

Lin, X., Y.-Y. Liang, B. Sun, M. Liang, Y. Shi, F. C. Brunicardi, Y. Shi and X.-H. Feng (2003). "Smad6 Recruits Transcription Corepressor CtBP To Repress Bone Morphogenetic Protein-Induced Transcription." Molecular and Cellular Biology **23**(24): 9081-9093.

Lo, S. J., C.-C. Lee and H.-J. Lai (2006). "The nucleolus: reviewing oldies to have new understandings." Cell Res **16**(6): 530-538.

Lönn, P., M. Vanlandewijck, E. Raja, M. Kowanetz, Y. Watanabe, K. Kowanetz, E. Vasilaki, C.-H. Heldin and A. Moustakas (2012). "Transcriptional Induction of Salt-inducible Kinase 1 by Transforming Growth Factor β Leads to Negative Regulation of Type I Receptor Signaling in Cooperation with the Smurf2 Ubiquitin Ligase." The Journal of Biological Chemistry **287**(16): 12867-12878.

Lopes, C. S. and F. Casares (2010). "*hth* maintains the pool of eye progenitors and its downregulation by Dpp and Hh couples retinal fate acquisition with cell cycle exit." Developmental Biology **339**(1): 78-88.

Luo, H., W. P. Hanratty and C. R. Dearolf (1995). "An amino acid substitution in the *Drosophila* hopTum-l Jak kinase causes leukemia-like hematopoietic defects." The EMBO Journal **14**(7): 1412-1420.

Makki, R., M. Meister, D. Pennetier, J.-M. Ubeda, A. Braun, V. Daburon, J. Krzemień, H.-M. Bourbon, R. Zhou, A. Vincent and M. Crozatier (2010). "A Short Receptor Downregulates JAK/STAT Signalling to Control the *Drosophila* Cellular Immune Response." PLoS Biology **8**(8): e1000441.

Mardon, G., N. M. Solomon and G. M. Rubin (1994). "dachshund encodes a nuclear protein required for normal eye and leg development in *Drosophila*." Development **120**(12): 3473-3486.

Marinho, J., F. Casares and P. S. Pereira (2011). "The *Drosophila Nol12* homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth." Development **138**(2): 349-357.

Marinho, J., T. Martins, M. Neto, F. Casares and P. S. Pereira (2013). "The nucleolar protein Viriato/Nol12 is required for the growth and differentiation progression activities of the Dpp pathway during *Drosophila* eye development." Dev Biol **377**(1): 154-165.

Martín-Blanco, E., J. C. Pastor-Pareja and A. García-Bellido (2000). "JNK and decapentaplegic signaling control adhesiveness and cytoskeleton dynamics during thorax closure in *Drosophila*." Proceedings of the National Academy of Sciences **97**(14): 7888-7893.

Massagué, J. (1999). "Wounding Smad." Nature Cell Biology **1**: E117.

Matsuda, S. and M. Affolter (2017). "Dpp from the anterior stripe of cells is crucial for the growth of the *Drosophila* wing disc." eLife **6**: e22319.

Mazars, A., F. Lallemand, C. Prunier, J. Marais, N. Ferrand, M. Pessah, G. Cherqui and A. Atfi (2001). "Evidence for a Role of the JNK Cascade in Smad7-mediated Apoptosis." Journal of Biological Chemistry **276**(39): 36797-36803.

McClure, K. D. and G. Schubiger (2005). "Developmental analysis and squamous morphogenesis of the peripodial epithelium in *Drosophila* imaginal discs." Development **132**(22): 5033-5042.

McStay, B. (2016). "Nucleolar organizer regions: genomic 'dark matter' requiring illumination." Genes & Development **30**(14): 1598-1610.

Menssen, A. and H. Hermeking (2002). "Characterization of the c-MYC-regulated transcriptome by SAGE: Identification and analysis of c-MYC target genes." Proceedings of the National Academy of Sciences **99**(9): 6274-6279.

Miyazawa, K. and K. Miyazono (2016). "Regulation of TGF-β Family Signaling by Inhibitory Smads." Cold Spring Harbor Perspectives in Biology.

Mochizuki, T., H. Miyazaki, T. Hara, T. Furuya, T. Imamura, T. Watabe and K. Miyazono (2004). "Roles for the MH2 Domain of Smad7 in the Specific Inhibition of

Transforming Growth Factor-β Superfamily Signaling." Journal of Biological Chemistry **279**(30): 31568-31574.

Montanaro, L., D. Treré and M. Derenzini (2008). "Nucleolus, Ribosomes, and Cancer." The American Journal of Pathology **173**(2): 301-310.

Morrison, D. K. and R. E. Cutler (1997). "The complexity of Raf-1 regulation." Current Opinion in Cell Biology **9**(2): 174-179.

Moustakas, A. and C.-H. Heldin (2012). "Induction of epithelial–mesenchymal transition by transforming growth factor β." Seminars in Cancer Biology **22**(5): 446-454.

Mu, Y., R. Sundar, N. Thakur, M. Ekman, S. K. Gudey, M. Yakymovych, A. Hermansson, H. Dimitriou, M. T. Bengoechea-Alonso, J. Ericsson, C.-H. Heldin and M. Landström (2011). "TRAF6 ubiquitinates TGFβ type I receptor to promote its cleavage and nuclear translocation in cancer." Nature Communications **2**: 330.

Mukherjee, T., J. C.-G. Hombría and M. P. Zeidler (2005). "Opposing roles for *Drosophila* JAK/STAT signalling during cellular proliferation." Oncogene **24**: 2503.

Mukherjee, T., U. Schäfer and M. P. Zeidler (2006). "Identification of *Drosophila* Genes Modulating Janus Kinase/Signal Transducer and Activator of Transcription Signal Transduction." Genetics **172**(3): 1683-1697.

Murakami, G., T. Watabe, K. Takaoka, K. Miyazono and T. Imamura (2003). "Cooperative Inhibition of Bone Morphogenetic Protein Signaling by Smurf1 and Inhibitory Smads." Molecular Biology of the Cell **14**(7): 2809-2817.

Myat, M. M. (2005). "Making tubes in the *Drosophila* embryo." Developmental Dynamics **232**(3): 617-632.

Nakao, A., M. Afrakhte, A. Morn, T. Nakayama, J. L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N.-E. Heldin, C.-H. Heldin and P. t. Dijke (1997). "Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling." Nature **389**: 631.

Nellen, D., R. Burke, G. Struhl and K. Basler (1996). "Direct and Long-Range Action of a DPP Morphogen Gradient." Cell **85**(3): 357-368.

Neto, M., D. Aguilar-Hidalgo and F. Casares (2016). "Increased avidity for Dpp/BMP2 maintains the proliferation of progenitors-like cells in the *Drosophila* eye." Developmental Biology **418**(1): 98-107.

Ng, J. and L. Luo (2004). "Rho GTPases Regulate Axon Growth through Convergent and Divergent Signaling Pathways." Neuron **44**(5): 779-793.

Niimi, T., M. Seimiya, U. Kloter, S. Flister and W. J. Gehring (1999). "Direct regulatory interaction of the eyeless protein with an eye-specific enhancer in the sine oculis gene during eye induction in *Drosophila*." Development **126**(10): 2253-2260.

Nijhout, H. F., L. M. Riddiford, C. Mirth, A. W. Shingleton, Y. Suzuki and V. Callier (2014). "The Developmental Control of Size in Insects." Wiley interdisciplinary reviews. Developmental biology **3**(1): 113-134.

Nolo, R., C. M. Morrison, C. Tao, X. Zhang and G. Halder (2006). "The *bantam* MicroRNA Is a Target of the Hippo Tumor-Suppressor Pathway." Current Biology **16**(19): 1895-1904.

Ogunjimi, A. A., D. J. Briant, N. Pece-Barbara, C. Le Roy, G. M. Di Guglielmo, P. Kavsak, R. K. Rasmussen, B. T. Seet, F. Sicheri and J. L. Wrana (2005). "Regulation of Smurf2 Ubiquitin Ligase Activity by Anchoring the E2 to the HECT Domain." Molecular Cell **19**(3): 297-308.

Oh, H. and K. D. Irvine (2008). "in vivo regulation of Yorkie phosphorylation and localization." Development (Cambridge, England) **135**(6): 1081-1088.

Ohashi, K., K. Nagata, M. Maekawa, T. Ishizaki, S. Narumiya and K. Mizuno (2000). "Rho-associated Kinase ROCK Activates LIM-kinase 1 by Phosphorylation at Threonine 508 within the Activation Loop." Journal of Biological Chemistry **275**(5): 3577-3582.

Orihara-Ono, M., E. Suzuki, M. Saito, Y. Yoda, T. Aigaki and C. Hama (2005). "The slender lobes gene, identified by retarded mushroom body development, is required for proper nucleolar organization in *Drosophila*." Developmental Biology **281**(1): 121-133.

Ostrin, E. J., Y. Li, K. Hoffman, J. Liu, K. Wang, L. Zhang, G. Mardon and R. Chen (2006). "Genome-wide identification of direct targetsof the *Drosophila* retinal determinationprotein Eyeless." Genome Research **16**(4): 466-476.

Ozdamar, B., R. Bose, M. Barrios-Rodiles, H.-R. Wang, Y. Zhang and J. L. Wrana (2005). "Regulation of the Polarity Protein Par6 by TGFß Receptors Controls Epithelial Cell Plasticity." Science **307**(5715): 1603-1609.

Padgett, R. W., R. D. St. Johnston and W. M. Gelbart (1987). "A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-*β* family." Nature **325**(6099): 81-84.

Pagliarini, R. A. and T. Xu (2003). "A Genetic Screen in *Drosophila* for Metastatic Behavior." Science **302**(5648): 1227-1231.

Pallavi, S. K. and L. S. Shashidhara (2005). "Signaling interactions between squamous and columnar epithelia of the *Drosophila* wing disc." Journal of Cell Science **118**(15): 3363-3370.

Panzer, S., D. Weigel and S. K. Beckendorf (1992). "Organogenesis in *Drosophila melanogaster*: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes." Development **114**(1): 49-57.

Papayannopoulos, V., A. Tomlinson, V. M. Panin, C. Rauskolb and K. D. Irvine (1998). "Dorsal-Ventral Signaling in the *Drosophila* Eye." Science **281**(5385): 2031-2034.

Pappu, K. and G. Mardon (2002). "Retinal specification and determination in *Drosophila*." Berlin, Germany: Springer-Verlag **In *Drosophila* Eye Development (ed. K. Moses)**: 5-17.

Pappu, K. S., E. J. Ostrin, B. W. Middlebrooks, B. T. Sili, R. Chen, M. R. Atkins, R. Gibbs and G. Mardon (2005). "Dual regulation and redundant function of two eye-specific enhancers of the *Drosophila* retinal determination gene *dachshund*." Development **132**(12): 2895-2905.

Park, S.-h., E.-H. Jung, G.-Y. Kim, B.-C. Kim, J. H. Lim and C.-H. Woo (2015). "Itch E3 Ubiquitin Ligase Positively Regulates TGF-β Signaling to EMT via Smad7 Ubiquitination." Molecules and Cells **38**(1): 20-25.

Parker, L., D. G. Stathakis and K. Arora (2004). Regulation of BMP and Activin Signaling in *Drosophila*. Invertebrate Cytokines and the Phylogeny of Immunity. A. Beschin and W. G. Müller, Springer Berlin Heidelberg. **34:** 73-101.

Pellegrin, S. and H. Mellor (2007). "Actin stress fibres." Journal of Cell Science **120**(20): 3491-3499.

Pereira, P. S., S. Pinho, K. Johnson, J. P. Couso and F. Casares (2006). "A 3′ cis-regulatory region controls wingless expression in the *Drosophila* eye and leg primordia." Developmental Dynamics **235**(1): 225-234.

Perrimon, N. and A. P. Mahowald (1986). "l(1)hopscotch, a larval-pupal zygotic lethal with a specific maternal effect on segmentation in *Drosophila*." Developmental Biology **118**(1): 28-41.

Peterson, A. J., P. A. Jensen, M. Shimell, R. Stefancsik, R. Wijayatonge, R. Herder, L. A. Raftery and M. B. O'Connor (2012). "R-Smad Competition Controls Activin Receptor Output in *Drosophila*." PLoS ONE **7**(5): e36548.

Peterson, A. J. and M. B. O'Connor (2013). "Activin receptor inhibition by Smad2 regulates *Drosophila* wing disc patterning through BMP-response elements." Development **140**(3): 649-659.

Pierce, S. B., C. Yost, J. S. Britton, L. W. M. Loo, E. M. Flynn, B. A. Edgar and R. N. Eisenman (2004). "dMyc is required for larval growth and endoreplication in *Drosophila*." Development **131**(10): 2317-2327.

Pignoni, F., B. Hu, K. H. Zavitz, J. Xiao, P. A. Garrity and S. L. Zipursky (1997). "The Eye-Specification Proteins So and Eya Form a Complex and Regulate Multiple Steps in *Drosophila* Eye Development." Cell **91**(7): 881-891.

Podos, S. D. and E. L. Ferguson (1999). "Morphogen gradients: new insights from DPP." Trends in Genetics **15**(10): 396-402.

Prasanth, Kannanganattu V. (2012). "Policing Cells under Stress: Noncoding RNAs Capture Proteins in Nucleolar Detention Centers." Molecular Cell **45**(2): 141-142.

Pulaski, L., M. Landström, C.-H. Heldin and S. Souchelnytskyi (2001). "Phosphorylation of Smad7 at Ser-249 Does Not Interfere with Its Inhibitory Role in Transforming Growth Factor-β-dependent Signaling but Affects Smad7-dependent Transcriptional Activation." Journal of Biological Chemistry **276**(17): 14344-14349.

Punzo, C., S. Plaza, M. Seimiya, P. Schnupf, S. Kurata, J. Jaeger and W. J. Gehring (2004). "Functional divergence between *eyeless* and *twin of eyeless* in *Drosophila melanogaster*." Development **131**(16): 3943-3953.

Quiring, R., U. Walldorf, U. Kloter and W. Gehring (1994). "Homology of the eyeless gene of *Drosophila* to the Small eye gene in mice and Aniridia in humans." Science **265**(5173): 785-789.

Raftery, L. A. and D. J. Sutherland (2003). "Gradients and thresholds: BMP response gradients unveiled in *Drosophila* embryos." Trends in Genetics **19**(12): 701-708.

Rahimi, R. A. and E. B. Leof (2007). "TGF-β signaling: A tale of two responses." Journal of Cellular Biochemistry **102**(3): 593-608.

Raska, I., K. Koberna, J. Malínský, H. Fidlerová and M. Masata (2004). "The nucleolus and transcription of ribosomal genes." Biology of the Cell **96**: 579–594.

Ready, D. F., T. E. Hanson and S. Benzer (1976). "Development of the *Drosophila* retina, a neurocrystalline lattice." Developmental Biology **53**(2): 217-240.

Reed, B. H., R. Wilk and H. D. Lipshitz (2001). "Downregulation of Jun kinase signaling in the amnioserosa is essential for dorsal closure of the *Drosophila* embryo." Current Biology **11**(14): 1098-1108.

Reiter, L. T., L. Potocki, S. Chien, M. Gribskov and E. Bier (2001). "A Systematic Analysis of Human Disease-Associated Gene Sequences In *Drosophila* melanogaster." Genome Research **11**(6): 1114-1125.

Ren, F., B. Wang, T. Yue, E.-Y. Yun, Y. T. Ip and J. Jiang (2010). "Hippo signaling regulates *Drosophila* intestine stem cell proliferation through multiple pathways." Proceedings of the National Academy of Sciences of the United States of America **107**(49): 21064-21069.

Ren, F., L. Zhang and J. Jiang (2010). "Hippo signaling regulates Yorkie nuclear localization and activity through 14-3-3 dependent and independent mechanisms." Developmental biology **337**(2): 303-312.

Restrepo, S., Jeremiah J. Zartman and K. Basler (2014). "Coordination of Patterning and Growth by the Morphogen DPP." Current Biology **24**(6): R245-R255.

Reynolds-Kenneally, J. and M. Mlodzik (2005). "Notch signaling controls proliferation through cell-autonomous and non-autonomous mechanisms in the *Drosophila* eye." Developmental Biology **285**(1): 38-48.

Rodrigues, A. B., T. Zoranovic, A. Ayala-Camargo, S. Grewal, T. Reyes-Robles, M. Krasny, D. C. Wu, L. A. Johnston and E. A. Bach (2012). "Activated STAT regulates growth and induces competitive interactions independently of Myc, Yorkie, Wingless and ribosome biogenesis." Development (Cambridge, England) **139**(21): 4051-4061.

Rogers, E. M., C. A. Brennan, N. T. Mortimer, S. Cook, A. R. Morris and K. Moses (2005). "Pointed regulates an eye-specific transcriptional enhancer in the *Drosophila hedgehog* gene, which is required for the movement of the morphogenetic furrow." Development **132**(21): 4833-4843.

Rudrapatna, V. A., E. Bangi and R. L. Cagan (2013). "A Jnk–Rho–Actin remodeling positive feedback network directs Src-driven invasion." Oncogene **33**: 2801.

Ruf, I. K., P. W. Rhyne, H. Yang, C. M. Borza, L. M. Hutt-Fletcher, J. L. Cleveland and J. T. Sample (2001). EBV Regulates c-MYC, Apoptosis, and Tumorigenicity in Burkitt's Lymphoma. Epstein-Barr Virus and Human Cancer. K. Takada, Springer Berlin Heidelberg. **258:** 153-160.

Safina, A., M. Q. Ren, E. Vandette and A. V. Bakin (2007). "TAK1 is required for TGF-β1-mediated regulation of matrix metalloproteinase-9 and metastasis." Oncogene **27**: 1198.

Sanchez, Carlos G., Felipe K. Teixeira, B. Czech, Jonathan B. Preall, Andrea L. Zamparini, Jessica R. K. Seifert, Colin D. Malone, Gregory J. Hannon and R. Lehmann (2016). "Regulation of Ribosome Biogenesis and Protein Synthesis Controls Germline Stem Cell Differentiation." Cell Stem Cell **18**(2): 276-290.

Sander, V., E. Eivers, R. H. Choi and E. M. De Robertis (2010). "*Drosophila* Smad2 Opposes Mad Signaling during Wing Vein Development." PLoS ONE **5**(4): e10383.

Santibañez, J. F. (2006). "JNK mediates TGF-β1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes." FEBS Letters **580**(22): 5385-5391.

Schlosser, I., M. Hölzel, M. Mürnseer, H. Burtscher, U. H. Weidle and D. Eick (2003). "A role for c-Myc in the regulation of ribosomal RNA processing." Nucleic Acids Research **31**(21): 6148-6156.

Schmidt, C. R., Y. J. Gi, T. A. Patel, R. J. Coffey, R. D. Beauchamp and A. S. Pearson (2005). "E-cadherin is regulated by the transcriptional repressor SLUG during Ras-mediated transformation of intestinal epithelial cells." Surgery **138**(2): 306-312.

Seimiya, M. and W. J. Gehring (2000). "The *Drosophila* homeobox gene optix is capable of inducing ectopic eyes by an eyeless-independent mechanism." Development **127**(9): 1879-1886.

Seong, H.-A., H. Jung and H. Ha (2010). "Murine protein serine/threonine kinase 38 stimulates TGF-β signaling in a kinase-dependent manner via direct phosphorylation of Smad proteins." Journal of Biological Chemistry.

Shen, W. and G. Mardon (1997). "Ectopic eye development in *Drosophila* induced by directed dachshund expression." Development **124**(1): 45-52.

Shi, X., F. Chen, J. Yu, Y. Xu, S. Zhang, Y.-G. Chen and X. Fang (2008). "Study of interaction between Smad7 and DNA by single-molecule force spectroscopy." Biochemical and Biophysical Research Communications **377**(4): 1284-1287.

Silies, M., Y. Yuva, D. Engelen, A. Aho, T. Stork and C. Klämbt (2007). "Glial Cell Migration in the Eye Disc." The Journal of Neuroscience **27**(48): 13130-13139.

Silver, S. J. and I. Rebay (2005). "Signaling circuitries in development: insights from the retinal determination gene network." Development **132**(1): 3-13.

Silvera, D., S. C. Formenti and R. J. Schneider (2010). "Translational control in cancer." Nat Rev Cancer **10**(4): 254-266.

Simonsson, M., C.-H. Heldin, J. Ericsson and E. Grönroos (2005). "The Balance between Acetylation and Deacetylation Controls Smad7 Stability." Journal of Biological Chemistry **280**(23): 21797-21803.

Smith, A. V. and T. L. Orr-Weaver (1991). "The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny." Development **112**(4): 997-1008.

Song, Y. and B. Lu (2011). "Regulation of cell growth by Notch signaling and its differential requirement in normal vs. tumor-forming stem cells in *Drosophila*." Genes & Development **25**(24): 2644-2658.

Souchelnytskyi, S., T. Nakayama, A. Nakao, A. Moren, C. H. Heldin, J. L. Christian and P. ten Dijke (1998). "Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-β receptors." J. Biol. Chem **273**: 25364–25370.

Staley, B. K. and K. D. Irvine (2012). "Hippo signaling in *Drosophila*: recent advances and insights." Developmental dynamics : an official publication of the American Association of Anatomists **241**(1): 3-15.

Strigini, M. and S. M. Cohen (1999). "Formation of morphogen gradients in the *Drosophila* wing." Seminars in Cell & Developmental Biology **10**(3): 335-344.

Strigini, M. and S. M. Cohen (2000). "Wingless gradient formation in the *Drosophila* wing." Current Biology **10**(6): 293-300.

Subramaniam, P. S., B. A. Torres and H. M. Johnson (2001). "SO MANY LIGANDS, SO FEW TRANSCRIPTION FACTORS: A NEW PARADIGM FOR SIGNALING THROUGH THE STAT TRANSCRIPTION FACTORS." Cytokine **15**(4): 175-187.

Sundar, R., S. K. Gudey, C.-H. Heldin and M. Landström (2015). "TRAF6 promotes TGFβ-induced invasion and cell-cycle regulation via Lys63-linked polyubiquitination of Lys178 in TGFβ type I receptor." Cell Cycle **14**(4): 554-565.

Tansey, W. P. (2014). "Mammalian MYC Proteins and Cancer." New Journal of Science **2014**: 27.

Tapon, N., K. F. Harvey, D. W. Bell, D. C. R. Wahrer, T. A. Schiripo, D. A. Haber and I. K. Hariharan (2002). "*salvador* Promotes Both Cell Cycle Exit and Apoptosis in *Drosophila* and Is Mutated in Human Cancer Cell Lines." Cell **110**(4): 467-478.

Tavares, A. L. P., M. E. Mercado-Pimentel, R. B. Runyan and G. T. Kitten (2006). "TGFβ-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart." Developmental Dynamics **235**(6): 1589-1598.

Tavares, L., A. Correia, M. A. Santos, J. B. Relvas and P. S. Pereira (2017). "dMyc is required in retinal progenitors to prevent JNK-mediated retinal glial activation." PLoS Genetics **13**(3): e1006647.

Tavares, L., E. Pereira, A. Correia, M. A. Santos, N. Amaral, T. Martins, J. B. Relvas and P. S. Pereira (2015). "*Drosophila* PS2 and PS3 integrins play distinct roles in retinal photoreceptors–glia interactions." Glia **63**(7): 1155-1165.

Tavsanli, B. C., E. J. Ostrin, H. K. Burgess, B. W. Middlebrooks, T. A. Pham and G. Mardon (2004). "Structure–function analysis of the *Drosophila* retinal determination protein Dachshund." Developmental Biology **272**(1): 231-247.

Tayler, T. D. and P. A. Garrity (2003). "Axon targeting in the *Drosophila* visual system." Current Opinion in Neurobiology **13**(1): 90-95.

Teleman, A. A. and S. M. Cohen (2000). "Dpp Gradient Formation in the *Drosophila* Wing Imaginal Disc." Cell **103**(6): 971-980.

Thompson, B. J. and S. M. Cohen (2006). "The Hippo Pathway Regulates the *bantam* microRNA to Control Cell Proliferation and Apoptosis in *Drosophila*." Cell **126**(4): 767-774.

Thompson, B. J., J. Mathieu, H.-H. Sung, E. Loeser, P. Rørth and S. M. Cohen (2005). "Tumor Suppressor Properties of the ESCRT-II Complex Component Vps25 in *Drosophila*." Developmental Cell **9**(5): 711-720.

Thomson, E., S. Ferreira-Cerca and E. Hurt (2013). "Eukaryotic ribosome biogenesis at a glance." Journal of Cell Science **126**(21): 4815-4821.

Tomlinson, A. (1985). "The cellular dynamics of pattern formation in the eye of *Drosophila*." Journal of Embryology and Experimental Morphology **89**(1): 313-331.

Tosello, V. and A. A. Ferrando (2013). "The NOTCH signaling pathway: role in the pathogenesis of T-cell acute lymphoblastic leukemia and implication for therapy." Therapeutic Advances in Hematology **4**(3): 199-210.

Treisman, J. E. and U. Heberlein (1998). "Eye Development in *Drosophila*: Formation of the Eye Field and Control of Differentiation." Current Topics in Developmental Biology **39**: 119-158.

Tsai, Y.-C. and Y. H. Sun (2004). "Long-range effect of upd, a ligand for Jak/STAT pathway, on cell cycle in *Drosophila* eye development." genesis **39**(2): 141-153.

Tschopp, O., Z.-Z. Yang, D. Brodbeck, B. A. Dummler, M. Hemmings-Mieszczak, T. Watanabe, T. Michaelis, J. Frahm and B. A. Hemmings (2005). "Essential role of protein kinase Bγ (PKBγ/Akt3) in postnatal brain development but not in glucose homeostasis." Development **132**(13): 2943-2954.

Tsuneizumi, K., T. Nakayama, Y. Kamoshida, T. B. Kornberg, J. L. Christian and T. Tabata (1997). "*Daughters against dpp* modulates dpp organizing activity in *Drosophila* wing development." Nature **389**(6651): 627-631.

Uhlirova, M. and D. Bohmann (2006). "JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in *Drosophila*." The EMBO Journal **25**(22): 5294-5304.

Uhlirova, M., H. Jasper and D. Bohmann (2005). "Non-cell-autonomous induction of tissue overgrowth by JNK/Ras cooperation in a *Drosophila* tumor model." Proceedings of the National Academy of Sciences of the United States of America **102**(37): 13123-13128.

Ungefroren, H., D. Witte and H. Lehnert (2017). "The role of small GTPases of the Rho/Rac family in TGF-β-induced EMT and cell motility in cancer." Developmental Dynamics: n/a-n/a.

Uttamsingh, S., X. Bao, K. T. Nguyen, M. Bhanot, J. Gong, J. L. K. Chan, F. Liu, T. T. Chu and L. H. Wang (2007). "Synergistic effect between EGF and TGF-β1 in inducing oncogenic properties of intestinal epithelial cells." Oncogene **27**: 2626.

Vaccari, T. and D. Bilder (2005). "The *Drosophila* Tumor Suppressor vps25 Prevents Nonautonomous Overproliferation by Regulating Notch Trafficking." Developmental Cell **9**(5): 687-698.

Vardouli, L., A. Moustakas and C. Stournaras (2005). "LIM-kinase 2 and Cofilin Phosphorylation Mediate Actin Cytoskeleton Reorganization Induced by Transforming Growth Factor-β." Journal of Biological Chemistry **280**(12): 11448-11457.

Ventura, J.-J., N. J. Kennedy, R. A. Flavell and R. J. Davis (2004). "JNK Regulates Autocrine Expression of TGF-β1." Molecular Cell **15**(2): 269-278.

Vining, M. S., P. L. Bradley, C. A. Comeaux and D. J. Andrew (2005). "Organ positioning in *Drosophila* requires complex tissue–tissue interactions." Developmental Biology **287**(1): 19-34.

Wang, H., K. Song, T. L. Krebs, J. Yang and D. Danielpour (2008). "Smad7 is inactivated through a direct physical interaction with the LIM protein Hic-5/ARA55." Oncogene **27**: 6791.

Wang, S., V. Tsarouhas, N. Xylourgidis, N. Sabri, K. Tiklová, N. Nautiyal, M. Gallio and C. Samakovlis (2009). "The tyrosine kinase Stitcher activates Grainy head and epidermal wound healing in *Drosophila*." Nature Cell Biology **11**: 890.

Weasner, B., C. Salzer and J. P. Kumar (2007). "*Sine oculis*, a member of the SIX family of transcription factors, directs eye formation." Developmental biology **303**(2): 756-771.

Wegiel, B., A. Bjartell, Z. Culig and J. L. Persson (2008). "Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival." International Journal of Cancer **122**(7): 1521-1529.

Wei, X., T. Shimizu and Z.-C. Lai (2007). "Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in *Drosophila*." The EMBO Journal **26**(7): 1772-1781.

Wilkes, M. C., H. Mitchell, S. G. Penheiter, J. J. Doré, K. Suzuki, M. Edens, D. K. Sharma, R. E. Pagano and E. B. Leof (2005). "Transforming Growth Factor-β Activation of Phosphatidylinositol 3-Kinase Is Independent of Smad2 and Smad3 and Regulates Fibroblast Responses via p21-Activated Kinase-2." Cancer Research **65**(22): 10431-10440.

Williamson, M. P. (1994). "The structure and function of proline-rich regions in proteins." Biochemical Journal **297**(2): 249-260.

Wright, G. L., I. G. Maroulakou, J. Eldridge, T. L. Liby, V. Sridharan, P. N. Tsichlis and R. C. Muise-Helmericks (2008). "VEGF stimulation of mitochondrial biogenesis: requirement of AKT3 kinase." The FASEB Journal **22**(9): 3264-3275.

Wrighton, K. H., X. Lin and X.-H. Feng (2008). "Critical regulation of TGFβ signaling by Hsp90." Proceedings of the National Academy of Sciences **105**(27): 9244-9249.

Wu, S., J. Huang, J. Dong and D. Pan (2003). "*hippo* Encodes a Ste-20 Family Protein Kinase that Restricts Cell Proliferation and Promotes Apoptosis in Conjunction with *salvador* and *warts*." Cell **114**(4): 445-456.

Wu, X., W. Zhang, J. Font-Burgada, T. Palmer, A. S. Hamil, S. K. Biswas, M. Poidinger, N. Borcherding, Q. Xie, L. G. Ellies, N. K. Lytle, L.-W. Wu, R. G. Fox, J. Yang, S. F. Dowdy, T. Reya and M. Karin (2014). "Ubiquitin-conjugating enzyme Ubc13 controls breast cancer metastasis through a TAK1-p38 MAP kinase cascade." Proceedings of the National Academy of Sciences **111**(38): 13870-13875.

Xu, J., S. Lamouille and R. Derynck (2009). "TGF-β-induced epithelial to mesenchymal transition." Cell research **19**(2): 156-172.

Xu, J., A. H. Wang, J. Oses-Prieto, K. Makhijani, Y. Katsuno, M. Pei, L. Yan, Y. G. Zheng, A. Burlingame, K. Brückner and R. Derynck (2013). "Arginine Methylation Initiates BMP-Induced Smad Signaling." Molecular Cell **51**(1): 5-19.

Xu, L., S. Chen and R. C. Bergan (2006). "MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer." Oncogene **25**: 2987.

Xu, N., G. Bagumian, M. Galiano and M. M. Myat (2011). "Rho GTPase controls *Drosophila* salivary gland lumen size through regulation of the actin cytoskeleton and Moesin." Development **138**(24): 5415-5427.

Xu, T. and G. M. Rubin (1993). "Analysis of genetic mosaics in developing and adult *Drosophila* tissues." Development **117**(4): 1223-1237.

Xue, G., D. F. Restuccia, Q. Lan, D. Hynx, S. Dirnhofer, D. Hess, C. Rüegg and B. A. Hemmings (2012). "Akt/PKB-Mediated Phosphorylation of Twist1 Promotes Tumor Metastasis via Mediating Cross-Talk between PI3K/Akt and TGF-β Signaling Axes." Cancer Discovery **2**(3): 248-259.

Xue, S. and M. Barna (2012). "Specialized ribosomes: a new frontier in gene regulation and organismal biology." Nat Rev Mol Cell Biol **13**(6): 355-369.

Yan, X., Z. Lin, F. Chen, X. Zhao, H. Chen, Y. Ning and Y. G. Chen (2009). "Human BAMBI cooperates with Smad7 to inhibit transforming growth factor-β signaling." J. Biol. Chem. **284**: 30097-30104.

Yanagisawa, M., K. Nakashima, K. Takeda, W. Ochiai, T. Takizawa, M. Ueno, M. Takizawa, H. Shibuya and T. Taga (2001). "Inhibition of BMP2-induced, TAK1 kinase-mediated neurite outgrowth by Smad6 and Smad7." Genes to Cells **6**(12): 1091-1099.

Yang, N., O. Higuchi, K. Ohashi, K. Nagata, A. Wada, K. Kangawa, E. Nishida and K. Mizuno (1998). "Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization." Nature **393**: 809.

Yao, L.-C., S. Phin, J. Cho, C. Rushlow, K. Arora and R. Warrior (2008). "Multiple modular promoter elements drive graded brinker expression in response to the Dpp morphogen gradient." Development **135**(12): 2183-2192.

Younossi-Hartenstein, A., U. Tepass and V. Hartenstein (1993). "Embryonic origin of the imaginal discs of the head of *Drosophila melanogaster*." Roux's archives of developmental biology **203**(1): 60-73.

Yu, L., M. C. Hébert and Y. E. Zhang (2002). "TGF-β receptor-activated p38 MAP kinase mediates Smad-independent TGF-β responses." The EMBO Journal **21**(14): 3749-3759.

Zecca, M., K. Basler and G. Struhl (1995). "Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing." Development **121**(8): 2265-2278.

Zehorai, E., Z. Yao, A. Plotnikov and R. Seger (2010). "The subcellular localization of MEK and ERK—A novel nuclear translocation signal (NTS) paves a way to the nucleus." Molecular and Cellular Endocrinology **314**(2): 213-220.

Zhang, H., J. P. Stallock, J. C. Ng, C. Reinhard and T. P. Neufeld (2000). "Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR." Genes & Development **14**(21): 2712-2724.

Zhang, L., F. Ren, Q. Zhang, Y. Chen, B. Wang and J. Jiang (2008). "The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control." Developmental cell **14**(3): 377-387.

Zhang, S., T. Fei, L. Zhang, R. Zhang, F. Chen, Y. Ning, Y. Han, X.-H. Feng, A. Meng and Y.-G. Chen (2007). "Smad7 Antagonizes Transforming Growth Factor β Signaling in the Nucleus by Interfering with Functional Smad-DNA Complex Formation." Molecular and Cellular Biology **27**(12): 4488-4499.

Zhang, Y., C. Chang, D. J. Gehling, A. Hemmati-Brivanlou and R. Derynck (2001). "Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase." Proceedings of the National Academy of Sciences of the United States of America **98**(3): 974-979.

Zhang, Y. E. (2017). "Non-Smad Signaling Pathways of the TGF-β Family." Cold Spring Harbor Perspectives in Biology **9**(2).

Zhao, B., K. Tumaneng and K.-L. Guan (2011). "The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal." Nature cell biology **13**(8): 877-883.

Zheng, X., C. T. Zugates, Z. Lu, L. Shi, J. m. Bai and T. Lee (2006). Baboon/dSmad2 TGF-β signaling is required during late larval stage for development of adult-specific neurons.

Zhou, B. P., J. Deng, W. Xia, J. Xu, Y. M. Li, M. Gunduz and M.-C. Hung (2004). "Dual regulation of Snail by GSK-3β-mediated phosphorylation in control of epithelial–mesenchymal transition." Nature Cell Biology **6**: 931.

Zhu, C. C., J. Q. Boone, P. A. Jensen, S. Hanna, L. Podemski, J. Locke, C. Q. Doe and M. B. O'Connor (2008). "*Drosophila* Activin-β and the Activin-like product Dawdle function redundantly to regulate proliferation in the larval brain." Development **135**(3): 513-521.

Zhu, H., P. Kavsak, S. Abdollah, J. L. Wrana and G. H. Thomsen (1999). "A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation." Nature **400**: 687.

Zhu, J., S. Palliyil, C. Ran and J. P. Kumar (2017). "*Drosophila* Pax6 promotes development of the entire eye-antennal disc, thereby ensuring proper adult head formation." Proceedings of the National Academy of Sciences of the United States of America **114**(23): 5846-5853.

Zhu, L., L. Wang, X. Luo, Y. Zhang, Q. Ding, X. Jiang, X. Wang, Y. Pan and Y. Chen (2012). "Tollip, an Intracellular Trafficking Protein, Is a Novel Modulator of the Transforming Growth Factor-β Signaling Pathway." The Journal of Biological Chemistry **287**(47): 39653-39663.

Zimmerman, K. and F. Alt (1990). "Expression and function of *myc* family genes." Crit Rev Oncog **2**(1): 75-95.

Zimmerman, K. A., G. D. Yancopoulos, R. G. Collum, R. K. Smith, N. E. Kohl, K. A. Denis, M. M. Nau, O. N. Witte, D. Toran-Allerand, C. E. Gee, J. D. Minna and F. W. Alt (1986). "Differential expression of *myc* family genes during murine development." Nature **319**(6056): 780-783.

Zoranovic, T., L. Grmai and E. A. Bach (2013). "Regulation of proliferation, cell competition, and cellular growth by the *Drosophila* JAK-STAT pathway." JAK-STAT **2**(3): e25408.

CHAPTER 2

Drosophila TGFβ/Activin is required for ribosome biogenesis and enhances dMyc-induced cell growth

Contents

Manuscript 1: **TGFβ/Activin signalling is required for ribosome biogenesis and cell growth in *Drosophila* salivary glands**

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TGFβ signaling is a large family of structurally related cell regulatory proteins, which are highly conserved along metazoans. In *Drosophila*, the activity of the TGFβ/Activin signaling branch has been linked to the regulation of cell growth and proliferation. However, the cellular and molecular basis for those functions are not fully understood. In this study we show that both the type II (RII) receptor Punt (Put) and the R-Smad Smad2 are strongly necessary for cell and tissue growth. The downregulation of *punt* or *smad2* in salivary glands caused alterations in nucleolar structure and functions. Moreover, gland cells with decreased TGFβ/Activin signaling accumulated intermediate pre-rRNA transcripts containing ITS1 (Internal Transcribed Sequences) regions accompanied by the nucleolar retention of ribosomal proteins. Therefore, our results show that TGFβ/Activin signaling plays an important role in ribosomal biogenesis. Remarkably, overexpression of Punt enhanced cell growth induced by *Drosophila* Myc, a well-characterized regulator of nucleolar functions. In this study, I have participated in experimental design, immunostaining experiments, transmission electron microscopy sample preparation and acquisition, sample preparation for qPCR and analyzes. Additionally, I have contributed in the revision of the manuscript by performing required immunostaining experiments and in the critical reading of the paper.

TGFβ/Activin signalling is required for ribosome biogenesis and cell growth in *Drosophila* salivary glands

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1. Abstract

Signaling by TGFβ superfamily factors plays an important role in tissue growth and cell proliferation. In *Drosophila*, the activity of the TGFβ/Activin signaling branch has been linked to the regulation of cell growth and proliferation, but the cellular and molecular basis for these functions are not fully understood. In this study, we show that both the RII receptor Punt (Put) and the R-Smad Smad2 are strongly required for cell and tissue growth. Knocking down the expression of Put or Smad2 in salivary glands causes alterations in nucleolar structure and functions. Cells with decreased TGFβ/Activin signaling accumulate intermediate pre-rRNA transcripts containing ITS1 (Internal Transcribed Spacer 1) regions accompanied by the nucleolar retention of ribosomal proteins. Thus, our results show that TGFβ/Activin signaling is required for ribosomal biogenesis, a key aspect of cellular growth control. Importantly, overexpression of Put enhanced cell growth induced by *Drosophila* Myc (dMyc), a well-characterized inducer of nucleolar hypertrophy and ribosome biogenesis.

2. Introduction

Tissue growth is a very complex process that requires interplay between multiple signaling pathways to ensure that an organ achieves its proper size and shape. Transforming Growth Factor β (TGFβ) and bone morphogenetic protein (BMP) signaling pathways play multiple roles during animal development through the regulation of cellular growth, proliferation, differentiation and survival (Massague 2012). At the cell surface, the secreted polypeptides of the TGFβ super-family (TGFβ, BMP, Activin, and Nodal) bind tetrameric complexes of type I (RI) and type II (RII) serine/threonine kinase receptors. Ligand binding allows the active RII receptors to phosphorylate serines and threonines within the GS domain of RI receptors, which in turn phosphorylate and activate Smads. Receptor-activated (R) Smads then associate with the common-mediator (Co) Smad and the complex is shuttled to the nucleus where it regulates target gene expression (Massague 2012). TGFβ signaling can either suppress or promote cell growth and proliferation, a phenomenon described as the TGFβ paradox in the context of cancer progression (Inman 2011, Salomon 2014). TGFβ is also an important promoter of epithelial–mesenchymal transition (EMT), where its activity leads to increased protein synthesis and cell size through activation of the PI3K, Akt, and mTOR complex 1 (Lamouille and Derynck 2007). Furthermore, the activity of TGFβ Receptor I kinase was shown to be required for glucose-induced hypertrophy in both fibroblasts and epithelial cells (Wu and Derynck 2009). Similarly to

high glucose, adding TGFβ to these cells caused an increase in protein synthesis and cell size (Wu and Derynck 2009). In a subsequent study, treatment with the anti-TGFβ1 neutralization antibody (1D11) was shown to protect mice from obesity and diabetes (Yadav, Quijano et al. 2011). Thus, the control of cell growth by TGFβ in different cell types and contexts is expected to play important roles in diabetes and cancer pathology.

The TGFβ pathway is evolutionarily conserved in *Drosophila*, where both the BMP and TGFβ/Activin branches are crucial regulators of developmental processes (Parker, Stathakis et al. 2004). Put is a common RII receptor for both signaling branches, and it heterodimerizes with branch-specific RI receptors to ensure pathway specificity. In the TGFβ/Activin branch, Put binds the RI receptor Baboon (Babo) that phosphorylates Smad2 (also known as Smox) in response to the Activin-β (Actβ), Dawdle (Daw), and Myoglianin (Myo) ligands (Brummel, Abdollah et al. 1999, Gesualdi and Haerry 2007, Zhu, Boone et al. 2008, Jensen, Zheng et al. 2009). The TGFβ/Activin pathway was shown to regulate axonal outgrowth and remodeling (Zheng, Wang et al. 2003, Parker, Ellis et al. 2006, Serpe and O'Connor 2006), as well as proliferation of neuroblasts and wing imaginal disc cells (Brummel, Abdollah et al. 1999, Zhu, Boone et al. 2008, Hevia and de Celis 2013).

In a recent eye-targeted double-RNAi screen we identified a genetic interaction between several *Drosophila* TGFβ signaling members (including Put, Baboon, and Smad2) and the nucleolar regulator Viriato (Vito)/Nol12 (Marinho, Martins et al. 2013). Previously, we had shown that Vito acts downstream of dMyc to ensure a coordinated nucleolar response during dMyc-stimulated growth (Marinho, Casares et al. 2011). Thus, Vito could play a role in dMyc-mediated increase in the rate of ribosome biogenesis in the nucleolus, one of the main mechanism by which dMyc drives growth (Grewal, Li et al. 2005). Since the mechanisms enabling TGFβ signaling to induce cell growth and proliferation are poorly understood, we pursued the analysis of the novel link between TGFβ signaling and nucleolar-based events. Here, we study the cell-autonomous functions of TGFβ/Activin signaling in cell growth, using the salivary gland as a model tissue. During larval stages, the salivary gland is an endoreplicative tissue where overall growth correlates directly with cell growth, and that allows easy characterization of subcellular structures.

3. Materials and Methods

3.1. Fly strains and husbandry

All crosses were raised at 25ºC under standard conditions and for synchronization, all the conditions were analyzed after a single day of egg collection. The following stocks (described in FlyBase, unless stated otherwise) were used: ey-Gal4, UAS-lacZ and the wild type strain w1118. UAS-CD4tdTomato was used to report salivary glands expression of ey-Gal4 (Figure S4A-C'). AB1-Gal4 and ptc-Gal4 were used as salivary glands alternative drivers with similar results to ey-Gal4 (Figure S4D-F'). The TGFβ RNAis were obtained from different collections: *put*RNAi#37279 (VDRC), *put*RNAi 7904R-3 (Nigfly), *smad2*RNAi (#2262R-2, Nigfly), and *mad*RNAi (#31315, TRiP). The following TGFβ pathway mutants were obtained from the Bloomington stock center: put^{135}, put^{10460}, $babo^{32}$, $babo^{K16912}$, tkv^{1} and tkv^{8}. Overexpression studies were done using UAS-Put (Ruberte, Marty et al. 1995) and UAS-dMyc (Datar, Jacobs et al. 2000). The protein trap strains used in these studies were RpL41YFP (#115-344 Cambridge Protein Trap YFP insertions) and RpS9YFP (#115-034 Cambridge Protein Trap YFP insertions).

3.2. Immunostaining

Eye-antennal imaginal discs and salivary glands were prepared for immunohistochemistry using standard protocols. As the growth conditions strongly affect salivary gland size, all the experiments were controlled by synchronization of L3 wandering larvae after a single day egg collection. To further control this issue, a controlled egg laying for 5 hours was set up and the salivary glands were analyzed 96h after Egg laying (96h-101h AEL, Figure S4G-J').

Primary antibodies used were: mouse anti-Armadillo N27A1 at 1:100 (Developmental Studies Hybridoma Bank, DSHB), mouse anti-Dlg at 1:1000 (4F3, Developmental Studies Hybridoma Bank, DSHB), rabbit anti-Viriato (Vito) at 1:250 (ABGent), rat anti-DCad at 1:100, mouse anti-AH6 at 1:10 (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-Fibrillarin at 1:250 (Abcam, #ab5821), mouse anti-Fibrillarin at 1:500 (Abcam, #ab4566), mouse anti-RpS6 at 1:100 (Cell Signaling, #2317), mouse anti-RpL11 at 1:100 (Abcam, # ab79352), mouse anti-RpL10A at 1:400 (Abcam, # ab55544), rabbit anti-RpL22 at 1:100 (kind gift from Dr. Vassie Ware). To stain for cellular limits Phalloidin conjugated with Rhodamine was used at a dilution of 1:1000. Appropriate Alexa-Fluor conjugated secondary antibodies were from Molecular Probes. Images were obtained with the Leica SP2 confocal system or Leica SP5 confocal

system and processed with Adobe Photoshop.

3.3. Size measurements and statistics

Salivary gland areas were measured using the Polygon selection tool of ImageJ 1.48r software (NIH, Bethesda, MA, USA), considering the limits stained by Arm, Dcad or RhPh and represented as Arbitrary Units. The cellular parameters shown in this study were measured using the Polygon selection tool of ImageJ 1.48r. The nucleolar area was determined using the nucleolar markers RpL41YFP, anti-AH6 or anti-Fibrillarin and DAPI staining was used to stain for the nuclear area. The results are presented as the ratio of the nucleolar area to the nucleus that it corresponds to. The intensity of the nucleolar components was determined using a fixed ROI circle and the mean intensity of each nucleolus was measured using ImageJ. To each measurement another nucleolar component was used as reference (for example, AH6 and Fibrillarin). For each genotype, 5-6 nuclei from the proximal region of at least 5-6 independent salivary glands were used. Statistical analysis and generation of the graphical output was done using the GraphPad Prism 5.0. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test, with a 95% confidence interval, after assessing the normality distribution of the data with D'Agostino-Pearson normality test.

3.4. Transmission electron microscopy (TEM)

Dissected third instar salivary glands were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 minutes and post-fixed with 4% osmium tetroxide. After washing, salivary glands were incubated with 0.5% uranyl acetate (30 minutes) and further dehydrated through a graded ethanol series (70% for 10 minutes, 90% for 10 minutes, and four changes of 100%). Salivary glands were then soaked in propylene oxide for 10 minutes and then in a mixture (1:1) of propylene oxide and Epon resin (TAAB Laboratories) for 30 minutes. This mixture was then replaced by 100% Epon resin for 24 hours. Finally, fresh Epon replaced the Epon and polymerization took place at 60°C for 48 hours. Ultrathin sections were obtained using an ultramicrotome, collected in copper grids and then double contrasted with uranyl acetate and lead citrate. In total, at least 16 independent cells of 5 independent salivary glands were analyzed for each genotype. Micrographs were taken using a TEM Jeol JEM-1400, with Orius SC 1000 digital camera (80 kV).

3.5. Quantitative real-time PCR (qPCR)

For qPCR experiments, all the RNAis were induced with the ey-Gal4 driver and the salivary glands of wondering L3 instar larvae were dissected. The number of salivary glands was determined according to its size to yield similar RNA concentrations, i.e. for w1118 control strain, a minimum of 30 salivary glands were dissected, for ey-Gal4; *put*RNAi^{37279} 50-60 salivary glands were dissected and 40-50 salivary glands were dissected for ey-Gal4; *smad2*RNAi$^{2262R-2}$.

The RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and treated with Turbo DNase I (Ambion). cDNA was generated by reverse transcription with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Quantitative real-time PCR analysis was performed in triplicate in 20 µL reactions containing iQ SYBR Green Supermix (BioRad), each gene-specific primer at 250 nM and 1 µl of cDNA template. Cycling conditions in a BioRad iQ5 instrument were 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing for 30 seconds at 53, 60 or 64°C depending on the primer set. Fold change relative to the expression of *CaMKII*, which has been used previously as a control for gene expression (Marinho, Casares et al. 2011) was calculated using the 2–^ΔCT method (Livak and Schmittgen 2001). Three to five biological replicates were analyzed for each primer set. The following primer pairs (5' to 3') were used:

CaMKII(control): Fw - TTACACCATCCCAACATAGTGC
Rev - CAAGGTCAAAAACAAGGTAGTGATAG;
28S: Fw - GGAGGATCTTCGATCACCTGATG
Rev - GCTGCTCAACCACTTACAACAC;
18S: Fw - TGGTCTTGTACCGACGACAG
Rev – GCTGCCTTCCTTAGATGTGG;
ITS1: Fw - TTATTGAAGGAATTGATATATGCC
Rev – ATGAGCCGAGTGATCCAC;
ETS: Fw - GCTCCGCGGATAATAGGAAT
Rev – ATATTTGCCTGCCACCAAAA;

4. Results

4.1. TGFβ/Activin signaling is required for tissue growth and nucleolar dynamics

To study possible cell-autonomous functions of TGFβ signaling in salivary gland cell growth, we down-regulated the expression of the RII receptor Put (Figure 2.1.). RNA interference (RNAi) was targeted to the post-mitotic salivary glands and eye imaginal discs using the ey-Gal4 driver (Marinho, Casares et al. 2011). The two *put*RNAi lines

we used (VDRC^{37279} and NIG-FLY$^{7904R-3}$) target non-overlapping regions of *put* and inhibited the progression of photoreceptor differentiation in the eye imaginal disc (Figure S2.1.A-C). This phenotype mimics mutant *put* phenotypes (Burke and Basler 1996), and confirms the specificity and efficiency of the RNAi knockdown. Importantly,

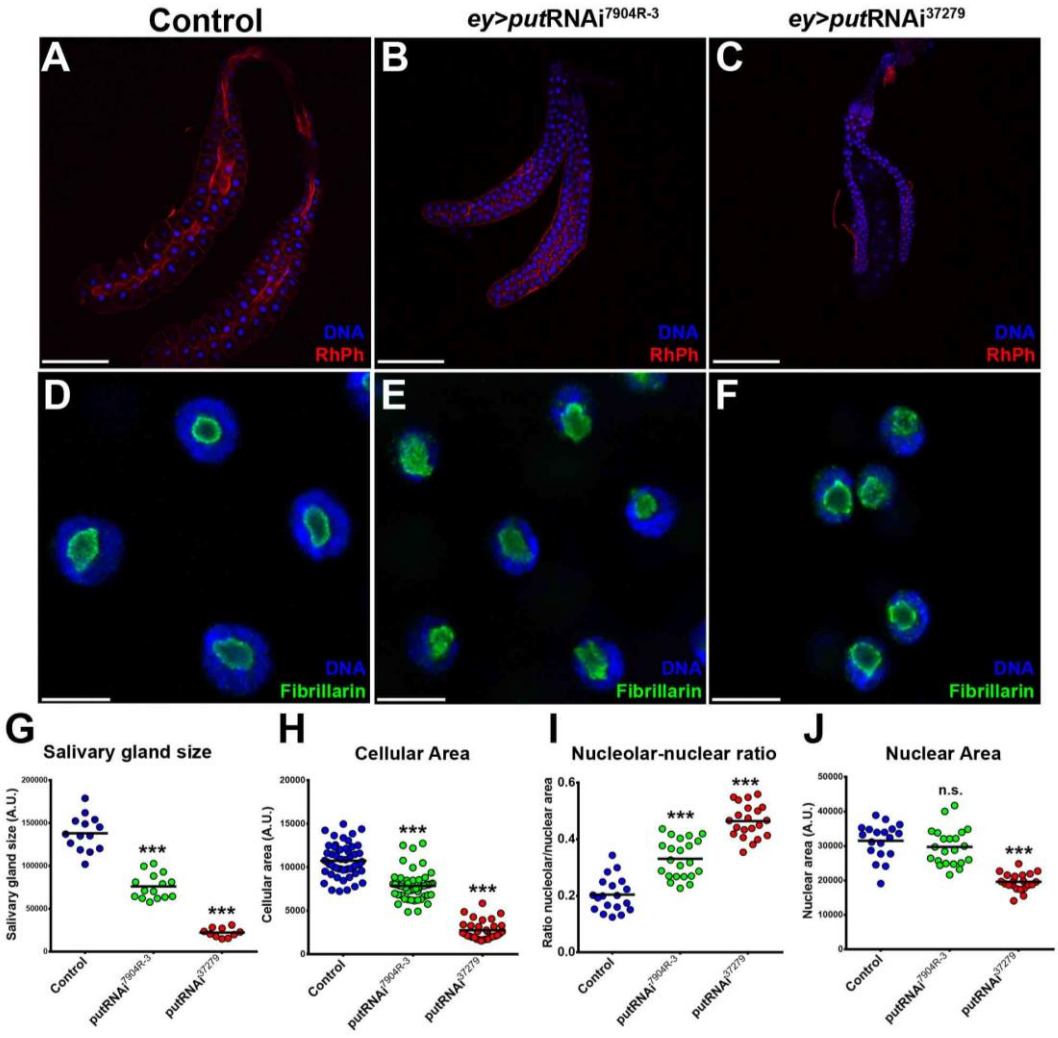

Figure 2.1. TGFβ/Activin signaling is required for tissue growth and nucleolar dynamics.

(A-C) Salivary glands show a substantial reduction in overall size upon *put* depletion. Low magnifications images of salivary glands from third instar *Drosophila* larvae expressing: (A) UAS-*lacZ* (control), (B) UAS-*put*RNAi$^{7904R-3}$ and (C) UAS-*put*RNAi^{37279} under the control of the *ey-Gal4* driver. Salivary glands of the indicated genotypes were stained for the cell limits with RhPh (red) and counterstained with DAPI (blue). (D-F) Put requirement for salivary glands growth is linked to an increase in nucleolar size: (D) Localization of the nucleolar marker Fibrillarin in control nuclei of salivary glands. (E) *put* RNAi$^{7904R-3}$ results in ectopic accumulation of Fibrillarin. (F) Strong *put*RNAi^{37279} induction results in expansion of the Fibrillarin at the nucleolus. (G) Scatter plot representative of *put* requirement for salivary gland growth. (H) Salivary gland growth deficit is linked to a decrease in the cellular area. (I) *put* depletion causes an increase in nucleolar/nuclear area ratio in the salivary glands. (J) Scatter plot showing nuclear area quantification of the described genotypes (n = 25-40; ***, P<1^10^{-4}). Scale bars: A-C = 200 μm, D-F = 20 μm

knocking down *put* expression in the salivary glands caused a strong reduction in cellular area (Figure 2.1.A-C, H) with a strong effect on tissue growth (Figure 2.1.G). In

particular, expression of the stronger *put*RNAi^{37279} caused a significant decrease in salivary gland area (84%, $P<1X10^{-4}$; Figure 2.1.C, G), and completely inhibited the onset of the photoreceptor differentiation (Figure S2.1.C). Our recent work established a genetic interaction between *put* and *vito*, which encodes for a regulator of nucleolar organization and tissue growth (Marinho, Martins et al. 2013). Since uncoordinated nucleolar hypertrophy has been associated with defective cell growth (Marinho, Casares et al. 2011, Hovhanyan, Herter et al. 2014), we evaluated whether Put controlled nucleolar dynamics. We stained salivary glands with anti-Fibrillarin, a nucleolar protein involved in pre-rRNA processing, or anti-AH6 to label nucleoli and DAPI for DNA (Figure 2.1. D-F, and not shown). Knocking down *put* caused an expansion of the nucleolar area, reflected in an increased ratio between nucleolar and nuclear areas. Expression of *put*RNAi$^{7904R-3}$ resulted in a 62% increase in the nucleolar/nuclear area ratio ($P<1X10^{-4}$), while expression of *put*RNAi^{37279} caused an even stronger increase (127%, $P<1X10^{-4}$; Figure 2.1.I). Thus, for *put* depletion in salivary glands, we observe an inverse correlation between increased nucleolar/nuclear ratio and diminished tissue growth ($R^2=0.99468$). This suggests that *put* controls nucleolar dynamics during salivary gland cell growth. Importantly, the weaker *put*RNAi$^{7904R-3}$ did not significantly affect nuclear size (Figure 2.1.J) suggesting that the reduced nuclear size caused by the stronger *put*RNAi^{37279} (Figure 2.1.J) is secondary, or a consequence of the observed nucleolar alterations.

The RII receptor Put heterodimerizes with RI receptors Tkv or Babo to mediate BMP and TGFβ/Activin signaling, respectively. To study the contribution of each signaling branch to nucleolar regulation, we knocked down the expression of the TGFβ/Activin-branch specific R-SMAD, Smad2 (Henderson and Andrew 1998, Brummel, Abdollah et al. 1999), and of the BMP-branch specific R-SMAD Mad (Sekelsky, Newfeld et al. 1995, Newfeld, Chartoff et al. 1996), either alone or in combination (Figure 2.2.). To validate the efficiency and specificity of the RNAi lines for Smad2 and Mad, we targeted their expression to the developing eye imaginal disc. In agreement with previous observations, down-regulation of TGFβ/Activin signaling by *smad2*RNAi affected growth of the eye disc (Figure S2.1.E) (Brummel, Abdollah et al. 1999), while down-regulation of BMP signaling by *mad*RNAi strongly interfered with both tissue growth and patterning (Figure S2.1.F) (Wiersdorff, Lecuit et al. 1996). Importantly, expression of *smad2*RNAi caused a strong increase in the nucleolar/nuclear area ratio

in salivary gland cells, which is not induced by *mad*RNAi expression (Figure 2F).

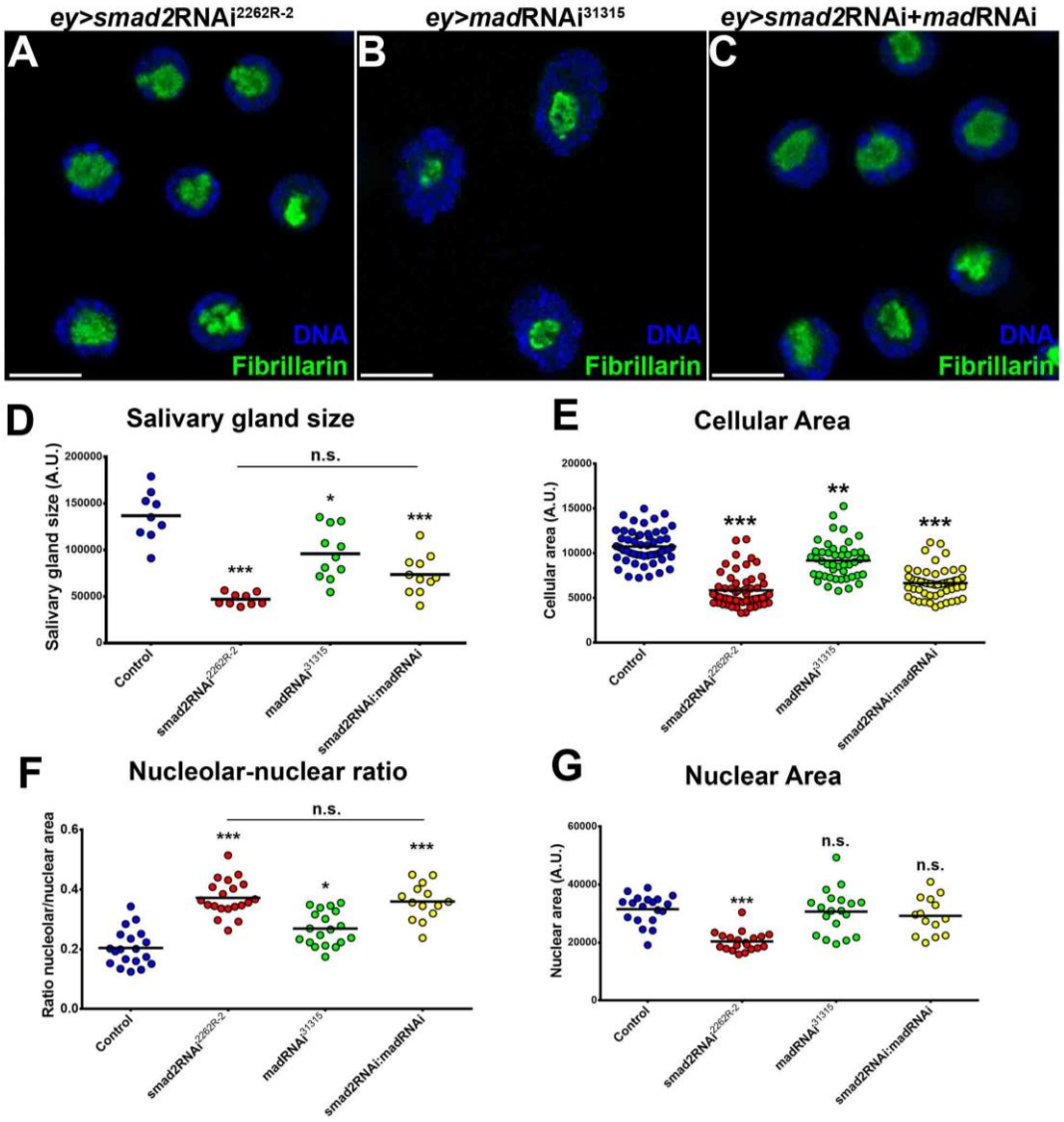

Figure 2.2. Activin R-Smad *smad2* is required for nucleolar dynamics.

(A-C) TGFβ R-Smads contribution for nucleolar activity. Fibrillarin expansion caused by (A) Activin R-Smad *smad2* depletion, (B) BMP R-Smad *mad* depletion and (C) co-depletion of both R-Smads presents a similar phenotype to *smad2*RNAi. In all panels DNA was labelled with DAPI and represented in blue. Scale bar correspond to 20 μm.

(D-G) Scatter plots showing the quantification of the different organ and cellular parameters after R-Smads depletion. *smad2*RNAi has a stronger effect on growth levels than *mad*RNAi, both in overall tissue size (D) and cellular area (E). Reduction of the Activin pathway activity increases the relative nucleolar area (F) with a mild impact on the nuclear size (G). (*n* = 25-40; n.s. means no statistical difference between samples; *, *P*<0.05; **, *P*<0.01 and ***, *P*<1^10^{-4})

Furthermore, co-expression of *mad*RNAi failed to enhance the increase in nucleolar size, or the decrease in cellular and tissue size, induced by *smad2*RNAi (Figure 2.2. and Figure S2.1. G-J). In addition, salivary glands of larvae mutant for *put* or for *babo*, the Activin-branch specific RI receptor, also displayed a nucleolar phenotype, not observed in mutants for *tkv*, the BMP-branch specific RI receptor (Figure 2.3. A-F).

Taken together, these results show that the TGFβ/Activin pathway is required for growth and normal nucleolar dynamics.

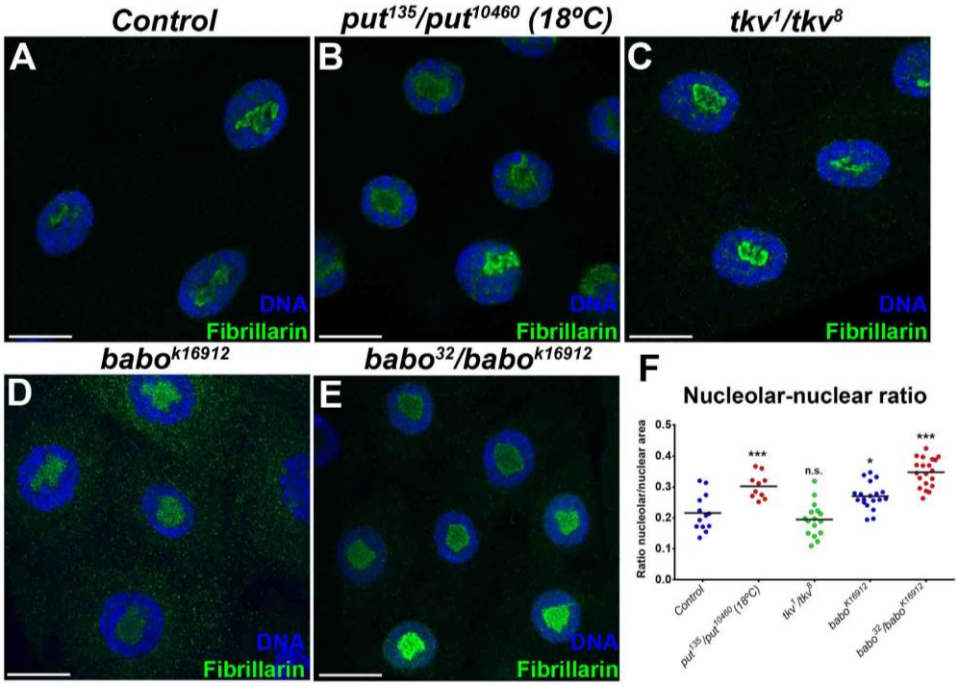

Figure 2.3. TGFβ/Activin mutants display nucleolar phenotypes.

(A-E) Reduced Activin activity induces nucleolar expansion. (A) Control nuclear and nucleolar staining (*w1118*). (B) *put* mutant combination (put^{135}/put^{10460}) grew at a permissive temperature (18ºC) present an expansion of the nucleolar area. (C) Dpp receptor *tkv* mutations (tkv^{1}/tkv^{8}) do not change the nucleolar/nuclear ratio. (D) A weak allele for the Activin receptor type I *baboon* ($babo^{k16912}$) shows a small expansion of the nucleolar area. (E) The heteroallelic combination of *baboon* ($babok^{16912}/babo^{32}$) shows a nucleolar expansion equivalent to *put* mutant combinations. All the nuclei were stained with DAPI (blue) and the nucleoli with Fibrillarin (green). (F) Scatter plot representing the nucleolar accumulation of the Fibrillarin in salivary glands of the indicated genotypes (*, p<0.05; ***, p<0.001).

4.2. TGFβ/Activin is required for the coordination of the ribosome biogenesis

The increased size ratio of the nucleolus apparently contradicts the reduction in tissue growth observed when the TGFβ/Activin pathway is attenuated. One possibility is that the increase in size is reflecting a defective, rather than a gain of nucleolar function – e.g. a defective production of ribosomes. The nucleolus plays a major role in cell growth through the coordination of three steps in ribosome biogenesis: transcription of pre-rRNA by Polymerase I, processing of pre-rRNA, and assembly of the large (60S) and small (40S) ribosome subunits (Boulon, Westman et al. 2010). Defects in the biogenesis of the large or small subunits (e.g. pre-RNA processing deficits) lead to nucleolar stress accompanied by alterations in the localization of ribosomal proteins and other nucleolar factors (Boulon, Westman et al. 2010). To study if TGFβ/Activin signaling regulates ribosome biogenesis in the salivary gland, we analyzed the

localization pattern of the ribosomal protein RpL41 (Wang, Huang et al. 2010). In the nuclei of control cells, RpL41 was mainly nucleolar restricted (Figure 2.4.A), as previously observed (Rugjee, Roy Chaudhury et al. 2013). Inhibition of TGFβ/Activin signaling activity by *put*RNAi caused a strong nucleolar accumulation of RpL41 (Figure 2.4.A-C, G). Similarly, we also observed an increase in the nucleolar localization of Vito (Figure 4D-F). Nucleolar enrichment of Vito was not homogenous, as we detected intra-nucleolar regions with higher Vito levels (Figure 2.4.D-F). These results prompted

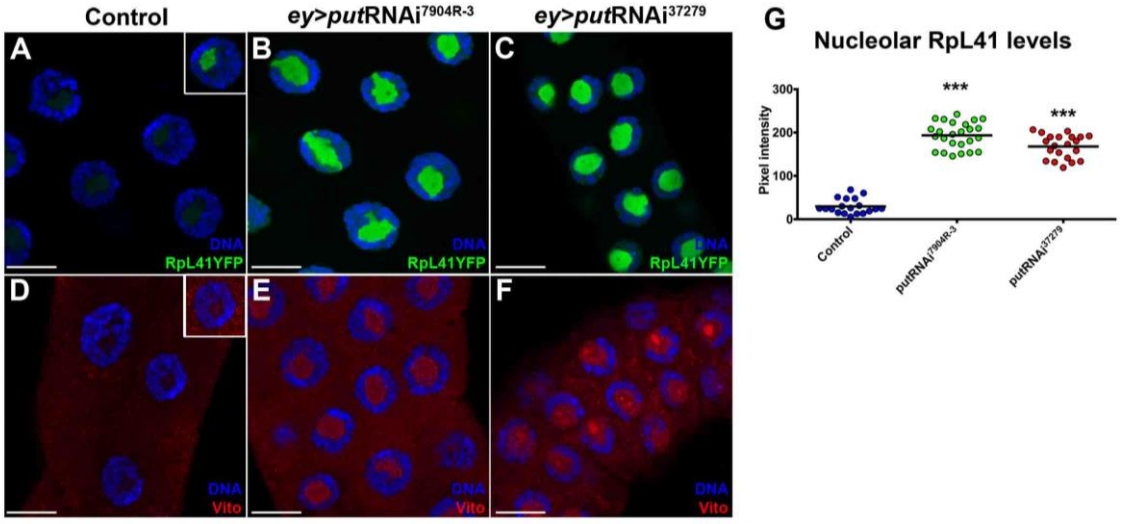

Figure 2.4. Inhibition of TGFβ/Activin signaling activity by *put*RNAi causes a strong nucleolar accumulation of RpL41 and Vito.

(A-D) Put regulates nucleolar size and accumulation of nucleolar components. (A) Localization of the large ribosome subunit RpL41YFP in control nuclei salivary glands. Inset shows nuclei with higher acquisition settings to determine the precise localization of RpL41YFP. (B) *put* RNAi$^{7904R-3}$ depletion results in ectopic accumulation of RpL41YFP. (C) Strong *put*RNAi^{37279} induction results in a growth deficit with several fold accumulation of RpL41YFP. (D-F) Vito accumulates at the nucleolus in *put* loss-of-function genotypes. (D) Vito localizes at the nucleolus in control where it strongly accumulates in (E) *ey>put*RNAi$^{7904R-3}$ and (F) *ey>put*RNAi^{37279}. In all panels DNA was labelled with DAPI and shown in blue. (G) Scatter plot representing the nucleolar accumulation of the RpL41YFP protein in salivary glands of the indicated genotypes (n = 25-40; ***, P<1^10^{-4}). Scale bar corresponds to 20 μm.

us to further evaluate the role of TGFβ/Activin signaling in nucleolar structure and ribosome biogenesis. In control cells, immunostaining with αRpL22, αRpL10A, and αRpS6 antibodies showed that these ribosomal proteins are mainly cytoplasmic, possibly reflecting a transient association with pre-ribosome subunits at the nucleolus (Figure 2.5.A-A'', C-C''). Interestingly, in *put*RNAi both RpL22 and Rpl10A are concentrated in the peripheral nucleoplasm and in the nucleolus (Figure 2.5.B-B''), while RpS6 accumulates in granular intra-nucleolar spots (Figure 2.5.D-D'). In comparison with control cells, RpL11 is also found at higher levels in these nucleolar granular spots, where it co-localizes with RpS6 (Figure 2.5.D''). This pattern of

nucleolar accumulation was not a general attribute of all ribosomal proteins. RpS9 is mainly cytoplasmic in control cells and its levels decrease in *put*RNAi without any evidence of nucleolar re-localization (Figure S2.2.).

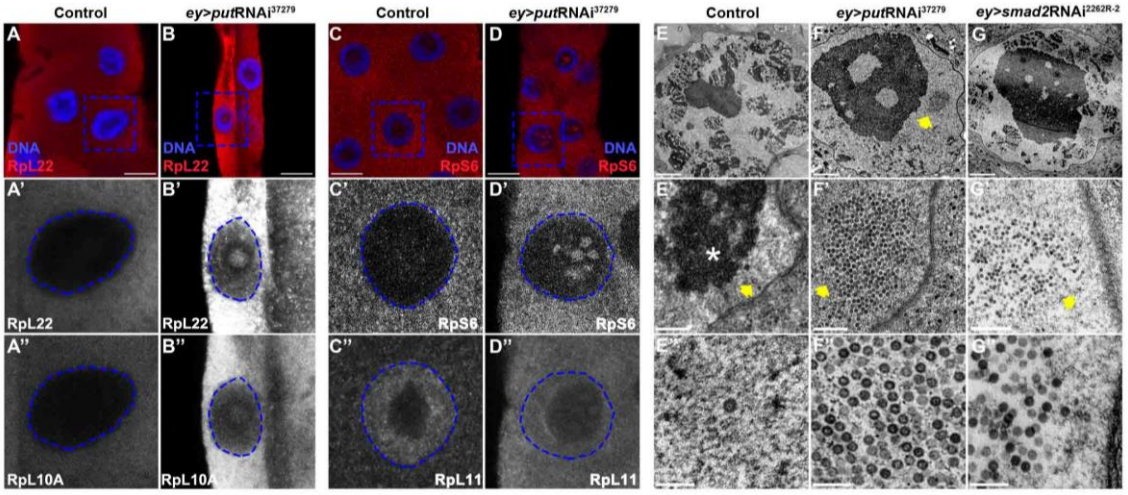

Figure 2.5. The TGFβ/Activin pathway is required for the coordination of the ribosome biogenesis.

(A-D) Analysis of Ribosome proteins (RPs) nucleolar localization. (A-A'') RpL22 and RpL10A are not detectable at the nucleolus in control nuclei. (B-B'') Depletion of *put* results in the accumulation of RpL22 and RpL10A at the nucleoplasm and nucleolus. (C-C'') RpS6 is not present at the nucleoplasm in control salivary glands while RpL11 has peripheral nucleoplasm localization. (D-D'') Decrease of TGFβ/Activin signaling results in nucleolar enrichment of RpS6 and RpL11. Blue squares represent the magnified area presented in A'-D''. Blue dashed circles represent the nuclear area of the respective nuclei. (E-G) Transmission electron micrographs of nuclear regions of independent salivary gland cells and examples of higher magnifications of the nucleoli (E''-G''). (E-E'') Higher magnifications of the control nucleoplasm reveal the nucleolus as the higher electrodense structure. Arrow points to an example of a single nuclear particle and asterisk represents the electrodense structure of the chromatin. In the absence of *put* (F-F''), or *smad2* (G-G''), the hypertrophied nucleolus presents vacuolar-like regions and clusters with a large number of particles (arrows). Scale bars: A-D = 20 μm, E-G = 2 μm, E'-G' = 200nm, E''-G'' = 20nm.

Ultrastructural TEM analysis of salivary gland cells where TGFβ/Activin signaling was inhibited by either *put*RNAi or *smad2*RNAi expression confirmed the presence of nucleolar hypertrophy in these genotypes (Figure 2.5.E-G). Further, low-contrast intranucleolar regions could be observed, although it is unclear if these regions correspond to the accumulation spots for Vito and RpS6 observed using confocal microscopy (Figure 2.4.F and 2.5.D). Moreover, we detected an accumulation of densely packed particles in the nucleoplasm when compared with control cells (*put*RNAi n=23 out of 23 cells from 7 independent salivary glands, Figure 2.5.F-F''; *smad2*RNAi n=10 out of 18 cells from 6 independent salivary glands, Figure 2.5.G-G''). Importantly, only individual particles were found occasionally in controls (Figure 2.5.E'') and are never found as clusters in our control samples (n=0 out of 16 cells analyzed from 5 independent salivary glands, Figure 2.5.E-E''). The size of these particles was on the scale expected for pre-ribosomal intermediates undergoing maturation in the

path from the nucleolus to the cytoplasm (Nissan, Galani et al. 2004). These particles also resemble the particles found when nucleolar stress was induced in *Drosophila* midgut cells by knock down of Nopp140 (He, James et al. 2014). Thus, our results suggest that ribosome biogenesis had been stalled, in which case we would expect to detect alterations in pre-rRNA processing. The rRNA genes are organized in tandem in several arrays that are transcribed as single units (Figure 2.6.A) (Phipps, Charette et al. 2011). After being transcribed by RNA polymerase I, the pre-rRNA is subjected to cleavage, 5' and 3' exonucleolytic digestion, and base modifications to yield the mature 28S, 18S and 5.8S rRNAs (Figure 2.6.A) (Phipps, Charette et al. 2011, Thomson, Ferreira-Cerca et al. 2013). Interestingly, in both *put*RNAi and *smad2*RNAi cells we detected a strong accumulation of uncleaved pre-rRNA intermediates containing the ITS1 region (Figure 2.6.B). To further distinguish whether this accumulation is derived from an increased transcription or an accumulation of the uncleaved pre-RNA intermediates, we quantified the relative abundance of pre-rRNA transcripts containing the ETS (External Transcribed Spacer) region. ETS-containing transcripts are short-

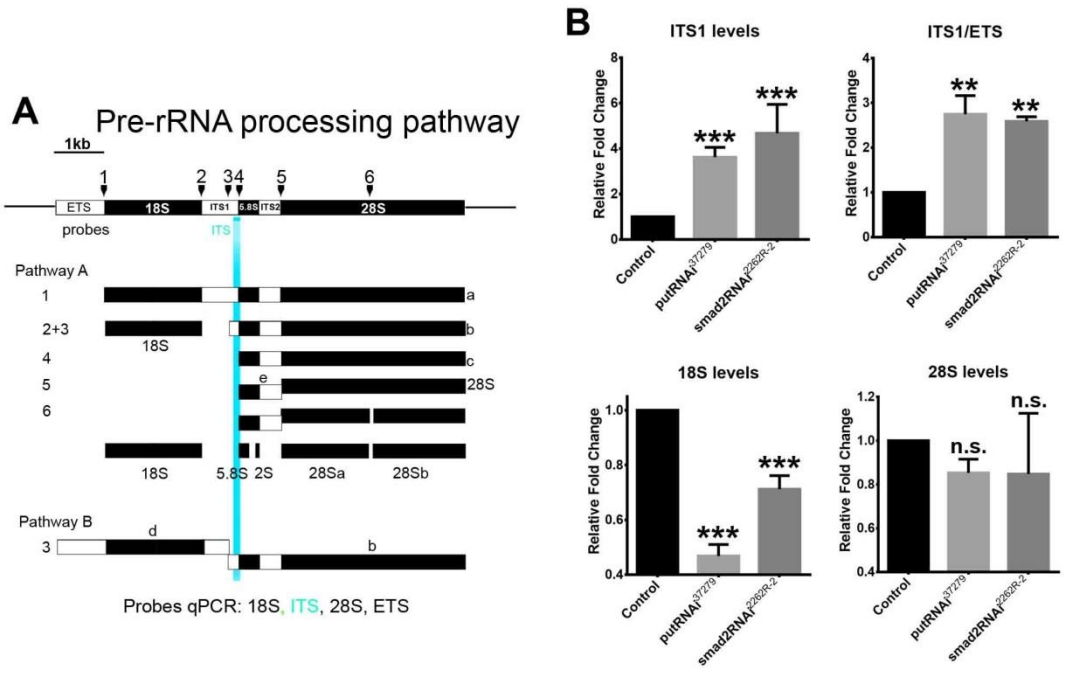

Figure 2.6. The TGFβ/Activin pathway is required for pre-rRNA processing.

(A) Diagram showing the several steps of the pre-rRNA processing. (B) The relative amounts of the ITS1 (target sequence labelled in light blue), ITS1/ETS ratio, 18S and 28S were measured by qPCR using RNA isolated from control larvae salivary glands or salivary glands from larvae expressing the *put*RNAi or *smad2*RNAi. Data are presented as fold change relative to control and indicate the mean + s.e.m (n=5). Data were normalised to the levels of CaMKII mRNA (n.s. means no statistical difference between samples; ***, p<0.001).

lived, as the ETS is the first region to be processed with fast kinetics, and can be used as a proxy for the pre-rRNA transcription rate by the RNA polymerase I. Thus, when the levels of ITS1 were normalized to ETS levels, both TGFβ RNAis present about a 3-fold increase of ITS1-containing intermediate precursors. Furthermore, the levels of the small ribosome subunit 18S rRNA were also significantly reduced in these cells while no significant differences were detected for the 28S rRNA (Figure 2.6.B). Together, these results point towards stalled ribosome biogenesis.

4.3. Put overexpression exacerbates Myc-induced nucleolar hypertrophy and cell growth

The ability of Myc to increase ribosome synthesis is an essential mechanism by which Myc promotes both cell growth and proliferation, as well as tumorigenesis (Ruggero 2009). In both mammalian and *Drosophila* cells, this mechanism requires coordination between nucleolar hypertrophy and the stimulation of pre-rRNA transcription and

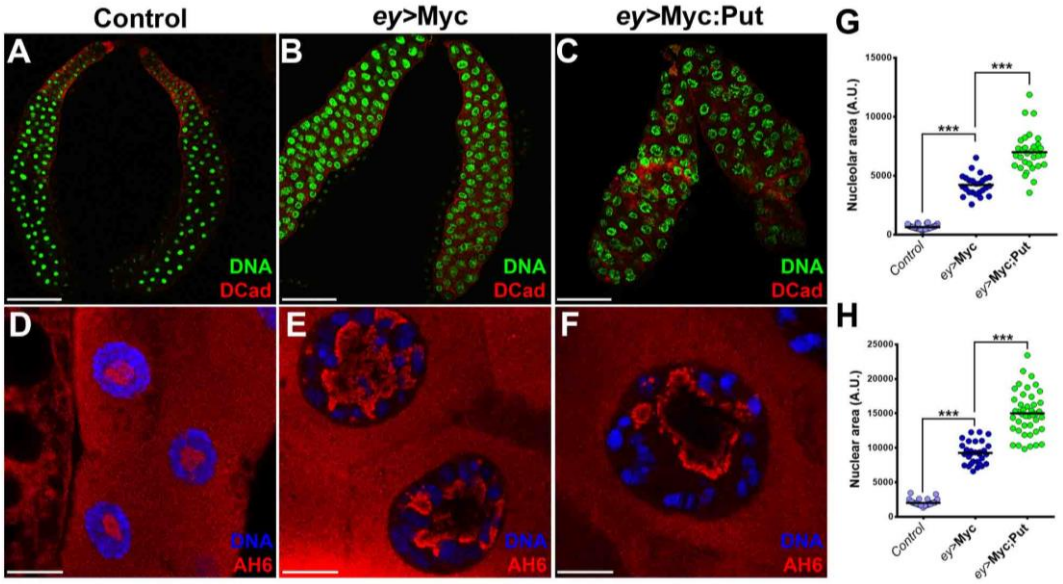

Figure 2.7. Put overexpression cooperates with dMyc to increase nucleolar hypertrophy and cell growth.
(A-C) *Drosophila* salivary gland growth is substantially increased by Myc overexpression (ey>Myc) and is enhanced by TGFβ stimulation. (A) Lower magnification of the control salivary gland. (B) dMyc overexpression results in larger salivary glands with increased nucleolar area. (C) Ectopic expression of Put potentiates dMyc induced nucleolar overgrowth. Salivary glands of the indicated genotypes were stained for the membrane marker DCad (red) and counterstained with DAPI (green). (D-F) Magnification of the nuclei of the indicated genotypes stained with a nucleolar marker AH6 (red) and counterstained with DAPI (blue): (D) Control nuclei showing the cytoplasmic and nucleolar localization of the nucleolar marker AH6. (E) Nuclear and nucleolar overgrowth induced by dMyc overexpression. (F) Nucleolar staining showing the synergistic effect between Put and Myc overexpression. (G) Quantification of nucleolar areas after overexpressing dMyc alone or together with Put. (H) Quantification of nuclear areas after overexpressing dMyc alone or together with Put. (n=40-65), ***, $P<1\text{^}10^{-4}$. Scale bars: A-C = 200 μm, D-F = 20 μm

processing (Arabi, Wu et al. 2005, Grandori, Gomez-Roman et al. 2005, Grewal, Li et al. 2005, Barna, Pusic et al. 2008, Marinho, Casares et al. 2011, Cowling, Turner et al. 2014). Overexpression of dMyc in salivary gland cells resulted in dramatic increases in nucleolar, nuclear and cellular sizes (Figure 2.7.A-B, D-E, G-H) (Pierce, Yost et al. 2004, Marinho, Casares et al. 2011). dMyc expression also increases ploidy in these cells, an effect that has been proposed to be secondary to the strong stimulation of cell growth (Maines, Stevens et al. 2004, Grewal, Li et al. 2005). It has been shown that the transcription factor E2F1 acts as a "growth sensor" coupling rates of endocycle progression to rates of cell growth (Zielke, Kim et al. 2011). Remarkably, we observed that overexpression of Put significantly enhanced the dMyc–stimulated nucleolar, nuclear and cellular growth (Figure 2.7.C, F, G-H). The overexpression of Put, on its own, was not sufficient to induce growth in the salivary glands (data not shown). In support of these observations, in the eye imaginal disc and resulting adult retinas the overexpression of Put was also able to synergize with Myc increasing overall tissue size (Figure S2.3.). These results suggest that TGFβ signaling cooperates with dMyc to control nucleolar function and mass accumulation.

5. Discussion and conclusions

Taken together, our results show that members of the Activin branch of the TGFβ signaling pathway (the RII receptor Put and the R-Smad Smad2) are autonomously required for cell and tissue growth in the *Drosophila* larval salivary gland. In this simple tissue model, cell growth control can be untangled from cell proliferation and ligand gradient control. Previously, the Activin RI receptor Babo and Smad2 were shown to be specifically required for cellular proliferation and overall growth of the wing imaginal disc (Hevia and de Celis 2013). Interestingly, the TGFβ/Activin branch was not found to affect any specific transition of the cell cycle or to cause extensive apoptosis in the wing disc (Hevia and de Celis 2013). Recently, TGFβ signaling was also shown to regulate mitochondrial metabolism in *Drosophila* (Ghosh and O'Connor 2014), and to promote the Warburg effect (aerobic glycolysis) in breast tumours (Guido, Whitaker-Menezes et al. 2012). These results, together with our previous report of a genetic interaction between members of the TGFβ signaling pathways and *vito*, a nucleolar regulator of growth (Marinho, Martins et al. 2013), lead us to focus on the possible regulation of basic mechanisms of cell growth by TGFβ/Activin. We found that interfering with TGFβ/Activin signaling caused changes in nucleolar biogenesis with increased relative areas and altered ultrastructure. Furthermore, this correlated with accumulation of unprocessed intermediate pre-rRNA transcripts, defects in ribosome

biogenesis with a significant decrease in 18S rRNA and very significant effects on the nuclear localization of ribosomal proteins. What are the mechanisms by which TGFβ regulates ribosome biogenesis? TGFβ signaling may regulate the transcription of targets with direct enzymatic roles in pre-RNA processing or ribosome biogenesis and nuclear export. The 5'-3' RNA exonuclease Vito is a strong candidate to fulfil that role. In the budding yeast, the Vito homologue Rrp17p acts as a functional link between late processing of pre-rRNA and nuclear export of pre-60S ribosomal subunits (Oeffinger, Zenklusen et al. 2009). When we interfered with Put function, Vito levels increased but Vito accumulated in intra-nucleolar spots and thus might not be available at the peripheral nucleoplasm to efficiently chaperone pre-ribosomal particles for nuclear export. That would explain the observed accumulation of RpL10A and RpL22 (and putative ribosomal particles detected by TEM) at the peripheral nucleoplasm. At the same time, Rrp17p is required for ITS1 processing (Oeffinger, Zenklusen et al. 2009, Sahasranaman, Dembowski et al. 2011), thus the misregulation of Vito function in salivary gland cells could cause the observed increase in pre-rRNA intermediate transcripts, and the observed accumulation of RpS6 in intra-nucleolar spots.

Could TGFβ regulate ribosome biogenesis through other novel mechanisms? In fact, in breast cancer cells a cancer-specific nuclear translocation of TβRI was shown to regulate nuclear mRNA processing (Chandra, Zang et al. 2012). Furthermore, in mammalian cells TGFβ signaling has also been shown to regulate directly the biogenesis of a set of miRNA at the post-transcriptional level (Davis, Hilyard et al. 2008, Davis, Hilyard et al. 2010). Surprisingly, R-Smads associated with the large Drosha/DGCR8/p68 microprocessor complex have been shown to bind pri-miRNAs and facilitate the cleavage of pri-miRNA to pre-miRNA by Drosha (Davis, Hilyard et al. 2008, Davis, Hilyard et al. 2010). Alternatively, TGFβ may regulate the expression or nucleolar recruitment of ribosomal proteins causing changes in nucleolar dynamics and indirectly affecting pre-rRNA processing. In fact, in Diamond–Blackfan anemia haploinsufficiency for several ribosomal genes has been shown to affect pre-ribosomal RNA (pre-rRNA) processing and thus to interfere with ribosome biogenesis (Ellis 2014). Despite the precise mechanism, we show for the first time that TGFβ/Activin signaling is required for normal assembly of the nucleolus and pre-rRNA processing.

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7. References

Arabi, A., S. Wu, K. Ridderstrale, H. Bierhoff, C. Shiue, K. Fatyol, S. Fahlen, P. Hydbring, O. Soderberg, I. Grummt, L. G. Larsson and A. P. Wright (2005). "c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription." Nat Cell Biol **7**(3): 303-310.

Barna, M., A. Pusic, O. Zollo, M. Costa, N. Kondrashov, E. Rego, P. H. Rao and D. Ruggero (2008). "Suppression of Myc oncogenic activity by ribosomal protein haploinsufficiency." Nature **456**(7224): 971-975.

Boulon, S., B. J. Westman, S. Hutten, F. M. Boisvert and A. I. Lamond (2010). "The nucleolus under stress." Mol Cell **40**(2): 216-227.

Brummel, T., S. Abdollah, T. E. Haerry, M. J. Shimell, J. Merriam, L. Raftery, J. L. Wrana and M. B. O'Connor (1999). "The *Drosophila* activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development." Genes & Development **13**(1): 98-111.

Burke, R. and K. Basler (1996). "Hedgehog-dependent patterning in the *Drosophila* eye can occur in the absence of Dpp signaling." Dev Biol **179**(2): 360-368.

Chandra, M., S. Zang, H. Li, L. J. Zimmerman, J. Champer, A. Tsuyada, A. Chow, W. Zhou, Y. Yu, H. Gao, X. Ren, R. J. Lin and S. E. Wang (2012). "Nuclear translocation of type I transforming growth factor beta receptor confers a novel function in RNA processing." Mol Cell Biol **32**(12): 2183-2195.

Cowling, V. H., S. A. Turner and M. D. Cole (2014). "Burkitt's lymphoma-associated c-Myc mutations converge on a dramatically altered target gene response and implicate Nol5a/Nop56 in oncogenesis." Oncogene **33**(27): 3519-3527.

Datar, S. A., H. W. Jacobs, A. F. de la Cruz, C. F. Lehner and B. A. Edgar (2000). "The *Drosophila* cyclin D-Cdk4 complex promotes cellular growth." Embo J **19**(17): 4543-4554.

Davis, B. N., A. C. Hilyard, G. Lagna and A. Hata (2008). "SMAD proteins control DROSHA-mediated microRNA maturation." Nature **454**(7200): 56-61.

Davis, B. N., A. C. Hilyard, P. H. Nguyen, G. Lagna and A. Hata (2010). "Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha." Mol Cell **39**(3): 373-384.

Ellis, S. R. (2014). "Nucleolar stress in Diamond Blackfan anemia pathophysiology." Biochim Biophys Acta **1842**(6): 765-768.

Gesualdi, S. C. and T. E. Haerry (2007). "Distinct signaling of *Drosophila* Activin/TGF-beta family members." Fly (Austin) **1**(4): 212-221.

Ghosh, A. C. and M. B. O'Connor (2014). "Systemic Activin signaling independently regulates sugar homeostasis, cellular metabolism, and pH balance in *Drosophila* melanogaster." Proc Natl Acad Sci U S A **111**(15): 5729-5734.

Grandori, C., N. Gomez-Roman, Z. A. Felton-Edkins, C. Ngouenet, D. A. Galloway, R. N. Eisenman and R. J. White (2005). "c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I." Nat Cell Biol **7**(3): 311-318.

Grewal, S. S., L. Li, A. Orian, R. N. Eisenman and B. A. Edgar (2005). "Myc-dependent regulation of ribosomal RNA synthesis during *Drosophila* development." Nat Cell Biol **7**(3): 295-302.

Guido, C., D. Whitaker-Menezes, C. Capparelli, R. Balliet, Z. Lin, R. G. Pestell, A. Howell, S. Aquila, S. Ando, U. Martinez-Outschoorn, F. Sotgia and M. P. Lisanti (2012). "Metabolic reprogramming of cancer-associated fibroblasts by TGF-beta drives tumor growth: connecting TGF-beta signaling with "Warburg-like" cancer metabolism and L-lactate production." Cell Cycle **11**(16): 3019-3035.

He, F., A. James, H. Raje, H. Ghaffari and P. DiMario (2014). "Deletion of *Drosophila* Nopp140 induces subcellular ribosomopathies." Chromosoma.

Henderson, K. D. and D. J. Andrew (1998). "Identification of a novel *Drosophila* SMAD on the X chromosome." Biochem Biophys Res Commun **252**(1): 195-201.

Hevia, C. F. and J. F. de Celis (2013). "Activation and function of TGFbeta signalling during *Drosophila* wing development and its interactions with the BMP pathway." Dev Biol **377**(1): 138-153.

Hovhanyan, A., E. K. Herter, J. Pfannstiel, P. Gallant and T. Raabe (2014). "*Drosophila* mbm is a nucleolar myc and casein kinase 2 target required for ribosome biogenesis and cell growth of central brain neuroblasts." Mol Cell Biol **34**(10): 1878-1891.

Inman, G. J. (2011). "Switching TGFbeta from a tumor suppressor to a tumor promoter." Curr Opin Genet Dev **21**(1): 93-99.

Jensen, P. A., X. Zheng, T. Lee and M. B. O'Connor (2009). "The *Drosophila* Activin-like ligand Dawdle signals preferentially through one isoform of the Type-I receptor Baboon." Mech Dev **126**(11-12): 950-957.

Lamouille, S. and R. Derynck (2007). "Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway." J Cell Biol **178**(3): 437-451.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Maines, J. Z., L. M. Stevens, X. Tong and D. Stein (2004). "*Drosophila* dMyc is required for ovary cell growth and endoreplication." Development **131**(4): 775-786.

Marinho, J., F. Casares and P. S. Pereira (2011). "The *Drosophila* Nol12 homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth." Development **138**(2): 349-357.

Marinho, J., T. Martins, M. Neto, F. Casares and P. S. Pereira (2013). "The nucleolar protein Viriato/Nol12 is required for the growth and differentiation progression activities of the Dpp pathway during *Drosophila* eye development." Dev Biol **377**(1): 154-165.

Massague, J. (2012). "TGFbeta signalling in context." Nat Rev Mol Cell Biol **13**(10): 616-630.

Newfeld, S. J., E. H. Chartoff, J. M. Graff, D. A. Melton and W. M. Gelbart (1996). "Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-beta responsive cells." Development **122**(7): 2099-2108.

Nissan, T. A., K. Galani, B. Maco, D. Tollervey, U. Aebi and E. Hurt (2004). "A pre-ribosome with a tadpole-like structure functions in ATP-dependent maturation of 60S subunits." Mol Cell **15**(2): 295-301.

Oeffinger, M., D. Zenklusen, A. Ferguson, K. E. Wei, A. El Hage, D. Tollervey, B. T. Chait, R. H. Singer and M. P. Rout (2009). "Rrp17p is a eukaryotic exonuclease required for 5' end processing of Pre-60S ribosomal RNA." Mol Cell **36**(5): 768-781.

Parker, L., J. E. Ellis, M. Q. Nguyen and K. Arora (2006). "The divergent TGF-beta ligand Dawdle utilizes an activin pathway to influence axon guidance in *Drosophila*." Development **133**(24): 4981-4991.

Parker, L., D. G. Stathakis and K. Arora (2004). "Regulation of BMP and activin signaling in *Drosophila*." Prog Mol Subcell Biol **34**: 73-101.

Phipps, K. R., J. Charette and S. J. Baserga (2011). "The small subunit processome in ribosome biogenesis-progress and prospects." Wiley Interdiscip Rev RNA **2**(1): 1-21.

Pierce, S. B., C. Yost, J. S. Britton, L. W. Loo, E. M. Flynn, B. A. Edgar and R. N. Eisenman (2004). "dMyc is required for larval growth and endoreplication in *Drosophila*." Development **131**(10): 2317-2327.

Ruberte, E., T. Marty, D. Nellen, M. Affolter and K. Basler (1995). "An absolute requirement for both the type II and type I receptors, punt and thick veins, for dpp signaling in vivo." Cell **80**(6): 889-897.

Ruggero, D. (2009). "The role of Myc-induced protein synthesis in cancer." Cancer Res **69**(23): 8839-8843.

Rugjee, K. N., S. Roy Chaudhury, K. Al-Jubran, P. Ramanathan, T. Matina, J. Wen and S. Brogna (2013). "Fluorescent protein tagging confirms the presence of ribosomal proteins at *Drosophila* polytene chromosomes." PeerJ **1**: e15.

Sahasranaman, A., J. Dembowski, J. Strahler, P. Andrews, J. Maddock and J. L. Woolford, Jr. (2011). "Assembly of Saccharomyces cerevisiae 60S ribosomal subunits: role of factors required for 27S pre-rRNA processing." EMBO J **30**(19): 4020-4032.

Salomon, D. (2014). "Transforming growth factor beta in cancer: Janus, the two-faced god." J Natl Cancer Inst **106**(2): djt441.

Sekelsky, J. J., S. J. Newfeld, L. A. Raftery, E. H. Chartoff and W. M. Gelbart (1995). "Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in *Drosophila melanogaste*r." Genetics **139**(3): 1347-1358.

Serpe, M. and M. B. O'Connor (2006). "The metalloprotease tolloid-related and its TGF-beta-like substrate Dawdle regulate *Drosophila* motoneuron axon guidance." Development **133**(24): 4969-4979.

Thomson, E., S. Ferreira-Cerca and E. Hurt (2013). "Eukaryotic ribosome biogenesis at a glance." J Cell Sci **126**(Pt 21): 4815-4821.

Wang, S., J. Huang, J. He, A. Wang, S. Xu, S. F. Huang and S. Xiao (2010). "RPL41, a small ribosomal peptide deregulated in tumors, is essential for mitosis and centrosome integrity." Neoplasia **12**(3): 284-293.

Wiersdorff, V., T. Lecuit, S. M. Cohen and M. Mlodzik (1996). "Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and

propagation of morphogenesis in the *Drosophila* eye." Development **122**(7): 2153-2162.

Wu, L. and R. Derynck (2009). "Essential role of TGF-beta signaling in glucose-induced cell hypertrophy." Dev Cell **17**(1): 35-48.

Yadav, H., C. Quijano, A. K. Kamaraju, O. Gavrilova, R. Malek, W. Chen, P. Zerfas, D. Zhigang, E. C. Wright, C. Stuelten, P. Sun, S. Lonning, M. Skarulis, A. E. Sumner, T. Finkel and S. G. Rane (2011). "Protection from obesity and diabetes by blockade of TGF-beta/Smad3 signaling." Cell Metab **14**(1): 67-79.

Zheng, X., J. Wang, T. E. Haerry, A. Y. Wu, J. Martin, M. B. O'Connor, C. H. Lee and T. Lee (2003). "TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain." Cell **112**(3): 303-315.

Zhu, C. C., J. Q. Boone, P. A. Jensen, S. Hanna, L. Podemski, J. Locke, C. Q. Doe and M. B. O'Connor (2008). "*Drosophila* Activin- and the Activin-like product Dawdle function redundantly to regulate proliferation in the larval brain." Development **135**(3): 513-521.

Zielke, N., K. J. Kim, V. Tran, S. T. Shibutani, M. J. Bravo, S. Nagarajan, M. van Straaten, B. Woods, G. von Dassow, C. Rottig, C. F. Lehner, S. S. Grewal, R. J. Duronio and B. A. Edgar (2011). "Control of *Drosophila* endocycles by E2F and CRL4(CDT2)." Nature **480**(7375): 123-127.

8. Supplementary Information

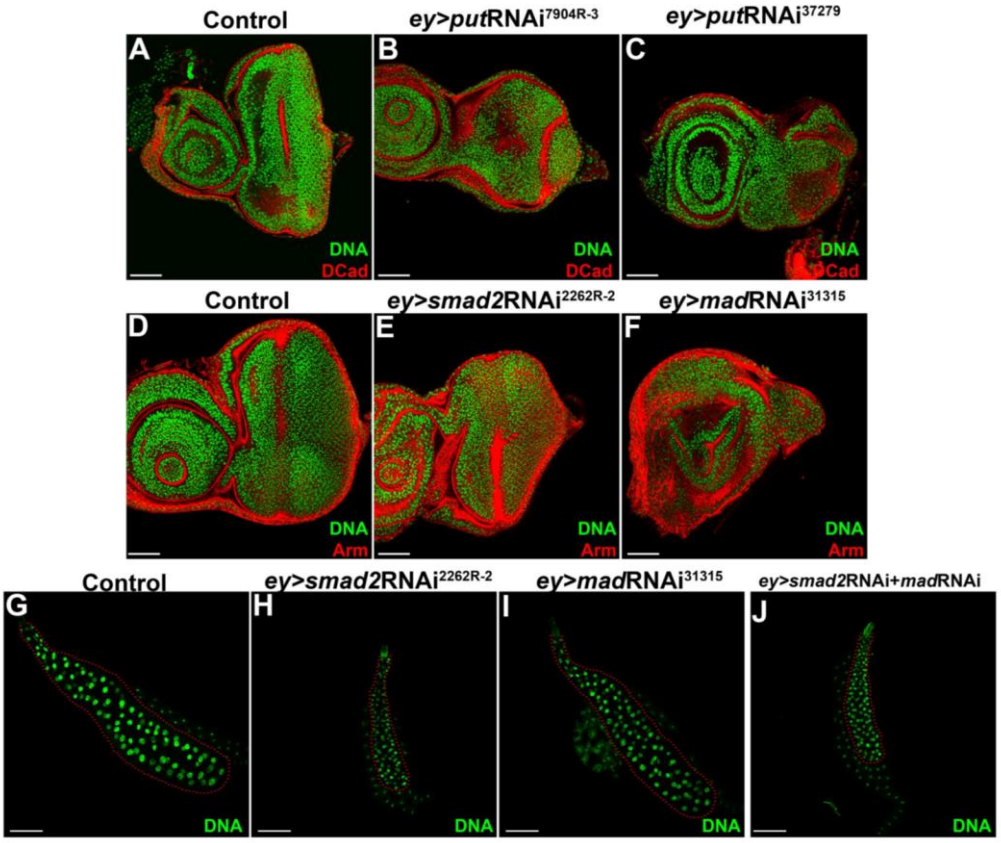

Figure S2.1. Eye and salivary gland phenotypes induced by RNAi for TGF-β signaling pathways. (A-C) put depletion impairs photoreceptors differentiation: (A) Control eye imaginal disc (*ey*>lacZ). (B) *put*RNAi$^{7904R-3}$ induction results in smaller imaginal discs with a severe delay in morphogenetic furrow progression. (C) Strong depletion of *put* affects eye imaginal disc growth and completely abolishes photoreceptor differentiation in L3 imaginal discs. The eye imaginal discs were stained with DAPI (green) and D-Cadherin (red) to label nuclei and cell membrane respectively. (D-F) 3rd instar larvae eye-antennal imaginal discs of R-Smad RNAi depletion genotypes: (D) Control eye imaginal disc (*ey*>lacZ). (E) *smad2*RNAi slightly affects eye imaginal disc size (*ey>smad2*RNAi). (F) *mad*RNAi strongly impairs eye primordia growth (ey>*mad*RNAi). The imaginal discs were labelled for DNA using DAPI (green) and Armadillo for cell limits (red). (G-J) Salivary glands of the R-Smads depletions: (G) Control salivary gland. (H) *smad2*RNAi salivary glands are smaller than controls. (I) Absence of *mad* does not affect salivary glands size. (J) Co-depletion of *smad2* and mad resembles the *smad2* depletion phenotype. All the salivary glands were stained with DAPI (green) and the limits are depicted by a red dashed line. Scale bars: A-F = 50 µm G-J = 200 µm

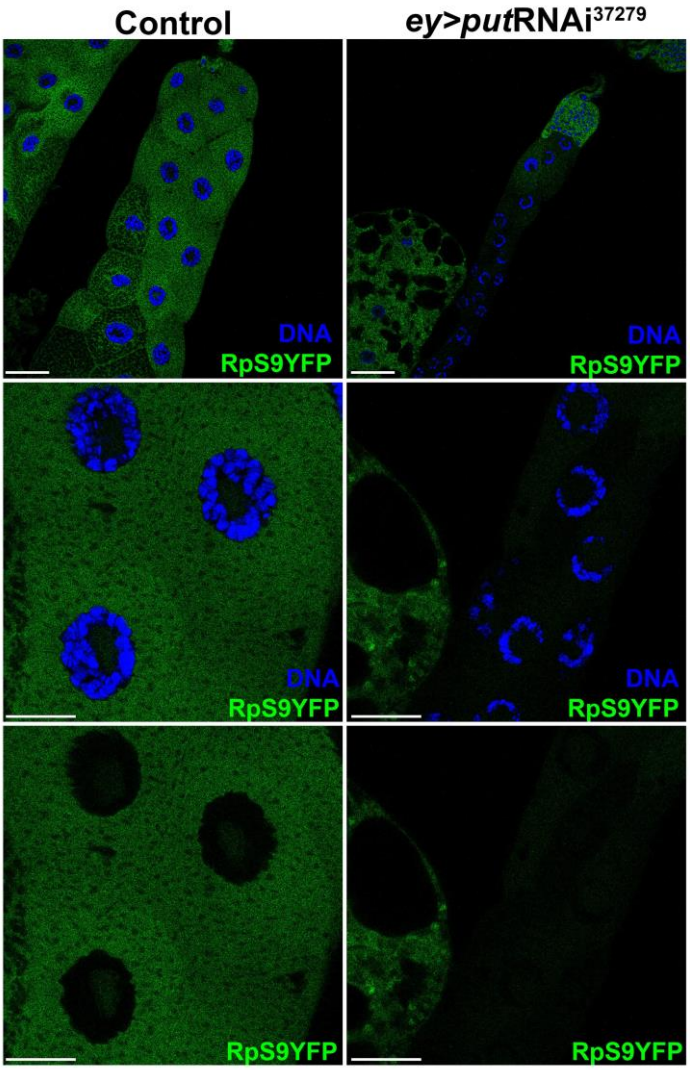

Figure S2.2. Inhibition of TGFβ /Activin signaling activity by *put*RNAi causes a decrease in RpS9 levels. Control salivary glands show cytoplasmic localization of RpS9YFP and a weak accumulation of RpS9 at the nucleolus (left panels). In *put* depleted salivary glands, RpS9YFP signal is severely reduced at the cytoplasm (right panels). Both conditions were stained with DAPI to label the nuclear area. Scale bars: Top panels = 50 µm, Bottom panels = 20 µm

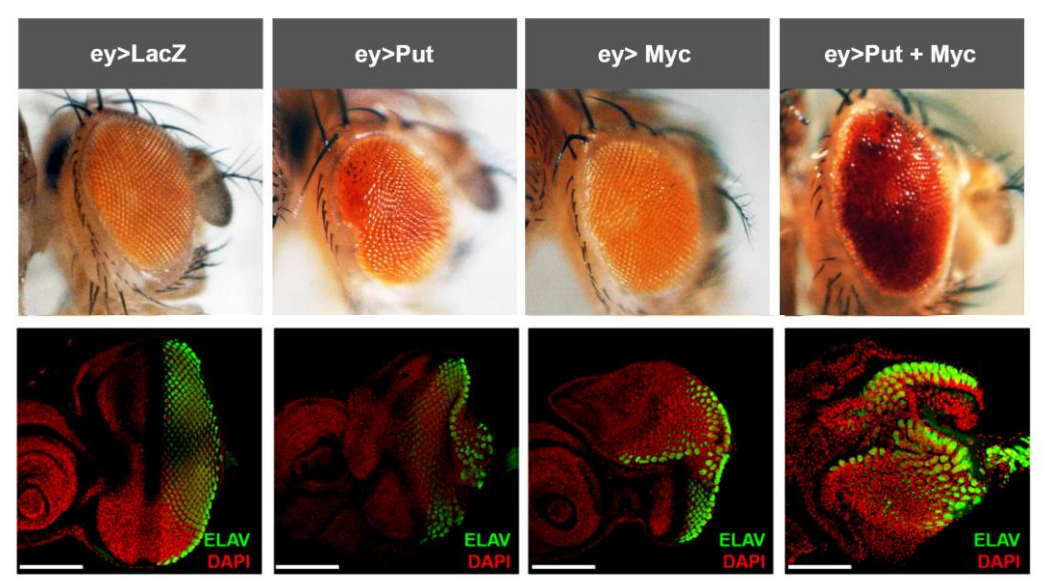

Figure S2.3. Overexpression of Punt synergizes with dMyc in retinal growth. Adult retinas from flies of the indicated genotypes (upper panel). Eye imaginal discs were stained with DAPI (red) and Elav (green) to label nuclei and photoreceptors respectively (lower panels). Scale bar corresponds to 50 µm.

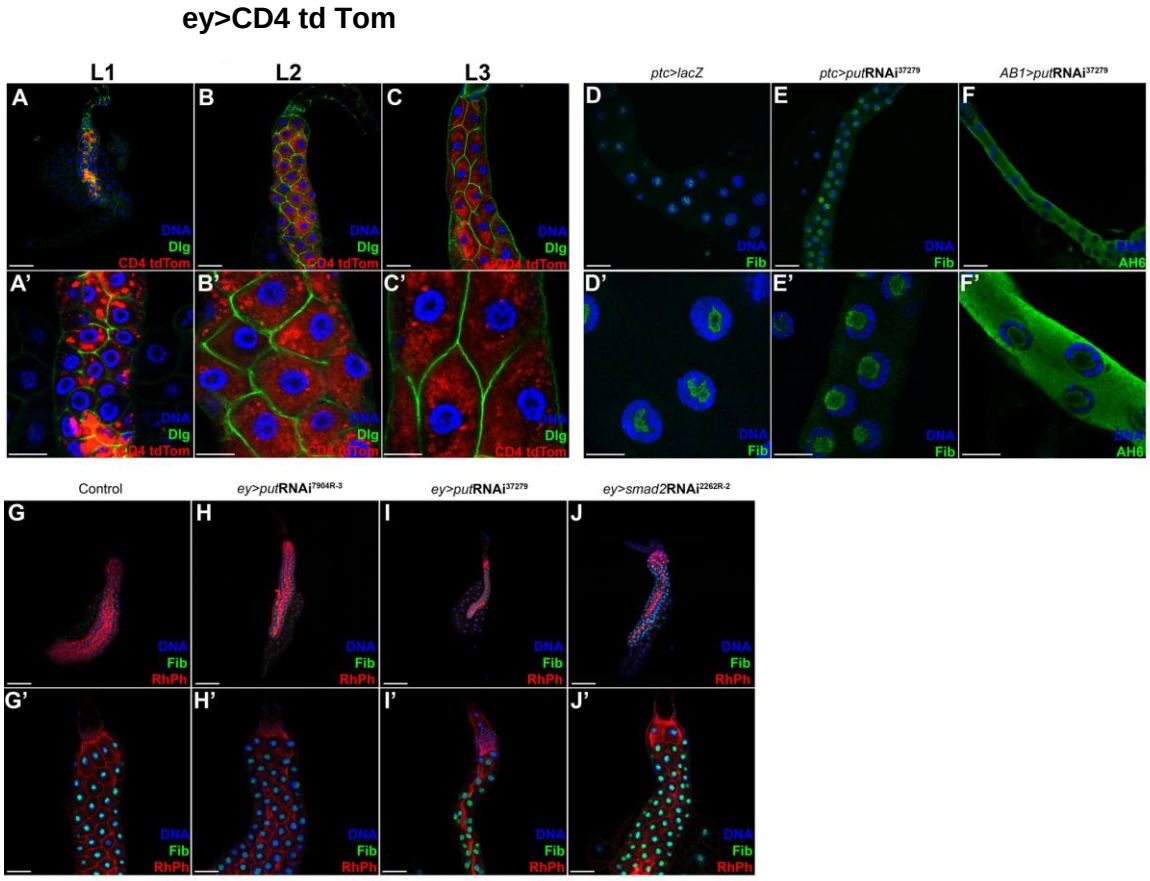

Figure S2.4. Salivary gland expression controls. (A-C) *ey*-Gal4 driven expression of a membrane marker CD4-tdTomato. *ey*-Gal4 expression in salivary glands starts at the beginning of larval development L1 (A-A') and is sustained during the subsequent larval stages, L2 and L3 (B-C'). A-C' were counterstained with DAPI to label the DNA and Dlg to reveal the cellular membrane. (D-F') Depletion of *put* with other salivary gland drivers result in a similar decrease in cellular size and an expansion of the nucleolar size. (D-D') *ptc*>lacZ presents a nucleolar size similar to wild type strains. *ptc*Gal4-driven put depletion results in smaller salivary glands (E) with an expansion on the nucleolar area (E', Fib in green labels the nucleolar area). A similar phenotype is obtained when *put* is depleted using the AB1-Gal4 driver (F,F', AH6 in green labels the nucleolar area). (G-J') TGFβ/Activin decreased activity causes growth deficits in synchronized larvae after restricted egg laying interval (96h-101h AEL). (G-G') Control salivary glands were dissected 96h to 101h after egg laying and stained for the nucleolus (Fib, green) and cellular membrane (RhPh, red). Depletion of *put* (H-I') or *smad2* (J-J') in restricted collections results in a growth deficit with expansion of the nucleolar size as presented in figure 1 and 2. Scale bars: A-F, G'-J' = 50 µm; A'-F' = 20 µm; G-J = 200 µm

CHAPTER 3

An eye-targeted double-RNAi screen reveals negative roles for the Archipelago ubiquitin ligase and CtBP in *Drosophila* Dpp-BMP2/4 signaling

Contents

Manuscript 2: **An eye-targeted double-RNAi screen reveals negative roles for the Archipelago ubiquitin ligase and CtBP in *Drosophila* Dpp-BMP2/4 signaling**

Nadia Eusebio[1,2], Paulo Pereira[1,2*]

TGFβ family plays two different roles during the four days of larval eye development. In the early eye primordium, Dpp promotes growth (mass accumulation) and cell survival, but later on it switches its function to induce a developmentally-regulated cell cycle arrest in the G1 phase, and neuronal photoreceptor differentiation. While some of the Dpp signaling targets required for retinal differentiation have been identified, the characterization of new effectors of cell growth and survival during early eye development is necessary. In order to investigate which genes are involved in those roles, we carried out an in vivo double-RNAi screen to identify genes functioning with *punt* (Type-II TGFβ receptor) during eye development. In this screen, we studied a gene set of about 251 genes previously implicated in eye development by expression profiling experiments. Here, we uncoved a link between the Dpp pathway and two Dpp negative regulators, CtBP and Archipelago (Ago). CtBP and Ago played important roles in the inhibition of Dpp activity promoting regulation of photoreceptor differentiation and eye disc growth. In this study, I participated in designing the experiments, I performed them entirely and analyzed the data. I also participated in the writing of the paper.

An eye-targeted double-RNAi screen reveals negative roles for the Archipelago ubiquitin ligase and CtBP in *Drosophila* Dpp-BMP2/4 signaling.

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1. Abstract

To regulate animal development, complex networks of signaling pathways maintain the correct balance between positive and negative growth signals, ensuring that tissues achieve proper sizes and differentiation patterns. In *Drosophila*, Dpp, a member of the TGFβ family, plays two main roles during larval eye development. In the early eye primordium, Dpp promotes growth and cell survival, but later on, it switches its function to induce a developmentally-regulated cell cycle arrest in the G1 phase and neuronal photoreceptor differentiation. To advance in the identification and characterization of regulators and targets of Dpp signaling required for retinal development, we carried out an *in vivo* eye-targeted double-RNAi screen to identify *punt* (Type II TGFβ receptor) interactors. Using a set of 251 genes associated with eye development, we identified Ago, Brk, CtBP and Dad as negative regulators of the Dpp pathway. Interestingly, both Brk and Ago are negative regulators of tissue growth and Myc activity, and we show that increased tissue growth ability, by overexpression of Myc or CyclinD-Cdk4 is sufficient to partially rescue *punt*-dependent growth and photoreceptor differentiation. Furthermore, we identify a novel role of CtBP in inhibiting Dpp-dependent Mad activation by phosphorylation, downstream or in parallel to Dad, the inhibitory Smad.

2. Introduction

Evolutionarily conserved Transforming Growth Factor β (TGFβ) signaling allows animal cells to drive developmental programmes through the regulation of cellular growth, proliferation, differentiation and morphogenesis. Its importance is reflected in the association of deregulation of this pathway with severe diseases and cancers (Massague, 2012). *Drosophila* Dpp is a ligand member of the TGFβ superfamily that signals through its type II receptors, Punt and Wit, which once activated and phosphorylated bind to type I receptors, Tkv and Sax (Restrepo et al., 2014). In turn, type I receptor phosphorylates the R-Smad, Mad, promoting its homodimerization and the formation of a trimeric complex with the common Co-Smad Medea. This complex is then translocated to the nucleus where it controls the expression of target genes (Dijke and Hill, 2004; Rahimi and Leof, 2007). This signaling pathway is negatively regulated by the I-Smad, Dad, which competes with R-Smads for receptors or Co-Smad interactions (Kamiya et al., 2008; Tsuneizumi et al., 1997). Dpp plays multiple roles in development, including the regulation of patterning and growth of the eye and wing imaginal discs (Akiyama and Gibson, 2015; Restrepo et al., 2014; Romanova-Michaelides et al., 2015). Dpp expression is required for early larval growth of the eye

imaginal disc and mutations that decrease *dpp* expression also result in severely reduced adult retinas (Blackman et al., 1991; Masucci et al., 1990; St Johnston et al., 1990). Supporting these early observations, cell-autonomous activation of the Dpp pathway, through the clonal expression of constitutively-active Tkv^{Q235D} receptor was shown to increase the proliferation of eye progenitor (Firth et al., 2010). Furthermore, Tkv and Mad were shown to act cooperatively with the transcriptional coactivator Yorkie to promote retina growth (Oh and Irvine, 2011). Tissue overgrowths driven by co-expression of retinal progenitors transcription factors Hth (TALE-class homeodomain) and Tsh (zinc finger) in the eye disc causes phosphorylation and activation of Mad and depend on Dpp/BMP2 signaling for growth (Neto et al., 2016). However, the underlying mechanisms of Dpp/BMP-induced growth of the eye disc are poorly understood. Interestingly, we showed that the type II receptor Punt interacts genetically with Vito in both eye disc growth and in the onset of photoreceptor differentiation (Marinho et al., 2013). Vito is a transcriptional target of Myc and encodes an 5' RNA exonuclease regulating rRNA and ribosome biogenesis in the nucleolus (Marinho et al., 2011). The second branch of the TGFβ signaling superfamily, the TGFβ/Activin pathway, has also been shown to be required for cell growth in the salivary glands through the control of ribosome biogenesis (Martins et al., 2017).

Retinal differentiation starts during the late L2 - early L3 larval stage within the morphogenetic furrow (MF), an epithelial indentation that advances from the posterior margin to the anterior region of the eye imaginal disc (Ready et al., 1976). The progression of the MF through the eye imaginal disc, and therefore differentiation of photoreceptors, require the secretion of Hedgehog (Hh) in and behind the MF (Treisman and Heberlein, 1998). Hh controls the expression of Dpp in the MF (Greenwood and Struhl, 1999; Heberlein et al., 1995; Heberlein et al., 1993), which is necessary to switch the progenitor cell state into the precursor state allowing the initiation and progression of retinal differentiation (Bessa et al., 2002; Lopes and Casares, 2010). At this stage, Dpp has been proposed to act by repressing, at long range, transcription of *hth* that is required to maintain cells in a proliferative and undifferentiated progenitor state. Progenitor cells anterior to the furrow divide asynchronously, and Dpp also promotes G1 arrest within the furrow (Firth and Baker, 2009; Horsfield et al., 1998; Penton et al., 1997).

In this work, we studied the regulation of Dpp-BMP2/4 signaling during imaginal eye disc development. We have used an eye-targeted double RNAi screen to identify novel genetic interactions between Dpp-BMP2/4 signaling and other proteins regulating cell growth and differentiation, such as the polyubiquitin ligase component, Ago, and the

transcriptional repressor CtBP. Our detailed characterization of these interactions showed that CtBP and Ago regulated eye development by different processes. Ago regulated eye disc development by promoting the critical size for eye differentiation and CtBP regulated differentiation through a negative regulation of Mad phosphorylation.

3. Material and Methods

3.1. Fly strains and genotypes

All crosses were raised at 25°C under standard conditions. Eye-targeted RNAi knockdown of *punt* was induced by crossing eyeless-Gal4 with UAS-*punt* RNAi, VDRC #37279. The overexpression of the several genes used in this work was performed using UAS-*dad*^{OE}, UAS-*punt*^{OE} (a gift from Konrad Basler), UAS-*CtBP*^{OE} FlyORF #F001239, UAS-*CycA*^{OE}, FlyORF #F001176, UAS-*CycB*^{OE}, FlyORF #F001154, UAS-*CycD*^{OE}, FlyORF #F001220, UAS-*CycD*^{OE} – *Cdk4*^{OE} (Datar et al., 2000b), UAS-*CycE*^{OE}, FlyORF #F001239 and UAS-*myc*^{OE} (a gift from Filipe Josué).

3.2. Generation of Mosaics

Flip-out *punt* RNAi clones were generated by crossing ywhs-flp122; act>y+>Gal4 UAS-GFP females with UAS *punt* RNAi^{37279} males. Clones of cells expressing *punt* RNAi were induced at 48–72 hours after egg laying by 1 hour heat shock at 37°C. Mitotic *CtBP* mutant clones were generated by crossing ey>flip;;*CtBP*^{KG07519} FRT82B/TM6B females with M (3) Ubi GFP FRT82B/TM6B males.

3.3. Double-RNAi screen and genetic interaction scores

All 365 UAS-RNAi (supplementary Table S4.1.) were obtained from VDRC, NIG-Fly stock center (http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp) and Transgenic RNAi Project (TRiP) at Harvard Medical School. Eye-targeted RNAi knockdown was induced by crossing males carrying an inducible UAS-RNAi construct with eyeless-Gal4-UAS-*punt* RNAi females. All crosses were done at 25°C. The flies were examined under a stereomicroscope (Stemi 2000, Zeiss) equipped with a digital camera (Nikon Digital Sight DS-2Mv), and several representative pictures for each transgenic line were taken, if significant alterations in eye size were detected. The genetic interactions of target genes with *punt* RNAi were evaluated by comparing the phenotypes of the double RNAis versus *punt* RNAi as reference. Phenotypes were classified as lethal, sublethal if only less of 10% of the pupae hatched, small (+) retina size, medium (++) retina size if there was a significant increase in the eye size, and strong (+++) if retina

size was similar to wild-type. Moreover, for the double RNAi genotypes that presented a significant increase in the retina size (+++), a supplementary evaluation was performed where adult eye size was evaluated using the following rankings: 0-25% if retinas were absent or severely reduced in size; 25-75% if retinas had moderate size reductions; and >75% if retinas had normal or nearly normal sizes.

3.4. Immunostaining

Immunohistochemistry of dissected eye-antennal discs was performed using standard protocols. Primary antibodies used were: rat anti-Elav 7E8A10 at 1:100 (DSHB Rat-Elav-7E8A10), rabbit anti-CtBP (kind gift of Dr. David Arnosti) at 1:5000 and rabbit anti-P-Smad1/5 41D10 at 1:100 (Cell Signaling 9516) Appropriate Alexa-Fluor conjugated secondary antibodies were from Molecular Probes. Images were obtained with the Leica SP5 confocal system and processed with Adobe Photoshop CS6.

3.5. Western Blot Analysis

For Western Blot analysis, eye imaginal discs were dissected from L3 larvae in lysis buffer (75mM HEPES pH 7.5, 1.5mM EGTA, 1.5 mM $MgCl_2$, 150 mM KCl, 15% glycerol and 0.1% NP-40) containing a complete protease (Roche) and phosphatase (Sigma) inhibitor cocktails. The eye imaginal discs were homogenized with a plastic pestle. Then, the homogenized was sonified twice for 10 sec. Lysates were clarified by centrifugation for 10 min at 4°C and boiled in 1×Laemmli buffer. Protein extracts were separated by 13% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked 1 h at room temperature with 5% milk in tris-buffered saline and then incubated overnight with primary antibodies at 4°C. Antibodies were diluted as follows: rabbit anti-CtBP (Dr. David Arnosti) at 1:10000 and mouse anti-tubulin β-5-1-2 (Santa Cruz Biotech) at 1:100000. Blots were detected using goat anti-rabbit and anti-mouse secondary antibodies and visualized with ECL Blotting Substrates 1:1 (Rio-Rad). A GS-800 calibrated densitometer system was used for quantitative analysis of protein levels.

3.6. Statistical Analysis

GraphPad Prism 5.0 was used for statistical analysis and for generating the graphical output. Statistical significance was determined using an unpaired two-tailed Student's t-test, with a 95% confidence interval, after assessing the normality distribution of the data with the D'Agostino-Pearson normality test.

4. Results

4.1. A *Drosophila* double-RNAi combinatorial screen identifies *punt* interactors during eye development

Eye-targeted knockdown of the type-II receptor *punt* using ey-Gal4 driven expression of UAS-*punt* RNAi causes absence or very strong reduction of adult retinal tissue in the majority of animals (Figure 3.1.; (Marinho et al., 2013; Martins et al., 2017)). Additionally, 42% of ey>*punt* RNAi animals die during pupal stage. In order to identify genes that cooperate with Dpp-BMP2/4 during eye development, we performed a combinatorial double-RNAi test for modifiers of the *punt* RNAi eye phenotype. Using a set of 365 lines, we were able to study 251 different genes (Figure 3.1.A and Table S3.1.). The core of this gene set was described previously (Marinho et al., 2013) and contain genes functionally classified as being involved in eye development, cell cycle, transcription, or translation. We also included several members of signaling pathways important for growth and patterning during eye development, such as Hedgehog (Hh), Notch and Wingless (Wg).

From the 365 RNAi lines tested in combination with ey>*punt* RNAi, 66 lines induced some rescue of the ey>*punt* RNAi absent eye phenotype (Table S3.1.). Within the remaining 299 lines, 232 lines did not modify the ey> *punt* RNAi phenotype and 67 lines enhanced it, having a lethal (58 lines) or sublethal (9 lines) phenotype. From the 66 lines that rescued the eye phenotype of *punt* knockdown, only the knockdown of five candidate genes was able to strongly rescue the phenotype (>75% of the normal retina size). Interestingly, we observed that knocking down either Dad, the I-Smad, or Brk (a transcriptional repressor of Dpp targets) rescued eye development when co-expressed with ey>*punt* RNAi (Figure 3.1.D and 3.1.E) (Bray, 1999; Kamiya et al., 2008). These results support the potential of the eye double-RNAi screen to identify Dpp regulators. The other three genes that presented a strong interaction with *punt* were *ago, CtBP* (Figure 3.1.F and 3.1.G) and *ND75* (data not show). To overcome potential off-target effects in our screen (Dietzl et al., 2007), we tested further available RNAi lines for *punt* interactors, obtaining very similar results to all genotypes (Figure S3.1.), with the exception of *ND75* (data not shown). Flies expressing RNAis against *dad*, *brk*, *ago*, and *CtBP* did not show significant defects in adult eyes (Figure S3.2.).

4.2. CtBP and Ago genetically interact with the Dpp pathway during eye development

Our genetic screen for *punt* interactors during eye development identified a strong genetic interaction with *CtBP* and *ago*. Photoreceptor differentiation was not observed in ey> *punt* RNAi eye imaginal discs (Figure 3.2.A and 3.2.B). However, differentiation was strongly rescued if *CtBP* RNAi or *ago* RNAi were co-expressed, even though a delay of the MF progression at the margins was observed (Figure 3.2.C and 3.2.D). This phenotype is also expressed by partial Dpp loss-of-function genotypes, including the hypomorph dpp^{blk} mutant (Marinho et al., 2013). Additionally, co-depletion of *punt* together with *ago* resulted in eye discs with increased tissue growth compared to *punt* depletion alone (Figure 3.2.D).

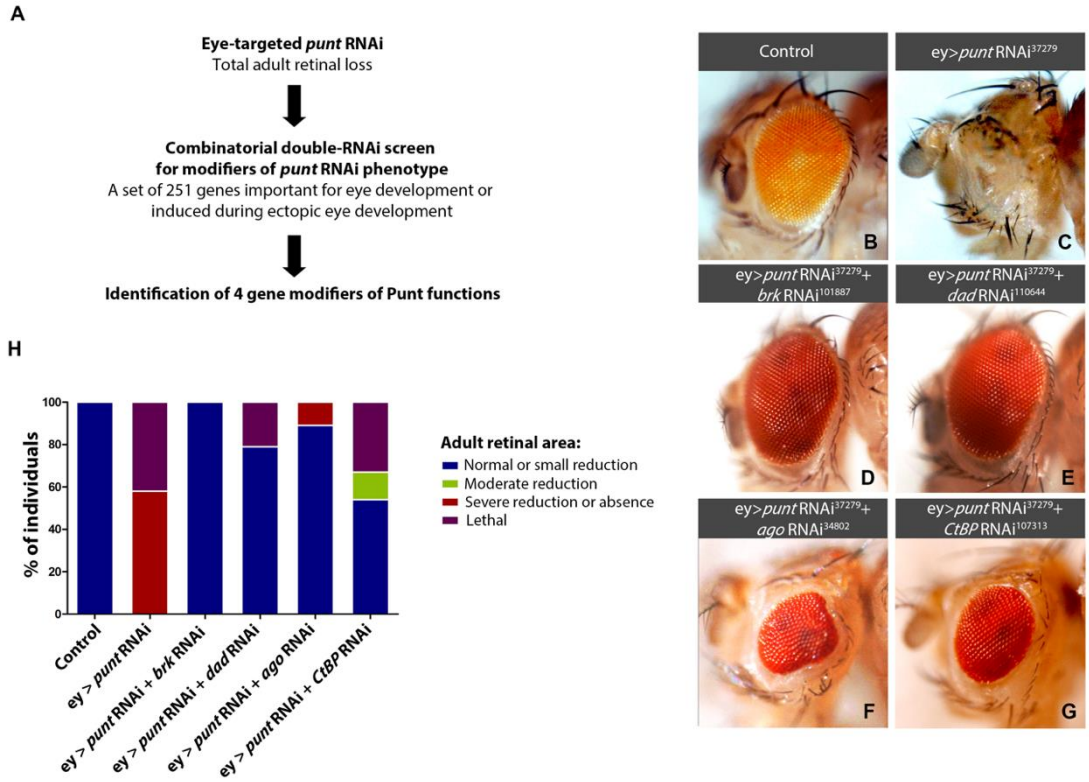

Figure 3.1. Identification of four gene modifiers of Punt loss-of-function phenotype.

(A) Eye-targeted double-RNAi screen approach for identification of genes functioning with *punt* during eye development. (B–G) Representative images of the adult eye phenotypes of the indicated genotypes. ey>*punt* RNAi^{37279} shows a very strong eye phenotype without retinal formation (C). However, the differentiation failure phenotype of ey>*punt* RNAi^{37279} is rescued by *brk* RNAi^{101887} (D), *dad* RNAi^{110644} (E), *ago* RNAi^{34802} (F) and *CtBP* RNAi^{107313} (G). (H) Percentage of individuals of the indicated genotypes presenting normal adult retinal area or small reduction of adult retinal area (+++), moderate reduction of adult retinal area (++), severe reduction or absence of adult retinal area (+ or no retina) and lethality in pupa (43<n<96).

The canonical TGFβ signaling pathway can be divided into two main branches, BMP2/4 and Activin, which are activated by specific ligands but share a requirement for the Punt type-II receptor. Therefore, for further validation we tested the interactions of

CtBP and *ago* with *tkv*, the Dpp BMP2/4 dedicated type-I receptor. Eye-targeted *tkv* knockdown delays progression of photoreceptor differentiation at the eye imaginal disc margins (Marinho et al., 2013), which mimicked the hypomorphic *dpp* mutant phenotype (Chanut and Heberlein, 1997) (Figure 3.2.E and 3.2.A), and a slight reduction in adult eye size (Marinho et al., 2013) (Figure S3.3.). Importantly, these phenotypes were rescued by co-expressing *CtBP* RNAi and *ago* RNAi (Figure 3.2.F and 3.2.G).

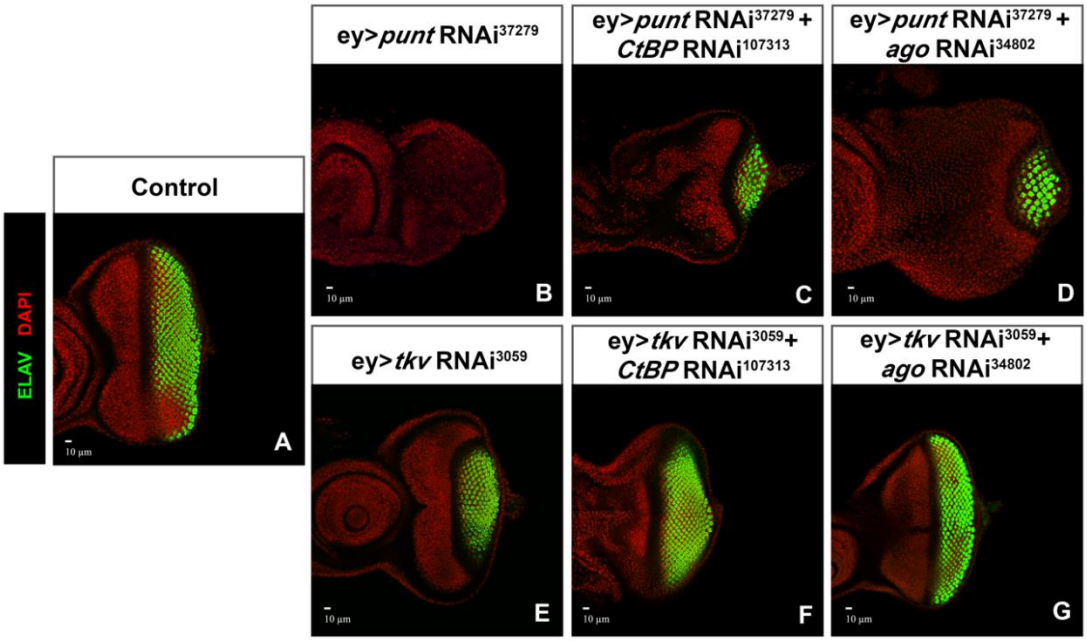

Figure 3.2. CtBP and Ago genetically interact with the Dpp-BMP2/4 signaling.

Downregulation of Dpp signaling using ey>*punt* RNAi^{37279} blocks photoreceptor differentiation (A, B). (C-D) The combinatorial RNAi downregulation of *punt* together with *CtBP* or *ago* partially rescues photoreceptor differentiation (ey>*punt* RNAi^{37279}+*CtBP* RNAi^{107313}; ey>*punt* RNAi^{37279}+*ago* RNAi^{34802}). (E) The downregulation of Dpp signaling using a RNAi for *tkv* leads to an impairment of differentiation progression at eye imaginal disc margins (ey>*tkv* RNAi^{3059}) (F, G). The combinatorial downregulation of *tkv* together with *CtBP* or *ago* by RNAi (ey>*tkv* RNAi^{3059}+*CtBP* RNAi^{107313}; ey> *tkv* RNAi^{3059}+*ago* RNAi^{34802}) rescues the differentiation delay at the margins induced by *tkv* RNAi. Eye discs were stained with DAPI (DNA, red) and anti-ELAV (photoreceptors, green). Scale bars correspond to 10 µm.

4.3. Loss of Archipelago function and conditions that stimulate tissue growth rescue initiation of photoreceptor differentiation caused by Punt depletion.

We observed that knocking down Ago function is sufficient for initiation and progression of photoreceptor differentiation in ey> *punt* RNAi eye discs (Figure 3.2.D and 3.3.A). Ago is an F-box protein that acts as the substrate-receptor component of a Skp/Cullin/F-box (SCF) E3 ubiquitin ligase (SCF-Ago) and targets Myc and CycE for degradation (Moberg et al., 2001; Moberg et al., 2004; Welcker and Clurman, 2008). Loss of Ago in imaginal discs causes an accumulation of Cyclin E and Myc, which drive cell growth and proliferation (Moberg et al., 2001; Moberg et al., 2004). We observed that rescue of *punt* RNAi eye phenotype by *ago* RNAi indeed correlated with increased Myc protein expression (Figure S3.4.). Next, we tested the hypothesis that knockdown of *ago* rescues Dpp BMP2-4 signaling through the detected Myc upregulation, given the previously described genetic interaction between overexpression of Myc and Punt in eye growth and differentiation (Martins et al., 2017). Indeed that was the case, as overexpression of Myc was also sufficient for a partial rescue of differentiation in all the eye discs and adults that we observed (Figure 3.3.B, 3.3.F, 3.3.I). Importantly, this rescue was specific as Myc could not rescue the small retina size caused by expression of a dominant-negative Jak/STAT ligand ($Dome^{\Delta cyt}$) (Tsai and Sun, 2004) (Figure S3.5). As Ago not only targets Myc for degradation but also CycE (Koepp et al., 2001; Moberg et al., 2001), we also tested if the overexpression of CycE ($CycE^{OE}$) rescued *punt* RNAi phenotype (Figure 3.3.C, 3.3.G and 3.3.I). Interestingly, both the overexpression of CycE or of the CycD-Cdk4 (Datar et al., 2000a) led to a weaker, but significant, rescue of *punt* RNAi phenotype while overexpression of the CycB or CycA failed to do so (Figure 3.3.). Taking all together, these results suggest that multiple condition that lead to growth stimulation could be sufficient to promote the onset and progression of the MF, enabling photoreceptor differentiation in eye discs with attenuated or compromised levels of Dpp signaling.

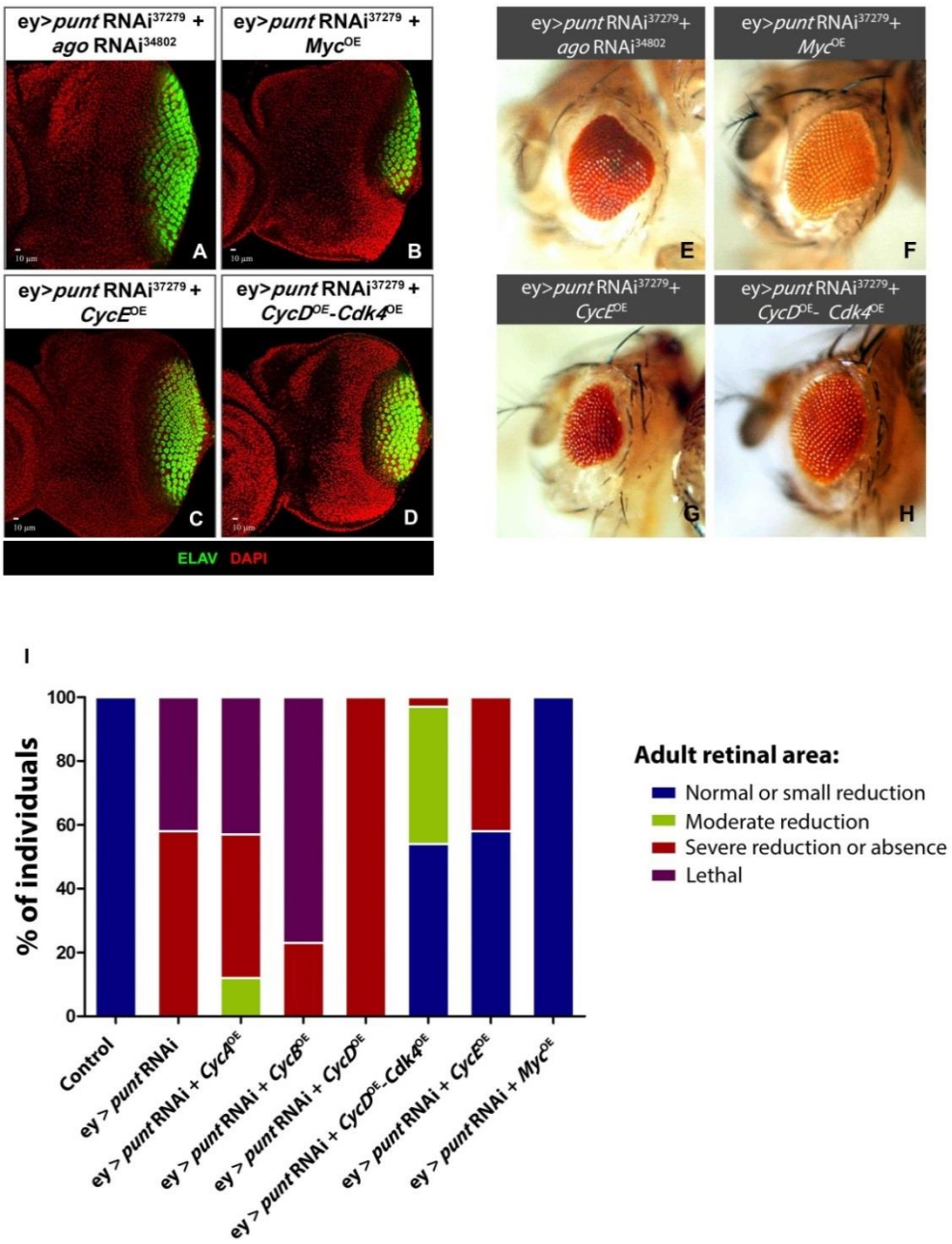

Figure 3.3. Overexpression of Myc, CycE, and CycD-Cdk4 rescue initiation of photoreceptor differentiation caused by Punt depletion.

(A-I) In a similar manner to *ago* RNAi (A, E), overexpression of *Myc* (B, F, I), *CycE* (C, G, I) and *CycD-Cdk4* (D, H, I) recover the initiation of photoreceptor differentiation in eye discs (A-D) and retinal formation (E-H) in the ey>*punt* RNAi^{37279} genetic background. (A-D) Eye imaginal discs of the indicated genotypes stained with DAPI (DNA, red) and anti-ELAV (photoreceptors, green). Scale bars correspond to 10 μm. (M) Percentage of individuals of the indicated genotypes presenting normal adult retinal area or small reduction of adult retinal area (+++), moderate reduction of adult retinal area (++), severe reduction or absence of adult retinal area (+ or no retina) and lethality in pupa (43<n<96).

4.4. CtBP is a negative regulator of Mad activation by phosphorylation

Drosophila CtBP was initially reported as a transcriptional co-repressor able to form complexes with other DNA-binding transcription factors, such as Hairy and Eyeless, to suppress transcription of their target genes (Bianchi-Frias et al., 2004; Hoang et al., 2010; Nibu et al., 1998; Poortinga et al., 1998). Interestingly, *hairy* was shown to be a Dpp target expressed ahead of the MF, where it is proposed to contribute to the pace of furrow movement by restricting expression of atonal, a pro-neural transcription factor

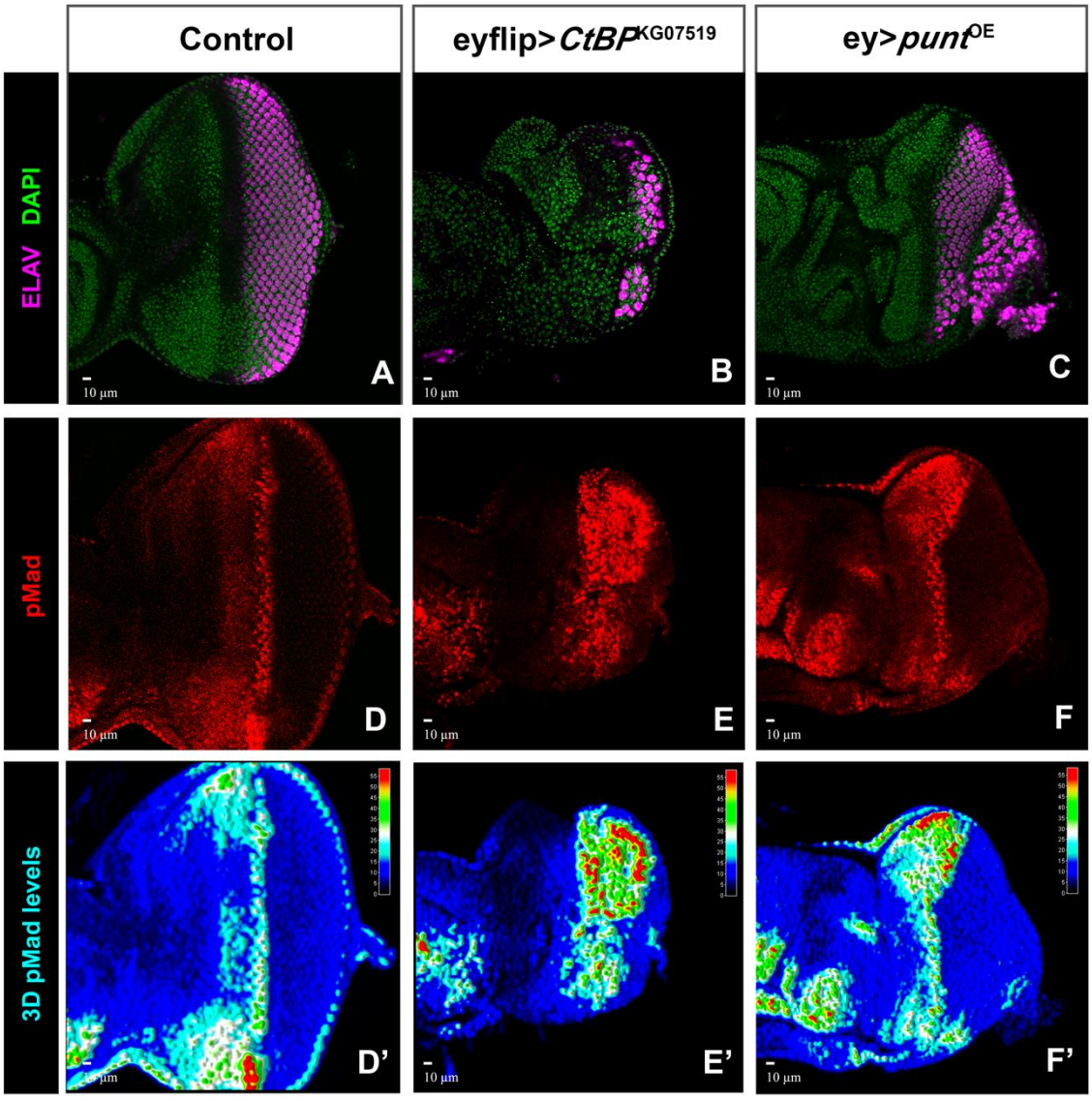

Figure 3.4. CtBP knockdown upregulates Mad activation by phosphorylation

(A-E') *CtBP*^{KG07519} mutant eye discs were generated by eyeless-flippase induction (eyflip>CtBP^{KG07519}). A broad and intense pattern of Mad activation (pMad) is detected (B, E, E'). The induction of Dpp signaling by ey>*punt*^{OE} leads to a precocious differentiation of the eye imaginal disc and pMad detection in regions anterior to the MF (C, F, F'). (D', E', F') 3D histograms of pMad patterns in eye discs of the indicated genotypes. Eye imaginal discs of the indicated genotypes stained with DAPI (DNA, green), anti-ELAV (photoreceptors, magenta) and anti-pMad (red). Scale bars correspond to 10 µm.

(Brown et al., 1995; Greenwood and Struhl, 1999; Spratford and Kumar, 2013). However, a different study could not identify any specific role for Hairy in the regulation of the MF (Bhattacharya and Baker, 2012). Furthermore, CtBP mutant adult retinas were reported to contain more ommatidia than wild-type (Hoang et al., 2010), and CtBP was described to interact with the transcription factor Danr, which contains a PXDLS motif and plays a role in specification and patterning through the regulation of atonal (Curtiss et al., 2007). As we showed above, CtBP works as a negative regulator of Dpp signaling in the eye disc. Thus, we aimed to distinguish whether CtBP works downstream of Mad activation working together with transcription factors regulated by phosphorylated Mad (pMad), like Hairy, or at the level of the Dpp pathway itself, upstream of Mad phosphorylation. For that aim we generated eye discs mostly composed of loss-of-function CtBP mutant cells, by early and extensive induction of mitotic CtBP mutant clones (*CtBP*^{KG07519}) using eyeless-flippase (eyflip>CtBP^{KG07519}) (Figure S3.6. and Figure 3.4.). Strong downregulation of CtBP expression is observed in these eye discs (Figure S4.6.), and we showed that *CtBP*^{KG07519} genetically interacts with *punt* RNAi loss-of-function (Figure S3.7.) validating the interaction identified with the UAS-RNAi lines. Importantly, in *CtBP* mutant discs, we observed a strong upregulation of pMad (Figure 3.4.D, 3.4.D', 3.4.E and 3.4.E'), with increased intensity and extensive broadening anterior to the differentiating cells when compared with control eye discs. As expected, overexpressing *punt* also caused pMad upregulation (Figure 3.4.D, 3.4.D', 3.4.F and 3.4.F'), albeit weaker, which can be attributed to a wider progression of the MF and a sustained downregulation of Mad activation in differentiated cells posterior to the MF. In both genotypes, ey>*punt*^{OE} and eyflip>*CtBP*^{KG07519}, retinal patterning was significantly affected (Figure 3.4.A-C). Remarkably, the overexpression of *CtBP* anterior to the MF, under control of the optix-

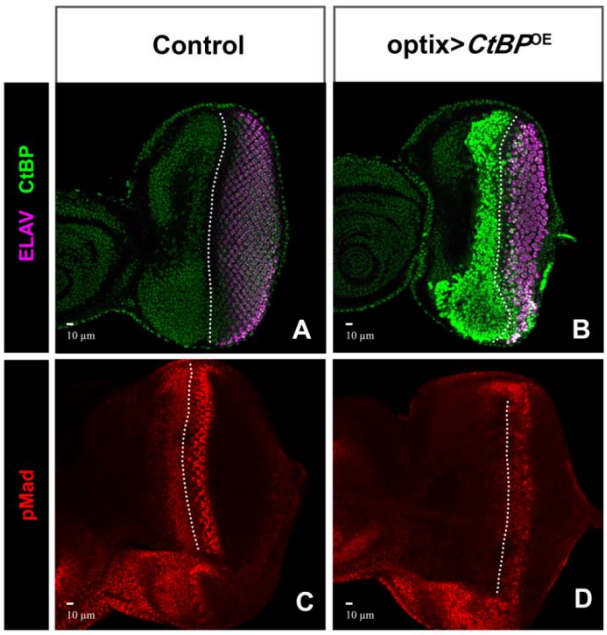

Figure 3.5. CtBP inhibits Mad activation.
(A-D) Eye imaginal discs of *optix*-Gal4>*LacZ* (control) (A and C) and *optix*>*CtBP*^{OE} (B and D) stained with anti-CtBP (green), anti-ELAV (photoreceptors, magenta), and anti-pMad (red). Scale bars correspond to 10 µm. The dashed line marks the morphogenetic furrow (MF).

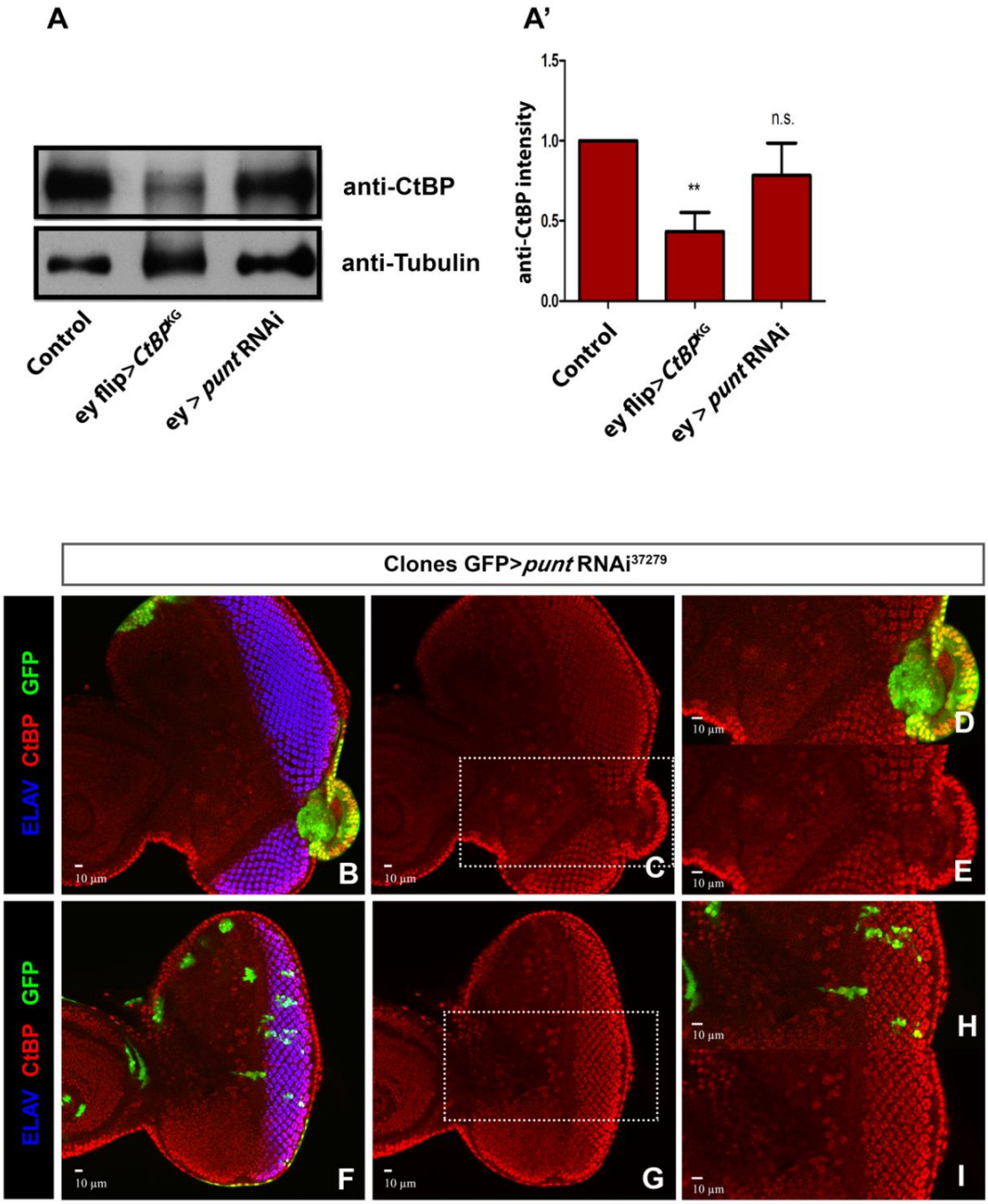

Figure 3.6. The knockdown of Punt does not alter CtBP levels.

(A) Immunoblotting analysis of CtBP in control, *ey*flip> *CtBP*^{KG07519} and ey>*punt* RNAi^{37279} imaginal eye discs lysates. CtBP expression is decreased in *CtBP*^{KG07519} mutant eye discs, however ey>*punt* RNAi^{37279} have similar CtBP protein levels to the control. (A') CtBP band intensities (relative to control) were quantified and the mean values are presented in a bar graph (n = 3). Data are normalized to the levels of control (n.s. means no statistical difference between samples; **p < 0.01; error bars represent SEM). (B-I) *punt* RNAi^{37279} clones were induced in the *Drosophila* eye disc at 48 hours (B, C, D, E) and 72 hours (F, G, H, I) after egg laying and analyzed at the wandering L3 stage. No alterations in CtBP expression are observed. Clones are marked positively by the presence of GFP (green). The imaginal eye discs were stained with anti-CtBP (red) and anti-ELAV (photoreceptors, blue). D-E and H-I show magnifications of the inset shown in C and G, respectively.

Gal4 driver, was sufficient to strongly downregulate Mad activation (Figure 3.5.), without inhibiting Mad protein expression (Figure S3.8.). As CtBP appears to be sufficient for inhibition of Mad activation, we tested if upregulation of CtBP expression levels could contribute to the absence of retinal differentiation in *punt* loss-of-function. However, we could not detect significant changes in CtBP expression when *punt* RNAi was induced using ey-Gal4 or in mitotic clones (Figure 3.6.). Overall, these results show that CtBP is a negative regulator of Dpp signaling in the eye disc acting upstream of Mad activation by phosphorylation.

4.5. CtBP cooperates with Dad for inhibition of Mad activation

An analysis of the interaction of *ago* and *CtBP* with the I-Smad *dad* (Kamiya et al., 2008; Tsuneizumi et al., 1997) revealed additional details on the role of both genes in Dpp signaling during eye disc growth and patterning. Overexpression of Dad inhibits differentiation in imaginal eye discs, as well as in adult eyes (Figure 3.7.B and E), resembling the phenotype caused by *punt* loss-of-function. However, the simultaneous overexpression of *dad* with *CtBP* RNAi (Figure 3.7.C and F) led to a partial recuperation of photoreceptor differentiation. Control imaginal eye discs (Figure 3.7.D') showed a sharp and intense pMad band close to the MF and a broader less intense anterior domain (Firth et al., 2010; Vrailas and Moses, 2006). As expected, in eye imaginal discs overexpressing *dad*, pMad was reduced to residual levels (Figure 3.7.E', Figure S3.9.), supporting the knockdown of Dpp-BMP2/4 signaling pathway by this I-Smad (Kamiya et al., 2008; Tsuneizumi et al., 1997). However, simultaneous overexpression of *dad* with *CtBP* RNAi partially rescued Mad activation in and anterior to MF (Figure 3.7.F'). Furthermore, we observed that simultaneous overexpression of both Dad and CtBP in the anterior domain enhanced the inhibition of Mad activation (Figure S3.10.). These results suggest that Dad requires the function of CtBP for efficient inhibition of Mad phosphorylation and that CtBP acts as a negative regulator of Dpp-BMP2/4 signaling pathway, possibly in parallel and/or downstream of Dad.

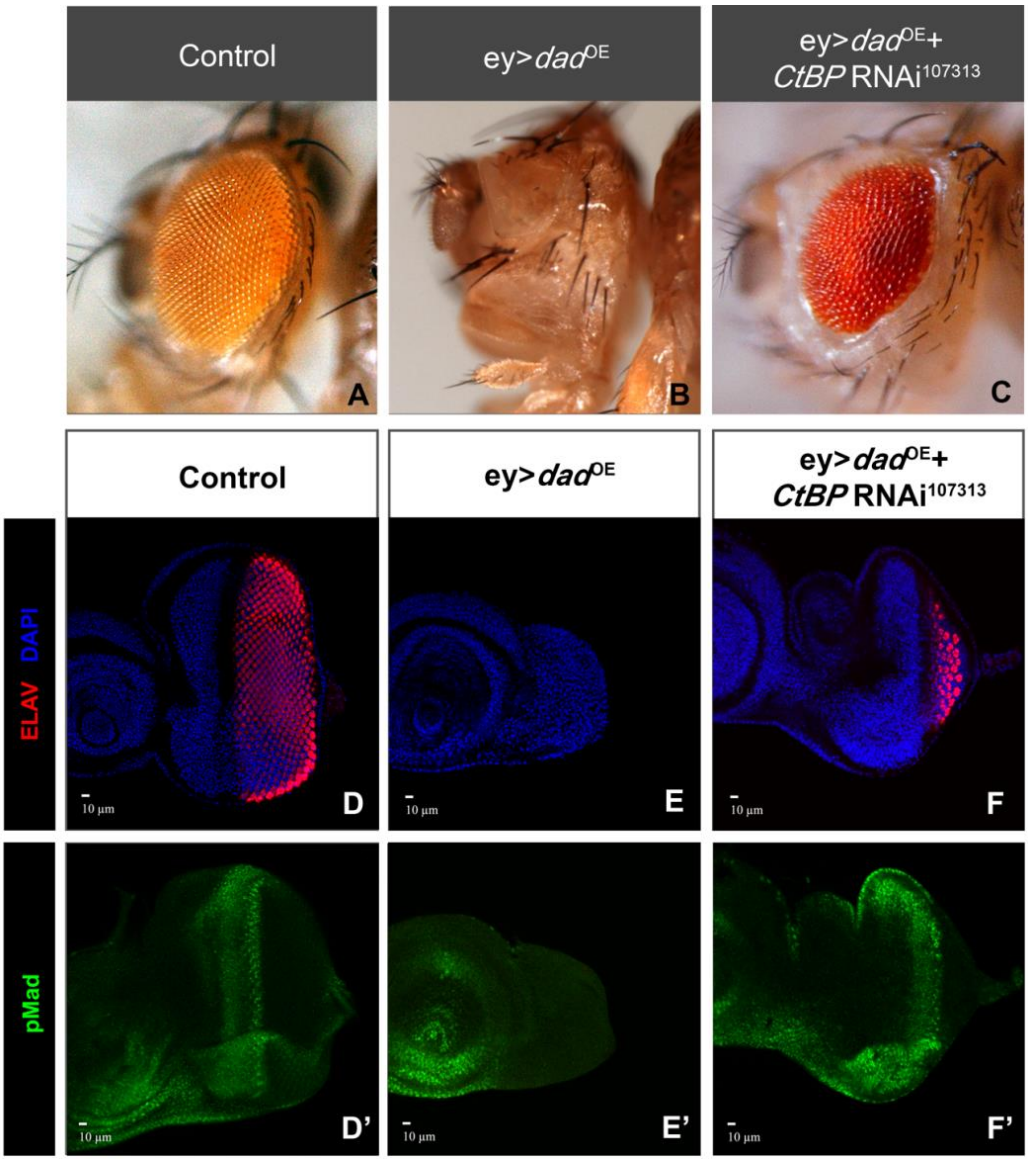

Figure 3.7. CtBP is required for Dad-mediated downregulation of Mad activation

(A–C) ey>*dad*^{OE} shows a very strong eye phenotype without retinal formation (B). However, retinal differentiation is rescued by co-expression of *CtBP* RNAi^{107313} (C). (D-F') The downregulation of Dpp signaling using ey>*dad*^{OE} inhibits photoreceptor differentiation in the eye disc (E). However, the co-expression of RNAi for *CtBP* together with overexpression of *dad* (ey> *dad*^{OE}+*CtBP* RNAi^{107313}) partially rescued differentiation (F). In ey>*dad*^{OE} eye discs, pMad is reduced to low basal levels (E'). Overexpression of Dad together with RNAi for *CtBP* rescued Mad activation (F'). Eye imaginal discs of the indicated genotypes were stained with DAPI (DNA, blue), anti-ELAV (photoreceptors, red), and anti-pMad (green). Scale bars correspond to 10 µm.

5. Discussion

The development of the *Drosophila* eye has served as a model system to study tissue patterning and cell-cell communication. Several key signaling pathways are conserved from flies to vertebrates, including TGFβ, Hh, Wg/Wnt, Notch, EGFR and JAK/STAT pathways (Zecca, Basler et al. 1995, Lee and Treisman 2001, Bach, Vincent et al.

2003, Reynolds-Kenneally and Mlodzik 2005). Dpp-BMP2/4 signaling plays an essential role in *Drosophila* development but we still have an incomplete knowledge of the regulation and functions of the Dpp-BMP2/4 during eye differentiation. Therefore, in this work we have taken advantage of an eye-targeted combinatorial screen to analyze the contribution of new Dpp-BMP2/4 genetic interactors. We studied a set of 251 genes with identified or putative functions in eye development (Marinho et al., 2013), and we identified four genes whose knockdown was able to significantly rescue eye-targeted loss-of-function for *punt* receptor: *brk*, *dad*, *ago* and *CtBP*. Co-induction of RNAi for each of these hits was able to rescue absence of retinal differentiation caused by *punt* RNAi expression (Figure 3.1.) suggesting that these four candidate genes act as negative modulators of Dpp-BMP2/4 signaling in the eye disc.

The transcription factor Brk is a negative repressor of Dpp signaling which competes with activated Mad, blocking the stimulation of Dpp target genes (Bray, 1999; Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Saller and Bienz, 2001). In the eye imaginal disc, Brk expression is detected at the very anterior region of the disc (Firth et al., 2010), and clonal *brk*-overexpression blocks the onset of the MF when clones are positioned along the disc margins (Baonza and Freeman, 2001), which represents a Dpp signaling loss-of-function phenotype. In the wing disc, the mechanisms by which Dpp controls patterning and growth have been intensively studied (Akiyama and Gibson, 2015; Barrio and Milan, 2017; Martin et al., 2017; Matsuda and Affolter, 2017; Sanchez Bosch et al., 2017). Dpp is expressed along the Anterior/Posterior boundary and the resulting gradient is required to establish distinct expression domains for targets (including *salm* and *omb*) involved in patterning. However, formation of a Dpp gradient is not required for its ability to repress *brk* transcription and promote tissue growth (Sanchez Bosch et al., 2017). Here, we demonstrate that also in the eye disc, the requirement for Punt function, and therefore Dpp signaling, in growth and retinal differentiation can be bypassed by removing Brk repression, leading to a differentiated eye.

We also identified Ago, the *Drosophila* orthologous of Fbw7 in mammals and the F-box specificity subunit of the SCF-Ago E3 ubiquitin ligase, as a negative regulator of Dpp signaling. Ago protein is involved in cell growth inhibition by ubiquitination of several proteins, such as Myc and CycE (Moberg et al., 2001; Moberg et al., 2004). Loss of Ago in imaginal discs causes an accumulation of CycE and Myc, which drive cell growth and proliferation (Moberg et al., 2004). The rescue of retinal differentiation induced by *ago* knockdown in a *punt* RNAi background was mimicked by overexpression of Myc. Interestingly, Myc was identified as a target of Brk repression in

the wing disc, where it was proposed that Dpp signaling inhibits Brk, thereby inducing expression of Myc that contributes significantly to Dpp-stimulated tissue growth (Doumpas et al., 2013). Thus, both Brk and Ago functions converge on the downregulation of Myc expression, at the transcriptional and post-transcriptional level, respectively. This supports the hypothesis that the mechanism for their genetic interaction with Punt that we identified in this study includes the contribution of Myc upregulation and tissue growth. We have also recently demonstrated that overexpression of Myc and Punt is able to enhance tissue growth and retinal differentiation in the eye disc (Martins et al., 2017). In here, we also show that other growth-stimulating conditions like overexpression of CycE or CycD-Cdk4 (Datar et al., 2000a) can partially rescue Punt knockdown. Overall, our results suggest that in the eye discs with reduced Dpp signaling, promoting tissue growth is sufficient to create the conditions required for the Dpp-dependent initiation of photoreceptor differentiation at the disc margins.

A third Dpp negative regulator identified in here was the I-Smad, Dad. In mammals the Dad orthologous, Smad 6 and 7, downregulate TGFβ signaling pathway by competing with R-Smads for receptors or for co-Smad interactions and also by targeting the receptors for degradation (Miyazawa and Miyazono, 2016). In *Drosophila*, Dad stably associates with Tkv receptor and thereby inhibits Tkv-induced Mad phosphorylation and nuclear translocation (Inoue et al., 1998; Kamiya et al., 2008). In the eye disc, overexpression of Dad in the Dpp-expression domain was shown to block the MF at the disc margins (Niwa et al., 2004), and pMad levels are upregulated in dad^{212} mutant clones (Ogiso et al., 2011). In here, we identify a significant role for Dad negative regulation of Dpp signaling in eye development, acting downstream of Punt receptor activity.

In this work we showed that knockdown of CtBP activity can compensate for a reduced level of Punt function, rescuing Dpp signaling to levels sufficient to restore retinal differentiation in a *punt* RNAi background. CtBP is a transcriptional repressor that functions as part of a complex containing enzymes that influence transcription by covalently modifying histones and influencing nucleosome packing and the binding of chromatin-associated proteins (Chen et al., 1999; Chinnadurai, 2002; Kim et al., 2005). Acting as a transcriptional co-repressor CtBP has been proposed to have both positive and negative contributions for Dpp/BMP signaling efficiency. On one hand, for a positive contribution, CtBP contributes to Shn/Mad/Med repression activity (Yao et al., 2008), which mediates Dpp-dependent Brk repression. However *brk* is not ectopically expressed in CtBP clones in the wing disc (Hasson et al., 2001) suggesting that CtBP

is not essential for Dpp signaling activation in that tissue. On the other hand, in mammalian cells CtBP interacts with Smad6 to repress BMP-dependent transcription (negative input), a nuclear Smad6 role that is independent of its binding to receptors (Lin et al., 2003). Our results suggest that the interaction between CtBP and I-Smad could be conserved in *Drosophila*, as the Dpp repression by Dad requires the function of CtBP. Additionally, we showed that CtBP works upstream of Mad activation, in parallel or downstream to Dad. Interestingly, we could not identify CtBP-interaction motifs (PxDLS) in Dad, the *Drosophila* I-Smad, suggesting that distinct molecular mechanisms support the *CtBP-dad* genetic interaction and the negative regulation of Mad activation exerted by CtBP expression in the eye disc (Figure 3.5. and 3.6.).

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7. References

Akiyama, T., Gibson, M.C., 2015. Decapentaplegic and growth control in the developing *Drosophila* wing. Nature 527, 375-378.

Baonza, A., Freeman, M., 2001. Notch signalling and the initiation of neural development in the *Drosophila* eye. Development 128, 3889-3898.

Barrio, L., Milan, M., 2017. Boundary Dpp promotes growth of medial and lateral regions of the *Drosophila* wing. eLife 6, e22013.

Bessa, J., Gebelein, B., Pichaud, F., Casares, F., Mann, R.S., 2002. Combinatorial control of *Drosophila* eye development by Eyeless, Homothorax, and Teashirt. Genes & Development 16, 2415-2427.

Bhattacharya, A., Baker, N.E., 2012. The role of the bHLH protein hairy in morphogenetic furrow progression in the developing *Drosophila* eye. PloS one 7, e47503.

Bianchi-Frias, D., Orian, A., Delrow, J.J., Vazquez, J., Rosales-Nieves, A.E., Parkhurst, S.M., 2004. Hairy Transcriptional Repression Targets and Cofactor Recruitment in *Drosophila*. PLOS Biology 2, e178.

Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T., Gelbart, W.M., 1991. An extensive 3′ cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in *Drosophila*. Development 111, 657-666.

Bray, S., 1999. DPP on the brinker. Trends Genet 15, 140.

Brown, N.L., Sattler, C.A., Paddock, S.W., Carroll, S.B., 1995. Hairy and emc negatively regulate morphogenetic furrow progression in the *Drosophila* eye. Cell 80, 879-887.

Campbell, G., Tomlinson, A., 1999. Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*. Cell 96, 553-562.

Chanut, F., Heberlein, U., 1997. Role of decapentaplegic in initiation and progression of the morphogenetic furrow in the developing *Drosophila* retina. Development 124, 559.

Chen, G., Fernandez, J., Mische, S., Courey, A.J., 1999. A functional interaction between the histone deacetylase Rpd3 and the corepressor Groucho in *Drosophila* development. Genes & Development 13, 2218-2230.

Chinnadurai, G., 2002. CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Mol. Cell 9, 213-224.

Curtiss, J., Burnett, M., Mlodzik, M., 2007. distal antenna and distal antenna-related function in the retinal determination network during eye development in *Drosophila*. Dev Biol 306, 685-702.

Datar, S.A., Jacobs, H.W., de la Cruz, A.F.A., Lehner, C.F., Edgar, B.A., 2000a. The *Drosophila* Cyclin D–Cdk4 complex promotes cellular growth.

Datar, S.A., Jacobs, H.W., de la Cruz, A.F.A., Lehner, C.F., Edgar, B.A., 2000b. The *Drosophila* Cyclin D–Cdk4 complex promotes cellular growth. The EMBO Journal 19, 4543-4554.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., Dickson, B.J., 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature 448, 151-156.

Dijke, P.t., Hill, C.S., 2004. New insights into TGF-β–Smad signalling. Trends in Biochemical Sciences 29, 265-273.

Doumpas, N., Ruiz-Romero, M., Blanco, E., Edgar, B., Corominas, M., Teleman, A.A., 2013. Brk regulates wing disc growth in part via repression of Myc expression. EMBO Reports 14, 261-268.

Firth, L.C., Baker, N.E., 2009. Retinal determination genes as targets and possible effectors of extracellular signals. Developmental Biology 327, 366-375.

Firth, L.C., Bhattacharya, A., Baker, N.E., 2010. Cell cycle arrest by a gradient of Dpp signaling during *Drosophila* eye development. BMC Developmental Biology 10, 28-28.

Greenwood, S., Struhl, G., 1999. Progression of the morphogenetic furrow in the *Drosophila* eye: the roles of Hedgehog, Decapentaplegic and the Raf pathway. Development 126, 5795-5808.

Hasson, P., Müller, B., Basler, K., Paroush, Z.e., 2001. Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. The EMBO Journal 20, 5725-5736.

Heberlein, U., Singh, C.M., Luk, A.Y., Donohoe, T.J., 1995. Growth and differentiation in the *Drosophila* eye coordinated by *hedgehog*. Nature 373, 709-711.

Heberlein, U., Wolff, T., Rubin, G.M., 1993. The TGFβ homolog *dpp* and the segment polarity gene *hedgehog* are required for propagation of a morphogenetic wave in the *Drosophila* retina. Cell 75, 913-926.

Hoang, C.Q., Burnett, M.E., Curtiss, J., 2010. *Drosophila* CtBP regulates proliferation and differentiation of eye precursors and complexes with Eyeless, Dachshund, Dan, and Danr during eye and antennal development. Developmental Dynamics 239, 2367-2385.

Horsfield, J., Penton, A., Secombe, J., Hoffman, F.M., Richardson, H., 1998. decapentaplegic is required for arrest in G1 phase during *Drosophila* eye development. Development 125, 5069-5078.

Inoue, H., Imamura, T., Ishidou, Y., Takase, M., Udagawa, Y., Oka, Y., Tsuneizumi, K., Tabata, T., Miyazono, K., Kawabata, M., 1998. Interplay of Signal Mediators of Decapentaplegic (Dpp): Molecular Characterization of Mothers against dpp, Medea, and Daughters against dpp. Molecular Biology of the Cell 9, 2145-2156.

Jaźwińska, A., Kirov, N., Wieschaus, E., Roth, S., Rushlow, C., 1999. The *Drosophila* Gene *brinker* Reveals a Novel Mechanism of Dpp Target Gene Regulation. Cell 96, 563-573.

Kamiya, Y., Miyazono, K., Miyazawa, K., 2008. Specificity of the inhibitory effects of Dad on TGF-β family type I receptors, Thickveins, Saxophone, and Baboon in *Drosophila*. FEBS Letters 582, 2496-2500.

Kim, J.-H., Cho, E.-J., Kim, S.-T., Youn, H.-D., 2005. CtBP represses p300-mediated transcriptional activation by direct association with its bromodomain. Nat Struct Mol Biol 12, 423-428.

Koepp, D.M., Schaefer, L.K., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., Elledge, S.J., 2001. Phosphorylation-Dependent Ubiquitination of Cyclin E by the SCF^{Fbw7} Ubiquitin Ligase. Science 294, 173-177.

Lin, X., Liang, Y.-Y., Sun, B., Liang, M., Shi, Y., Brunicardi, F.C., Shi, Y., Feng, X.-H., 2003. Smad6 Recruits Transcription Corepressor CtBP To Repress Bone Morphogenetic Protein-Induced Transcription. Molecular and Cellular Biology 23, 9081-9093.

Lopes, C.S., Casares, F., 2010. *hth* maintains the pool of eye progenitors and its downregulation by Dpp and Hh couples retinal fate acquisition with cell cycle exit. Developmental Biology 339, 78-88.

Marinho, J., Casares, F., Pereira, P.S., 2011. The *Drosophila Nol12* homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth. Development 138, 349-357.

Marinho, J., Martins, T., Neto, M., Casares, F., Pereira, P.S., 2013. The nucleolar protein Viriato/Nol12 is required for the growth and differentiation progression activities of the Dpp pathway during *Drosophila* eye development. Developmental Biology 377, 154-165.

Martin, M., Ostale, C.M., de Celis, J.F., 2017. Patterning of the *Drosophila* L2 vein is driven by regulatory interactions between region-specific transcription factors expressed in response to Dpp signalling. Development.

Martins, T., Eusebio, N., Correia, A., Marinho, J., Casares, F., Pereira, P.S., 2017. TGFβ/Activin signalling is required for ribosome biogenesis and cell growth in *Drosophila* salivary glands. Open Biology 7.

Massague, J., 2012. TGFβ signalling in context. Nat Rev Mol Cell Biol 13, 616-630.

Masucci, J.D., Miltenberger, R.J., Hoffmann, F.M., 1990. Pattern-specific expression of the *Drosophila* decapentaplegic gene in imaginal disks is regulated by 3' cis-regulatory elements. Genes & Development 4, 2011-2023.

Matsuda, S., Affolter, M., 2017. Dpp from the anterior stripe of cells is crucial for the growth of the *Drosophila* wing disc. eLife 6, e22319.

Miyazawa, K., Miyazono, K., 2016. Regulation of TGF-β Family Signaling by Inhibitory Smads. Cold Spring Harbor Perspectives in Biology.

Moberg, K.H., Bell, D.W., Wahrer, D.C.R., Haber, D.A., Hariharan, I.K., 2001. Archipelago regulates Cyclin E levels in *Drosophila* and is mutated in human cancer cell lines. Nature 413, 311-316.

Moberg, K.H., Mukherjee, A., Veraksa, A., Artavanis-Tsakonas, S., Hariharan, I.K., 2004. The *Drosophila* F Box Protein Archipelago Regulates dMyc Protein Levels In Vivo. Current Biology 14, 965-974.

Neto, M., Aguilar-Hidalgo, D., Casares, F., 2016. Increased avidity for Dpp/BMP2 maintains the proliferation of progenitors-like cells in the *Drosophila* eye. Developmental Biology 418, 98-107.

Nibu, Y., Zhang, H., Bajor, E., Barolo, S., Small, S., Levine, M., 1998. dCtBP mediates transcriptional repression by Knirps, Krüppel and Snail in the *Drosophila* embryo. The EMBO Journal 17, 7009-7020.

Niwa, N., Hiromi, Y., Okabe, M., 2004. A conserved developmental program for sensory organ formation in *Drosophila* melanogaster. Nature genetics 36, 293-297.

Ogiso, Y., Tsuneizumi, K., Masuda, N., Sato, M., Tabata, T., 2011. Robustness of the Dpp morphogen activity gradient depends on negative feedback regulation by the inhibitory Smad, Dad. Development, growth & differentiation 53, 668-678.

Oh, H., Irvine, K.D., 2011. Cooperative Regulation of Growth by Yorkie and Mad through *bantam*. Developmental Cell 20, 109-122.

Penton, A., Selleck, S.B., Hoffmann, F.M., 1997. Regulation of cell cycle synchronization by decapentaplegic during *Drosophila* eye development. Science (New York, N.Y.) 275, 203-206.

Poortinga, G., Watanabe, M., Parkhurst, S.M., 1998. *Drosophila* CtBP: a Hairy-interacting protein required for embryonic segmentation and Hairy-mediated transcriptional repression. The EMBO Journal 17, 2067-2078.

Rahimi, R.A., Leof, E.B., 2007. TGF-β signaling: A tale of two responses. Journal of Cellular Biochemistry 102, 593-608.

Ready, D.F., Hanson, T.E., Benzer, S., 1976. Development of the *Drosophila* retina, a neurocrystalline lattice. Developmental Biology 53, 217-240.

Restrepo, S., Zartman, Jeremiah J., Basler, K., 2014. Coordination of Patterning and Growth by the Morphogen DPP. Current Biology 24, R245-R255.

Romanova-Michaelides, M., Aguilar-Hidalgo, D., Jülicher, F., Gonzalez-Gaitan, M., 2015. The wing and the eye: a parsimonious theory for scaling and growth control? Wiley Interdisciplinary Reviews: Developmental Biology 4, 591-608.

Saller, E., Bienz, M., 2001. Direct competition between Brinker and *Drosophila* Mad in Dpp target gene transcription. EMBO Reports 2, 298-305.

Sanchez Bosch, P., Ziukaite, R., Alexandre, C., Basler, K., Vincent, J.-P.B., 2017. Dpp controls growth and patterning in *Drosophila* wing precursors through distinct modes of action. eLife 6, e22546.

Spratford, C.M., Kumar, J.P., 2013. Extramacrochaetae imposes order on the *Drosophila* eye by refining the activity of the Hedgehog signaling gradient. Development 140, 1994-2004.

St Johnston, R.D., Hoffmann, F.M., Blackman, R.K., Segal, D., Grimaila, R., Padgett, R.W., Irick, H.A., Gelbart, W.M., 1990. Molecular organization of the decapentaplegic gene in *Drosophila melanogaster*. Genes & Development 4, 1114-1127.

Treisman, J.E., Heberlein, U., 1998. Eye Development in *Drosophila*: Formation of the Eye Field and Control of Differentiation. Current Topics in Developmental Biology 39, 119-158.

Tsai, Y.C., Sun, Y.H., 2004. Long-range effect of upd, a ligand for Jak/STAT pathway, on cell cycle in *Drosophila* eye development. Genesis (New York, N.Y. : 2000) 39, 141-153.

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L., Tabata, T., 1997. *Daughters against dpp* modulates dpp organizing activity in *Drosophila* wing development. Nature 389, 627-631.

Vrailas, A.D., Moses, K., 2006. Smoothened, thickveins and the genetic control of cell cycle and cell fate in the developing *Drosophila* eye. Mechanisms of development 123, 151-165.

Welcker, M., Clurman, B.E., 2008. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer 8, 83-93.

Yao, L.-C., Phin, S., Cho, J., Rushlow, C., Arora, K., Warrior, R., 2008. Multiple modular promoter elements drive graded brinker expression in response to the Dpp morphogen gradient. Development 135, 2183-2192.

8. Supplementary Information

8.1. Fly strains and genotypes

All crosses were raised at 25°C under standard conditions. Eye-targeted RNAi knockdown of *punt* was induced by crossing eyeless-Gal4 with UAS-*punt* RNAi, VDRC #37279. The expression of the several genes used in this work was performed using UAS-*dad* ^{OE} (a gift from Konrad Basler), UAS-*CtBP*^{OE} FlyORF #F001239, UAS-*myc*^{OE} (a gift from Filipe Josué) and UAS *dome*$^{\Delta cyt}$. To characterize the expression pattern of Dpp, we used DppZ 3.0 fly line.

8.2. Generation of Mosaics

Flip-out *dad*^{OE} clones were generated by crossing ywhs-flp122; act>y+>Gal4 UAS-GFP females with UAS *dad*^{OE} males. Clones of cells expressing *dad*^{OE} were induced at L2 instar larval stage by 1 hour heat shock at 37°C. Mitotic CtBP mutant clones were generated by crossing ey>flip;;CtBPKG07519 FRT82B/TM6B females with M (3) Ubi GFP FRT82B/TM6B males.

8.3. Immunostaining

Immunohistochemistry of dissected eye-antennal discs was performed using standard protocols. Primary antibodies used were: rat anti-Elav 7E8A10 at 1:100 (DSHB Rat-Elav-7E8A10), rabbit anti-CtBP (kind gift of Dr. David Arnosti) at 1:5000, rabbit anti-P-Smad1/5 41D10 at 1:100 (Cell Signaling 9516), and guinea pig anti-Mad at 1:100 (kind gift of Dr. Newfeld), rabbit anti-Myc d1-717 at 1:500 (Santa Cruz sc-28207). and mouse anti-βgalactosidase (Promega Z378A). Rhodamine phalloidin dye (Thermo Fisher Scientific) was used to stain F-actin filaments. Appropriate Alexa-Fluor conjugated secondary antibodies were from Molecular Probes. Images were obtained with the Leica SP5 confocal system and processed with Adobe Photoshop CS6.

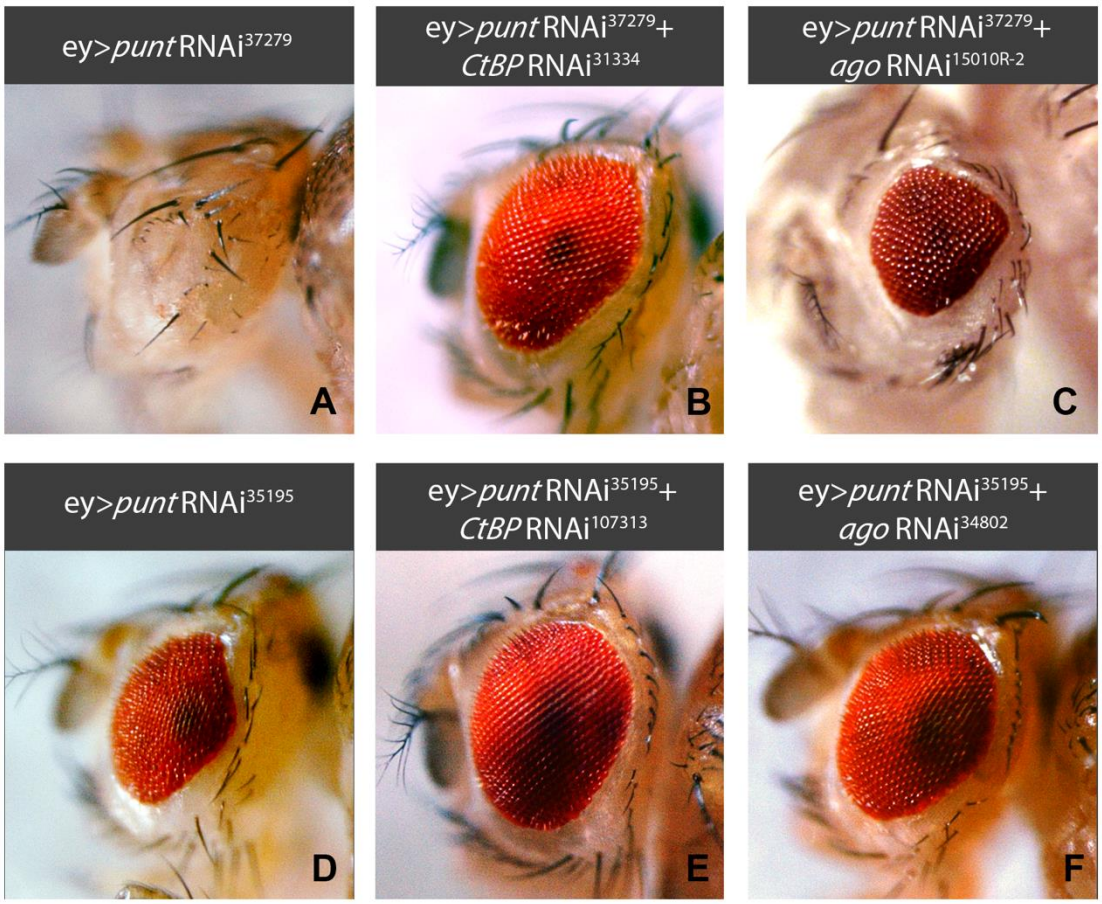

Figure S3.1. Validation of the identified genetic interactions with distinct RNAi lines

(A-F) Representative images of the adult eye phenotypes of the indicated genotypes. ey>*punt* RNAi^{37279} shows a very strong eye phenotype without retinal formation (A). A second RNAi line for *CtBP* (*CtBP* RNAi^{31334}) (B) or for *ago* (*ago* RNAi$^{15010R-2}$) (C) in combination with ey>*punt* RNAi^{37279} recue the phenotype induced by a *punt* loss-of-function. Likewise, the differentiation failure phenotype induced by a second RNAi line for *punt* (ey>*punt* RNAi^{35195}) (D) is rescued by *CtBP* RNAi^{107313} (E) or by *ago* RNAi^{34802} (F).

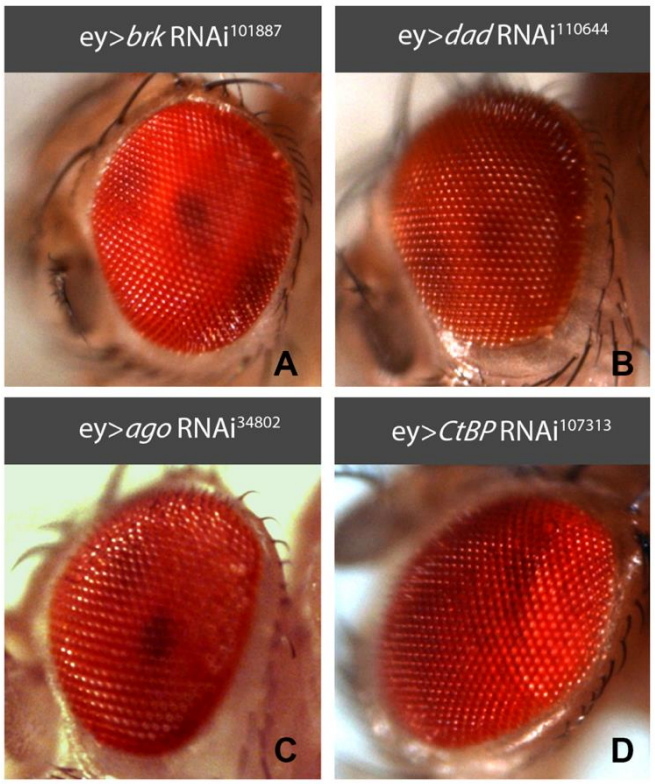

Figure S3.2. Flies expressing RNAis against *dad*, *brk*, *ago*, and *CtBP* did not show significant defects in adult eyes

(A-D) Representative images of the adult eye phenotypes of the indicated genotypes.

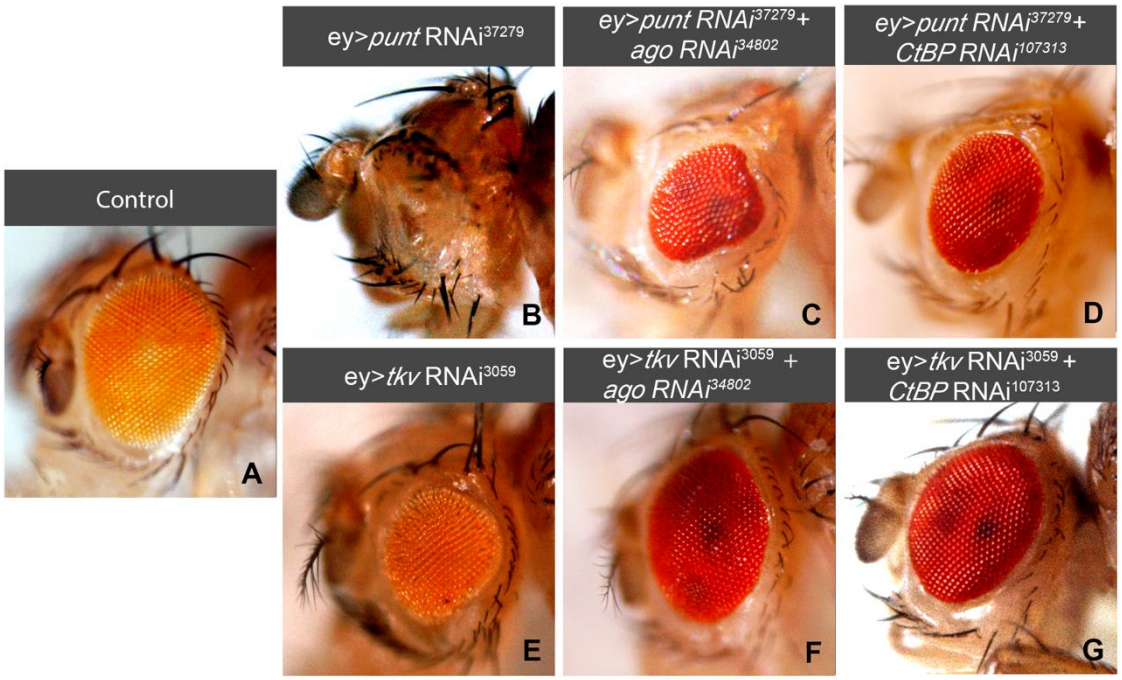

Figure S3.3. Adult eye phenotypic analysis of the strongest *punt* modifiers genes.

(A-G) Adult eyes of the individual and double RNAis of the indicated genotypes. A strong downregulation of Dpp signaling using ey>*punt* RNAi^{37279} results in adult eyes without differentiation (B). The combinatorial downregulation of *punt* together with *ago* or *CtBP* partially rescue eye differentiation (ey>*punt* RNAi^{37279}+*ago* RNAi^{34802}; ey>*punt* RNAi^{37279}+*CtBP* RNAi^{107313}) (C, D). The downregulation of Dpp signaling using a RNAi for *tkv* inhibits differentiation of adult eyes (ey>*tkv* RNAi$^{3059)}$ (E). However, the combinatorial downregulation of *tkv* together with *ago* or *CtBP* partially rescue the differentiation of adult eyes (ey> *tkv* RNAi^{3059}+*ago* RNAi^{34802}; ey>*tkv* RNAi^{3059}+*CtBP* RNAi^{107313}) (F, G).

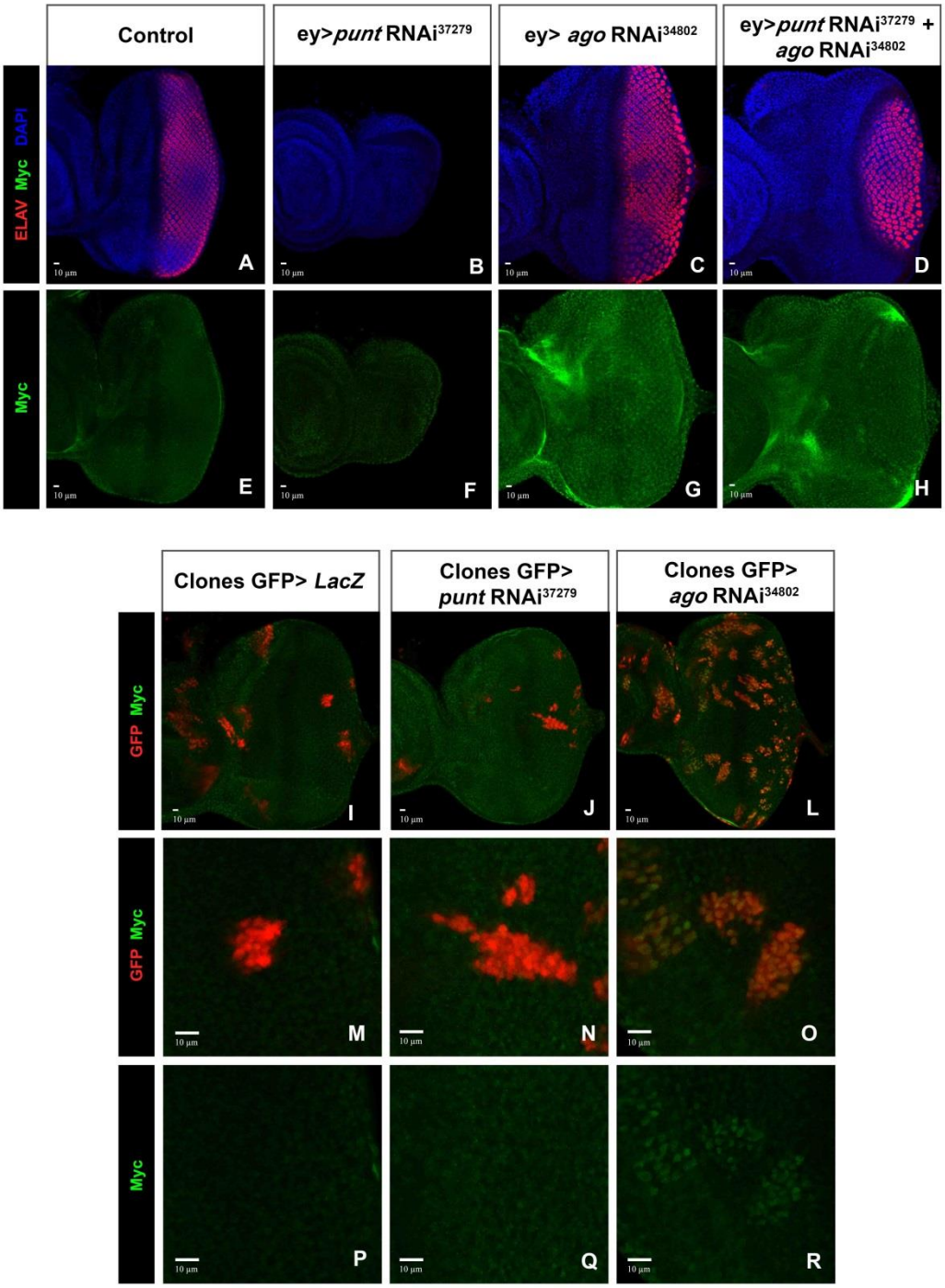

Figure S3.4. The RNAi for *ago* in a *punt* loss-of-function background induces an increase of Myc levels.
Eye imaginal discs of the indicated genotypes stained with DAPI (DNA, blue), ELAV (photoreceptors, red) and anti-Myc (green). Control (ey>*LacZ*) eye discs exhibit a Myc nuclear staining (A, A'). The levels and pattern of Myc in ey>*punt* RNAi^{37279} are similar to control (B, B'). However, in the double RNAi for *ago* together with *punt* (C, C'), an increase of Myc levels is observed related to control and ey>*punt* RNAi^{37279}. (J, N, Q) Clones of *punt* RNAi^{37279} and (L, O, R) *ago* RNAi^{34802} were induced in the *Drosophila* eye disc at 48 hours after egg laying and analyzed at the wandering L3 stage. No alterations in Myc expression are observed *punt* RNAi^{37279} clones but an increase of Myc intensity levels is observed in *ago* RNAi^{34802}. Clones are marked positively by the presence of GFP (red). The imaginal eye discs were stained with anti-Myc (green). N-O and Q-R show magnifications of the inset shown in J and L, respectively. Scale bars correspond to 10 µm.

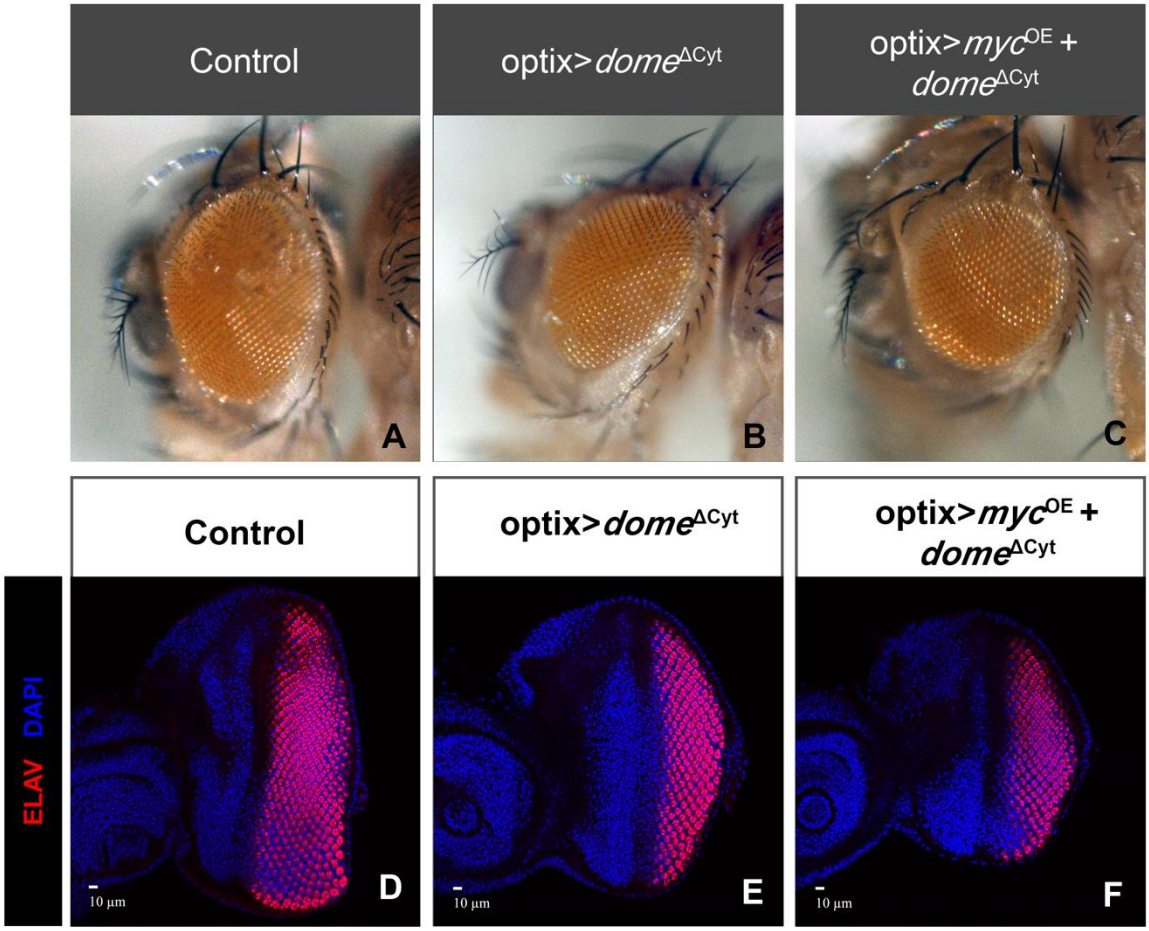

Figure S3.5. The overexpression of *myc* does not alter *dome* knockdown-induced phenotype.
(A–F) The downregulation of JAK/STAT signaling using ey>*dome*$^{\Delta Cyt}$ slightly reduces eye retinal formation (B) and eye disc growth and photoreceptor differentiation (E). The overexpression of the *myc* is not sufficient to rescue the phenotype induced by ey>*dome*$^{\Delta Cyt}$ (C and F). (D-F) Eye discs were stained with DAPI (DNA, blue) and anti-ELAV (photoreceptors, red). Scale bars correspond to 10 µm.

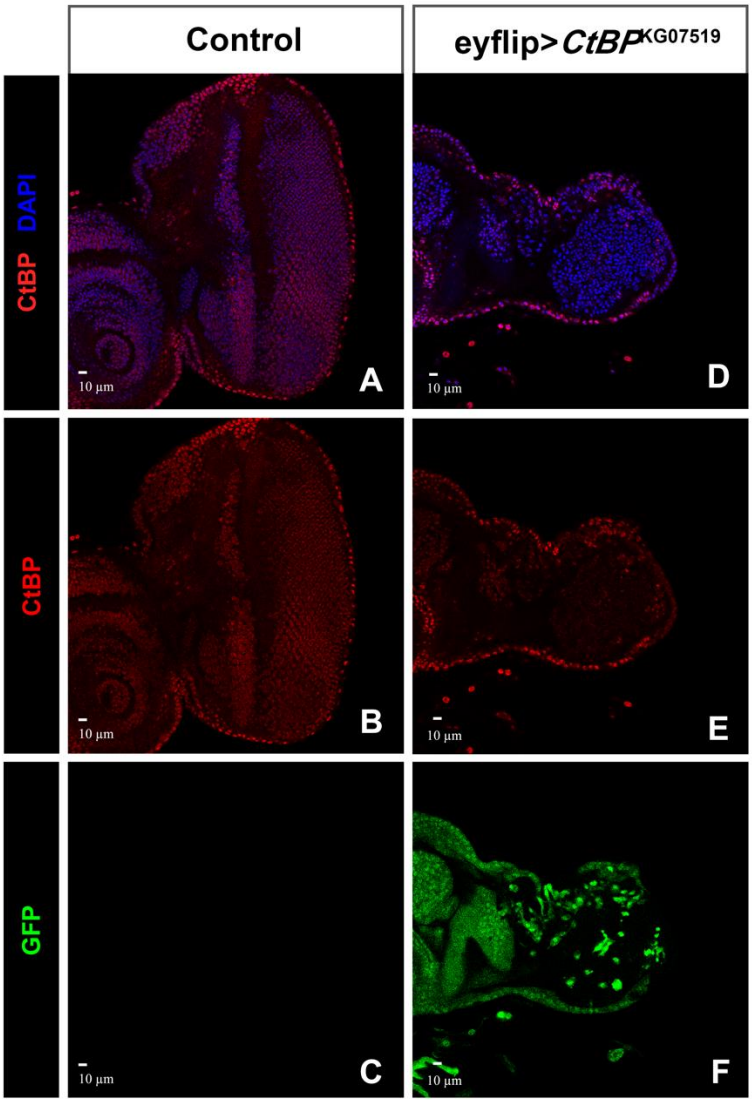

Figure S3.6. CtBP expression is downregulated in *CtBP*^{KG07519} mutant clones induced by eyeless-flippase. (A, B, D, E) Representative images of eye imaginal discs of the indicated genotypes stained with anti-CtBP (red) and DAPI (DNA, blue). (C, F) *CtBP*^{KG07519} mutant clones are labelled by absence of GFP (green) expression. Scale bars correspond to 10 µm.

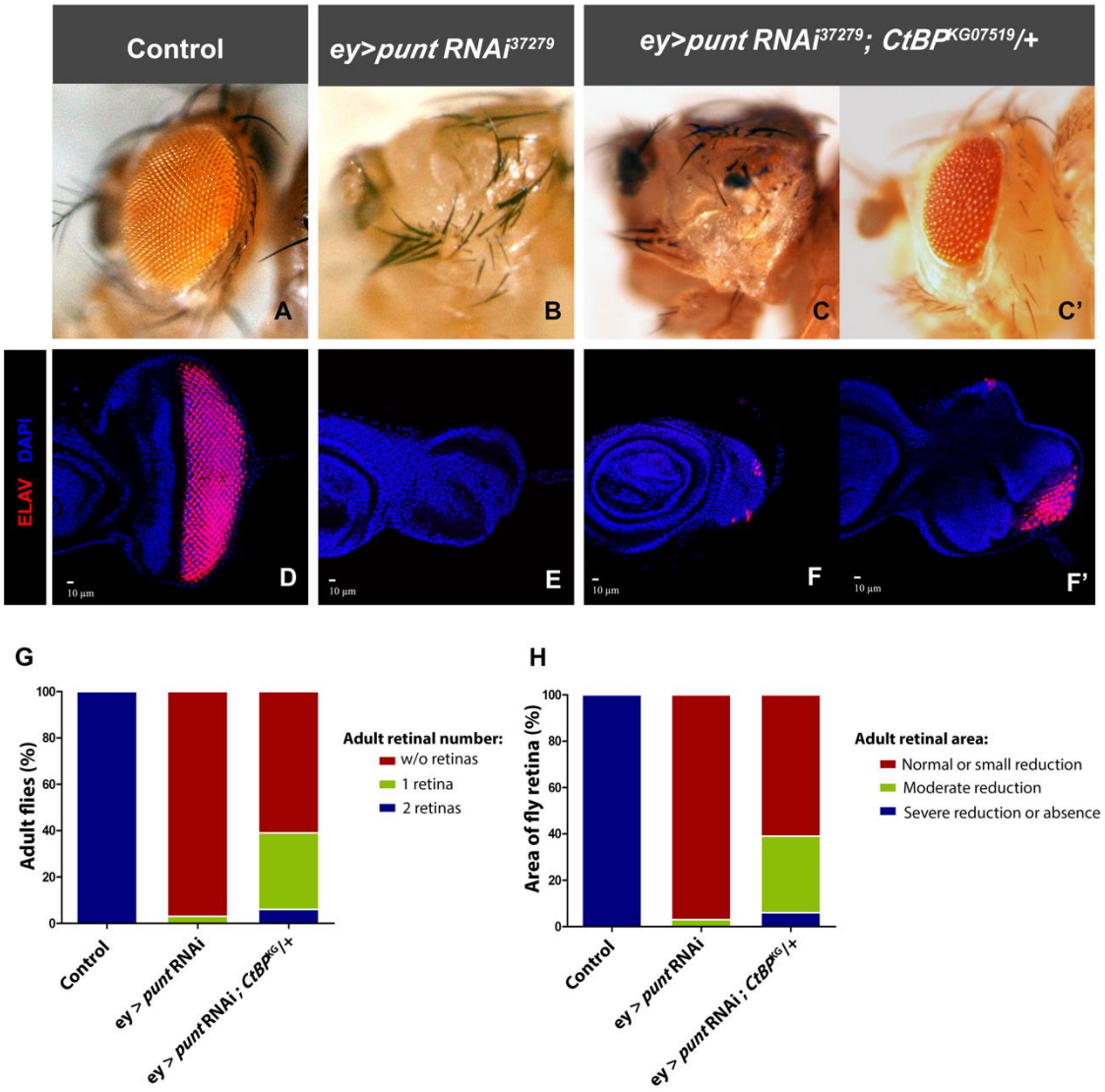

Figure S3.7. CtBP is a negative regulator of Dpp-BMP2/4 signaling.

(A–C') Adult eyes of the indicated genotypes. ey>*punt* RNAi^{37279} shows a very strong eye phenotype without retinal formation (B). However, the differentiation failure phenotype of ey>*punt* RNAi^{37279} is partially restored in a heterozygous *CtBP* ^{KG07519} mutant background (C and C'). (D-F') Eye imaginal discs of the indicated genotypes stained with DAPI (DNA, blue) and anti-ELAV (photoreceptors, red). Scale bars correspond to 10 µm. A strong downregulation of Dpp signaling using ey>*punt* RNAi^{37279} inhibits photoreceptors differentiation (E). The heterozygous *CtBP* ^{KG07519} mutant partially restores photoreceptors differentiation in a *punt* loss-of-function background (F and F'). (G) Percentage of adult flies with two retinas, one retina or without (w/o) retinas (43<n<96). (H) Percentage of adult flies presenting normal or small reduction of adult retinal area (+++), moderate reduction of adult retinal area (++) and severe reduction or absence of adult retinal area (+ or no retina) (43<n<96).

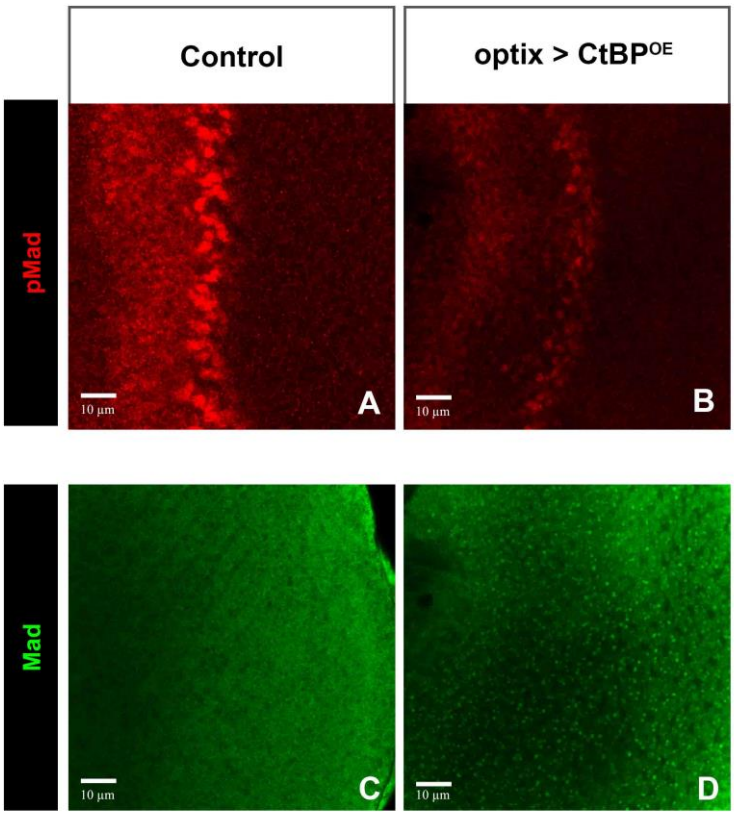

Figure S3.8. Dad downregulates Mad phosphorylation but not Mad levels.
(A-D) Eye imaginal discs of *optix*-Gal4 (control) (A and C) and *optix>CtBP*^{OE} (B and D) stained with anti-pMad (red) and anti-Mad (green). Scale bars correspond to 10 µm.

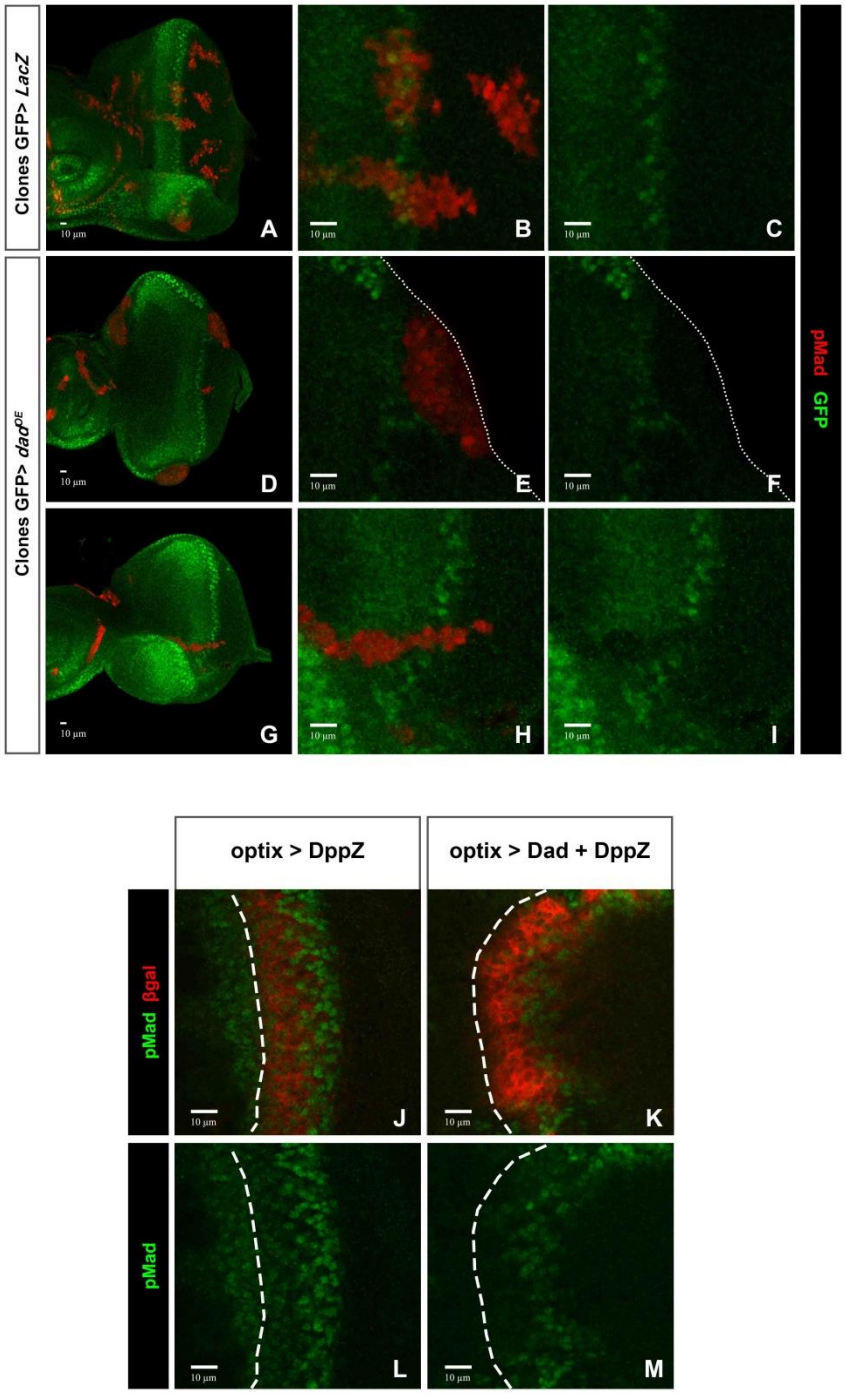

Figure S3.9. Dad regulates Dpp-BMP2/4 signaling pathway inhibiting Mad phosphorylation.

(A-I) *dad*^{OE} clones were induced in the *Drosophila* eye disc at 48 hours (D, E, F) and 72 hours (G, H, I) after egg laying and analyzed at the wandering L3 stage. A reduction of pMad levels is observed in clones overexpressing *dad.* Clones are marked positively by the presence of GFP (red). The dotted line marks the eye disc margin. (J-M) In *optix> DppZ +dad*^{OE} eye discs, pMad levels are severely reduced, however, Dpp is still observed. Eye imaginal discs of *optix*-Gal4>*DppZ* (J and L) and *optix> DppZ +dad*^{OE} (K and M) stained with anti-pMad (green) and βgal (red). The dashed line marks the morphogenetic furrow (MF).The imaginal eye discs were stained with anti-pMad (green).

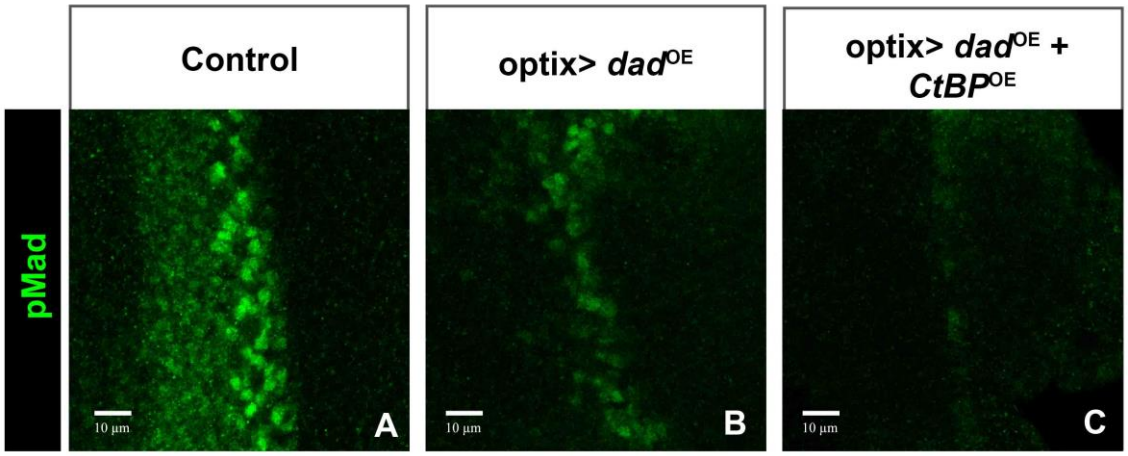

Figure S3.10. Dad and CtBP synergize for an efficient inhibition of Mad phosphorylation.
(A-D) Eye imaginal discs of *optix*-Gal4 (control) (A), *optix>dad*^{OE} (B) and *optix>dad*^{OE} + *CtBP*^{OE} (C) stained with anti-pMad (green). Scale bars correspond to 10 µm.

Table S3.1. Classification of observed double RNAi versus *punt* RNAi phenotype modifications.
UAS-RNAi lines indicating the correspondent target gene (CG) and the reference of Bloomington Stock Center, Vienna Drosophila RNAi Center and National Institute of Genetics. Eyes of adult flies of ey>*punt* RNAi + gene of interest RNAi are classified according to the phenotype observed comparing to *punt* loss-of-function phenotype: no modification (N), weak rescue (+), medium rescue (++) and strong rescue (+++). Lethal means absence of adult flies. Sublethal means presence of only 10% or less of adult flies.

Classification of observed double RNAi vs *punt*RNAi phenotype modifications	
N	No modification
+	Weak rescue
++	Medium rescue
+++	Strong rescue
Lethal	100% of adult fly lethality
Sublethal	>90% of adult fly lethality

Gene ID	Symbol	Double RNAi ey Gal4>punti37279 RNAi + Gene of interest RNAi	Bloomington, NIG-Fly or Vienna stock center number
CG1168	7B2	no	#30816
CG4376	Actn	no	#34874
CG11062	Actβ	no	#29597
CG11062	Actβ	no	#108663
CG11062	Actβ	no	#11062R-1
CG15010	ago	+	#31501

CG15010	ago	+++	#34802
CG15010	ago	++	#15010R-2
CG42783	aPKC	no	#2907
CG42783	aPKC	sublethal	#105624
CG42783	aPKC	lethal	#25946
CG42783	aPKC	lethal	#35001
CG1643	Atg5	no	#104461
CG7508	ato	no	#48675
CG5670	Atpα	lethal	#12330
CG7926	axin	+	#7748
CG8224	babo	no	#25933
CG8224	babo	+	#106092
CG8224	babo	+	#8224R-3
CG3274	Bap170	+	#34582
CG4303	Bap60	lethal	#103634
CG4303	Bap60	lethal	#12675
CG4303	Bap60	lethal	#31337
CG4722	bib	no	#8892
CG4608	bnl	no	#5730
CG5206	bom	sublethal	#27047
CG9653	brk	+++	#101887
CG9654	brk	+++	#2919
CG5942	brm	+	#37720
CG5680	bsk	+	#34138
CG5680	bsk	+	#34139
CG7563	CalpA	no	#35261
CG4209	CanB	no	#21611
CG12530	Cdc42	no	#100794
CG5363	cdk1	+	#41838
CG10192	CG10192	+	#18031
CG10211	CG10211	no	#12352
CG10347	CG10347	+	#16025
CG1079	CG1079	no	#7477
CG10802	CG10802	no	#39765
CG10916	CG10916	no	#31379
CG11095	CG11095	no	#37911
CG1116	CG1116	no	#18161
CG11168	CG11168	no	#18170
CG11237	CG11237	no	#38462
CG11347	CG11347	+	#41186
CG11533	CG11533	+	#45121
CG11658	CG11658	no	#16255
CG11982	CG11982	no	#38623

CG12214	CG12214	no	#31689
CG12814	CG12814	no	#9066
CG13046	CG13046	no	#41496
CG13349	CG13349	no	#23874
CG13894	CG13894	no	#32078
CG13897	CG13897	no	#39733
CG13917	CG13917	no	#32082
CG13937	CG13937	no	#3373
CG14207	CG14207	no	#44831
CG14275	CG14275	+	#7642
CG14715	CG14715	+	#12828
CG14815	CG14815	no	#42332
CG14946	CG14946	+	#38306
CG14948	CG14948	+	#45821
CG15436	CG15436	no	#39986
CG15443	CG15443	no	#40877
CG1550	CG1550	no	#26129
CG1620	CG1620	no	#12681
CG17765	CG17765	no	#32404
CG18112	CG18112	no	#43415
CG18273	CG18273	lethal	#107818
CG18275	CG18275	lethal	#39348
CG18507	CG18507	no	#7705
CG18815	CG18815	+	#33414
CG1882	CG1882	no	#41405
CG2054	CG2054	no	#7609
CG2064	CG2064	no	#8729
CG2206	CG2206	no	#30843
CG2310	CG2310	no	#1337
CG2865	CG2865	+	#20924
CG2989	CG2989	no	#46285
CG3033	CG3033	lethal	#7086
CG31038	CG31038	no	#25656
CG31072	CG31072	+	#1252
CG31712	CG31712	no	#21401
CG31937	CG31937	no	#3449
CG31997	CG31997	no	#12928
CG32223	CG32223	no	#46771
CG3257	CG3257	no	#34578
CG3365	CG3365	no	#43763
CG34104	CG34104	no	#31140
CG34104	CG34104	+	#33738

CG3618	CG3618	no	#6977
CG3619	CG3619	no	#3720
CG3731	CG3731	lethal	#40466
CG3814	CG3814	no	#4671
CG4080	CG4080	no	#9026
CG4303	CG4303	lethal	#12675
CG4330	CG4330	no	#11078
CG4449	CG4449	+	#26539
CG4778	CG4778	no	#7652
CG4952	CG4952	no	#2942
CG5022	CG5022	no	#8262
CG5189	CG5189	no	#40318
CG5282	CG5282	+	#5387
CG5315	CG5315	no	#40935
CG5315	CG5315	no	#31800
CG5455	CG5455	no	#35010
CG5731	CG5731	no	#15543
CG5740	CG5740	no	#22197
CG5835	CG5835	no	#33358
CG5888	CG5888	no	#12413
CG6014	CG6014	no	#31067
CG6084	CG6084	no	#27551
CG6294	CG6294	no	#46349
CG6329	CG6329	no	#13319
CG6340	CG6340	no	#34159
CG6412	CG6412	no	#44327
CG6583	CG6583	no	#44880
CG6999	CG6999	no	#41828
CG7686	CG7686	lethal	#33649
CG7891	CG7891	no	#26085
CG7920	CG7920	no	#21577
CG8058	CG8058	no	#13314
CG8216	CG8216	no	#23270
CG8319	CG8319	+	#15723
CG8616	CG8616	no	#38249
CG9027	CG9027	no	#37794
CG9075	CG9076	lethal	#42202
CG9170	CG9170	+	#29066
CG9266	CG9266	no	#47802
CG9338	CG9338	no	#8609
CG9526	CG9526	no	#51450
CG3937	cher	+	#26307
CG3937	cher	no	#35755

CG4108	Chmp1	no	#21788
CG10546	Cralbp	no	#31258
CG6383	crb	no	#34999
CG3510	CycB	sublethal	#43772
CG3938	CycE	+	#47941
CG3938	CycE	no	#47942
CG3938	CycE	no	#52662
CG5201	dad	+	#42840
CG5201	dad	no	#26235
CG5201	dad	+++	#33759
CG5201	dad	+++	#110644
CG16987	daw	no	#105309
CG16987	daw	no	#16987R-2
CG6169	Dcp2	+	#34806
CG4792	Dcr-1	no	#28598
CG7583	dCtBP	+++	#37608
CG7583	dCtBP	+++	#107313
CG7583	dCtBP	+++	#31334
CG3619	Dl	lethal	#34322
CG1725	dlg1	no	#41136
CG1725	dlg1	sublethal	#41134
CG1725	dlg1	no	#109274
CG32146	dlp	+	#10299
CG9885	dpp	no	#31530
CG9885	dpp	lethal	#33618
CG8730	drosha	no	#23772
CG8730	drosha	no	#108026
CG4952	ds	no	#8609
CG12238	e(y)3	no	#105946K
CG6611	ect	no	#14003
CG7383	eg	no	#29629
CG7383	eg	no	#7157
CG7383	eg	no	#35234
CG1464	ey	lethal	#32486
CG1464	ey	++	#106628
CG9554	eya	no	#43911
CG3665	Fas2	lethal	#34084
CG3665	Fas2	sublethal	#8392
CG9888	Fib	lethal	#104372
CG17697	fz	+	#43075
CG17697	fz	+	#43077
CG6706	GABA-B-R2	no	#1784

CG17035	GXIVsPLA2	no	#44441
CG4637	Hh	no	#1402
CG11228	Hpo	+	#27661
CG11228	Hpo	no	#33614
CG9623	If	no	#27544
CG9623	If	no	#27544
CG10504	Ilk	no	#35374
CG10504	Ilk	no	#16062
CG6632	Ing3	no	#109799
CG16827	ItgaPS4	no	#28535
CG33967	kibra	no	#31755
CG33967	kibra	no	#28683
CG2666	kkv	no	#42610
CG4717	kni	no	#2980
CG4761	knrl	+	#47216
CG4761	knrl	no	#47217
CG10236	LanA	no	#28071
CG10236	LanA	no	#28071
CG10533	Lcp65Af	+	#23530
CG12052	lola	no	#12573
CG12399	mad	lethal	#31315
CG12399	mad	no	#110517
CG12399	mad	+	#12399R-1
CG1775	med	lethal	#19688
CG1775	med	no	#19689
CG1771	mew	no	#27543
CG10145	mspo	+	#15194
CG5588	Mtl	no	#28622
CG10798	myc	lethal	#2947
CG10798	myc	lethal	#2948
CG1560	mys	no	#29619
CG1560	mys	no	#27735
CG1560	mys	lethal	#103704
CG1560	mys	no	#33642
CG1560	mys	no	#1560 R-2
CG1560	mys	no	#28601
CG3936	N	lethal	#27229
CG3936	N	lethal	#27228
CG3936	N	no	#7078
CG2286	ND-75	+++	#52047
CG2286	ND-75	lethal	#27739
CG2286	ND-75	lethal	#33910
CG2286	ND-75	lethal	#33911

CG7421	Nopp140	no	#27995
CG7421	Nopp140	no	#45582
CG7421	Nopp140	lethal	#45583
CG8663	nrv3	no	#44486
CG3983	Ns1	lethal	#29622
CG14789	O-fut2	no	#41361
CG7467	osa	no	#7810
CG3479	osp	no	#3010
CG10279	p68	lethal	#110102
CG10279	p68	no	#46908
CG10279	p68	no	#46909
CG40411	Parp	no	#35792
CG1800	pasha	no	#107445
CG1800	pasha	no	#40118
CG31794	Pax	no	#28695
CG8114	Pbl	no	#35349
CG8114	Pbl	no	#109305
CG8114	Pbl	no	#35350
CG30483	Prosap	no	#21216
CG14039	qtc	no	#17349
CG6434	qua	+	#100856
CG6433	qua	+	#27623
CG2248	Rac1	no	#28985
CG2248	Rac1	no	#34910
CG8556	Rac2	+	#28926
CG8556	Rac2	no	#50349
CG8556	Rac2	no	#50350
CG3000	rap	no	#25550
CG9375	ras	++	#v28129
CG9375	ras	+	#106642
CG9375	ras	no	#29319
CG8739	Rbo	no	#105391
CG8739	Rbo	no	#105391
CG10800	Rca1	lethal	#35489
CG6831	rhea	sublethal	#32999
CG6831	rhea	no	#33913
CG8416	Rho1	lethal	#29002
CG8416	Rho1	no	#9910
CG8416	Rho1	lethal	#27727
CG8416	Rho1	no	#32383
CG8416	Rho1	no	#109420
CG8416	Rho1	sublethal	#51953

CG8416	Rho1	lethal	#12734,
CG8416	Rho1	no	#29003
CG8416	Rho1	lethal	#9909
CG5701	RhoBTB	no	#100815
CG32555	RhoGAP	no	#6429
CG32555	RhoGAP	no	#31070
CG7823	RhoGDI	no	#4154
CG7823	RhoGDI	no	#46155
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CG9635	RhoGEF2	no	#110577
CG9635	RhoGEF2	no	#34643
CG9635	RhoGEF2	no	#31239
CG43976	RhoGEF3	no	#31580
CG9366	RhoL	no	#102461
G9774	Rok	no	#104675
CG9774	Rok	+	#3793
CG1475	Rpl135	lethal	#24921
CG7434	Rpl135	lethal	#34828
CG1475	RpL13A	lethal	#43760
CG7434	RpL22	lethal	#34828
CG3314	RpL7A	lethal	#43760
CG4125	rst	no	#951
CG12190	RYBP	no	#105283
CG12190	RYBP	+	#52138
CG1891	sax	no	#42457
CG1891	sax	no	#46350
CG1891	sax	+	#46356
CG1891	sax	no	#JF03431
CG17579	sca	no	#44527
CG8095	Scab	no	#27546
CG5595	Sce	lethal	#106328
CG5595	Sce	+	#27465
CG5505	scny	no	#11152
CG5505	scny	no	#105989,
CG3182	sei	no	#3606
CG6584	SelR	no	#26000,
CG5661	Sema-5c	no	#9428,
CG5661	Sema-5c	no	#29436,
CG4173	Sep2	no	#26413
CG18076	shg	no	#28336
CG18076	shot	no	#28336
CG10706	SK	+	#28155
CG10212	smc2	lethal	#10713

CG11561	smo	+	#9542
CG2262	Smox	lethal	#26756
CG2262	Smox	no	#105687
CG2262	Smox	no	#2262R-2
CG2262	Smox	no	#2262R-1
CG34421	Snoo	no	#31934
CG7233	Snoo	no	#7233R-4
CG34421	Snoo	no	#7233R-1
CG11121	so	no	#104386,
CG31048	Sqh	lethal	#32439
CG31048	Sqh	lethal	#33892
CG31048	Sqh	lethal	#31542
CG7873	Src	+	#35252
CG7873	Src	no	#10078
CG11895	stan	no	#1665
CG7954	stck	no	#52537
CG7954	stck	no	#100582
CG1395	stg	+	#17760
CG5407	Sur-8	no	#27410
CG10808	Syngr	no	#8784
CG10390	Taf6L	no	#37563
CG5723	Ten-m	no	#29390
CG5041	Tfb4	no	#12559
CG7525	Tie	no	#27087
CG3278	Tif-IA	lethal	#103777
CG11527	tig	lethal	#31570
CG11527	tig	no	#28257
CG11527	tig	no	#100036
CG14026	tkv	+	#3059
CG14026	tkv	no	#35166
CG11186	toy	no	#15919
CG18214	trio	no	#27732
CG33950	trol	+	#24549
CG12840	Tsp42El	no	#11331
CG2713	ttm50	lethal	#5586
CG13345	tum	lethal	#28982
CG13345	tum	lethal	#17145
CG13401	U26	no	#43571
CG3299	Vinc	no	#25965
CG32418	vito	lethal	#34548
CG32418	vito	lethal	#34549
CG32418	vito	lethal	#102513

CG16858	vkg	no	#106812
CG14029	vri	++	#5650
CG14029	vri	no	#40862
CG14029	vri	no	#25989
CG2759	w	no	#30033
CG42677	Wb	no	#29559
CG4889	wg	+	#13351
CG4889	wg	lethal	#13352
CG4889	wg	+	#32994
CG4889	wg	no	#33902
CG10776	wit	no	#25949
CG10776	wit	no	#42244
CG10776	wit	+	#41906
CG4969	Wnt6	sublethal	#26669
CG4969	Wnt6	+	#27610
CG12072	Wts	no	#27662
CG4005	yki	sublethal	#34067
CG4005	yki	no	#31965
CG8536	β4GalNAcTA	no	#4867

CHAPTER 4

Dad regulates cell growth and differentiation

Contents

Dad is a nucleolar protein required for cell growth and differentiation

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1. Abstract

The expression of *dad* is regulated by TGFβ signaling through the binding of the R-Smad, Mad, directly to the *dad* enhancer. However, Dad also plays an essential role in the negative-feedback regulation of TGFβ signaling, through the competition with R-Smads for receptors or Co-Smad interactions. Our data suggest that Dad localizes in the nucleolus and controls cell growth and differentiation through the regulation of Mad activation. The N-terminal domain of Dad is important for an efficient inhibition of TGFβ signaling and it determines the subcellular localization of this I-Smad. Particularly, the N-terminal domain of Dad is required for an efficient binding of this protein to the cell membrane. Finally, a strong upregulation of TGFβ signaling is able to induce Dad phosphorylation, which blocks Dad functions as I-Smad.

2. Introduction

The Transforming Growth Factor β (TGFβ) signaling pathway is involved in several cellular processes in both the adult organism and the whole development, including cell proliferation, cell differentiation and survival/or apoptosis. The canonical TGFβ signaling pathway can be divided into two main branches, BMP and Activin. TGFβ signaling pathway initiates with the binding of a ligand to a type II receptor (Punt or Wit). Once phosphorylated, type II receptor binds to type I receptor (Tkv and Sax from BMP branch and Babo from Activin branch). Then, type I receptor phosphorylates their specific R-Smad, Mad in the BMP branch and Smad2 (or Smox) in the Activin branch. R-Smad activation promotes its homodimerization and induces the formation of a trimeric complex with the common Co-Smad Medea. This complex is then translocated to the nucleus where it controls the expression of several target genes (Dijke and Hill, 2004; Rahimi and Leof, 2007). This signaling pathway is regulated by the I-Smad, Daughters against dpp (Dad), through the competition with R-Smads for receptors or Co-Smad interactions (Tsuneizumi, Nakayama et al. 1997, Kamiya, Miyazono et al. 2008). In vertebrates, there are two different I-Smads, Smad6 and Smad7. Smad7 inhibits Activin signaling through the competition with R-Smad2/R-Smad3 and BMP signaling through the competition with Smad1/Smad5 (Miyazono, Kamiya et al. 2010). The other inhibitory Smad, Smad6 targets BMP branch, antagonizing R-Smad1/R-Smad5 activation by competing with R-Smad1/R-Smad5 binding to the activated type I receptor (Feng and Derynck 2005, Massagué, Seoane et al. 2005, Miyazono, Kamiya et al. 2008).

I-Smads have highly conserved Mad homology 2 (MH2) domains with the other Smads but lack the phosphorylation site, presented in R-Smads and required for their activation by the type I receptors. In mammals, the N domains of I-Smads have been described as very important for its functions. The N-domain of Smad7 is important for an efficient inhibition of TGFβ signaling and also determines its subcellular localization (Itoh, Landström et al. 1998, Hanyu, Ishidou et al. 2001). In Smad6, the MH1-linker region also plays an important role in the inhibition of BMP signaling (Lin, Liang et al. 2003). Remarkably, MH2 domain is essential for the inhibition of BMP and TGFβ signaling by both Smad6 and Smad7 (Hanyu, Ishidou et al. 2001). The MH2 domain of Smad7 mediates the interaction of this Smad with the type I receptor, enabling the competition with R-Smads for receptor activation (Hayashi, Abdollah et al. 1997, Nakao, Afrakhte et al. 1997, Mochizuki, Miyazaki et al. 2004). Moreover, the MH2 domain of Smad7 has high affinity for DNA binding and it is required for nuclear localization (Zhang, Fei et al. 2007, Shi, Chen et al. 2008). Similar to the other I-Smads, Dad has a conserved MH2 domain and a linker domain but the MH1 domain is a divergent amino-termini that share regions of similarity within the Inhibitory Smad subgroup. The MH2 domain of Dad is conserved in Smad6 and Smad7 sequences in 41.1% and 36.7%, respectively. However, the N-terminal domain (MH1 domain and linker regions) is conserved only in 26.1% of the sequence with both Smad6 and Smad7.

To elucidate the importance of Dad in the repression of TGFβ signaling and to determine how Dad efficiently inhibits this signaling, we examined the activity of Dad in both imaginal discs and salivary glands. Dad localizes in several compartments of *Drosophila* cells, including nucleolus, nucleus, cytosol and membrane. Particularly, the presence of Dad in the nucleolus is sufficient to inhibit Mad activation. Additionally, our present findings revealed that the N-terminal domain of Dad is required for the efficient inhibition of TGFβ signaling. We also found that the N-terminal domain determine the subcellular localization of Dad. Intriguingly, the strong upregulation of TGFβ signaling induced Dad phosphorylation in *Drosophila* salivary glands and blocked the inhibitory function of Dad on TGFβ signaling.

3. Materials and Methods

3.1. Fly lines

All crosses were raised at 25°C under standard conditions. The following stocks (described in FlyBase, unless stated otherwise) were used: ey-Gal4, Ms-1096, UAS-

lacZ, w1118, Dad 001A (#42669), UAS-*punt* (a gift from Konrad Basler), UAS-*tkv* (Tom Bunch lab), UAD-Dad (a gift from Basler lab), UAS-tkv^{QD} (#36536), Dad 271.68 (#10305), Dad1E4 (#271268), UAS-*dad* RNAi (#110644, #5201R-1) and UAS-*punt* RNAi (#37279). The RNAi was validated by testing other lines.

3.2. Dad antibody production

Rabbit polyclonal antisera were generated using peptides composed of amino acids DRSPDQGQVQPVDRC for anti-Dad, by ABGENT Biotechnology Company. Specificity of anti-Dad antisera was confirmed on wild type, *dad* mutant and overexpression *dad* strains.

3.3. Immunostaining

Eye-antennal imaginal discs and salivary glands were prepared for immunohistochemistry using standard protocols. Primary antibodies used were: rat anti-Elav 7E8A10 at 1:100 (DSHB Rat-Elav-7E8A10), mouse anti-Fibrillarin 38F3 at 1:500 (Abcam ab4566), rat anti-*Drosophila* E-cadherin at 1:100, rabbit anti-P-Smad1/5 41D10 at 1:100 (Cell Signaling 9516), rabbit anti-GFP at 1:1000 (Molecular Probes A11122) and mouse anti-Coracle at 1:100 (DSHB C615.16). To stain for cellular limits phalloidin conjugated with rhodamine was used at a dilution of 1:1000. Appropriate Alexa-Fluor conjugated secondary antibodies were from Molecular Probes. Images were obtained with the Leica SP5 confocal system and processed with Adobe Photoshop.

3.4. Generation of UAS GFP-Dad and UAS GFP-Dad Δ113 transgenic strains

Tagged constructs were made using the Gateway Cloning System (Life Technologies). Dad and Dad Δ113 coding sequences were amplified from *Drosophila* cDNA clone LD47465 (GenBank accession AY095185), using Dad flanking primers designed accordingly to the manufacturer´s protocol (see Table below), and cloned into pENTR. The GFPtagged constructs were obtained using LR clonase II to mediate the recombination into pTGW to transfect *Drosophila* flies (BestGene Inc).

Purpose		Primers
Cloning of Dad variants	Dad full length	5’ CACCATGATATTCCCAAGAGAAAAG 3’
		5’ CCGCAGATGACTAAAGTGAAC 3’
	Dad Δ113	5’ CACCATGGATGTGTTGCCGCCGCCT 3’
		5’ CCGCAGATGACTAAAGTGAAC 3’

3.5. Western Blot Analysis and immunoprecipitation

For Western Blot analysis, salivary glands were dissected from L3 larvae in lysis buffer (75mM HEPES pH 7.5, 1.5mM EGTA, 1.5 mM MgCl2, 150 mM KCl, 15% glycerol and 0.1% NP-40) containing a complete protease (Roche) and phosphatase (Sigma) inhibitor cocktails. The eye imaginal discs were homogenized with a plastic pestle. Then, the homogenized was sonified twice for 10 sec. Lysates were clarified by centrifugation for 10 min at 4°C and boiled in 1×Laemmli buffer. For immunoprecipitation assays, GFP-Dad was immunoprecipitated using the GFP-Trap_MA system (Chromotec GmbH, Planegg-Martinsried, Germany) according to the manufacturer's instructions. For dephosphorylation treatment Lambda phosphatase (NEB) was used accordingly to the manufacture instructions. Protein extracts were separated by 8% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked 1 h at room temperature with 5% milk in tris-buffered saline and then incubated overnight with primary antibodies at 4°C. Antibodies were diluted as follows: rabbit anti-GFP at 1:1000, rabbit anti-Punt (Abcam) at 1:1000 and mouse anti-tubulin β-5-1-2 (Santa Cruz Biotech) at 1:100000. Blots were detected using goat anti-rabbit and anti-mouse secondary antibodies and visualized with ECL Blotting Substrates 1:1 (Rio-Rad). A GS-800 calibrated densitometer system was used for quantitative analysis of protein levels.

3.6. Dad phosphorylated peptides identification by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI/TOF-TOF) Mass Spectrometry

Following Coomassie Blue-stained, the band approximately at 100 KDa was excised from the gel, washed with 50% acetonitrile in 50 mM ammonium bicarbonate, digested for 3 h with 20 ng of trypsin at 37 °C, and analyzed on a MALDI mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer, SCIEX) as described in (Ferreirinha, Correia et al. 2016). Proteins were identified by Peptide Mass Fingerprint using the Mascot software v2.5.1 (Matrix Science, London, UK). Protein searches were performed against the UniProt protein sequence database for the *Drosophila melanogaster* taxonomic selection (2017_09, UP000000803 reviewed proteome, canonical proteins). The established search parameters were: up two missed cleavages allowed, cysteine carbamidomethylation as a fixed modification and phosphorylation as a variable modification. The peptide tolerance was 20 ppm. Protein scores greater than 56 were considered to be significant ($p < 0.05$).

3.7. FRAP

Dissected third-instar salivary glands were placed in glass bottom dishes from MaTeck with Schneider's media. Glands were used for no more than 1h after dissection. FRAP was performed using a 40× objective and a fully open pinhole (154.3 µm). Six prebleach scans were performed using 30% maximal laser 488nm before using one frame for bleach with 100% of 405 nm laser. First postbleach scanning was performed every 0.769 s for 10 times, following by a second postbleach using 20 scans for 20 min. Acquisition after bleach was also performed with 488 nm laser. Approximately 10 salivary glands were subjected to FRAP for each experimental group. For the purpose of presentation, FRAP traces were normalized by the level of fluorescence measured prior to bleaching, after subtracting the background fluorescence.

3.8. Size measurements and statistics

Salivary glands and imaginal eye discs areas were measured using the Polygon selection tool of ImageJ 1.46J software (NIH, Bethesda, MA, USA) from at least 8 salivary glands or imaginal eye discs for each genotype. GraphPad Prism 5.0 was used for statistical analysis and generating the graphical output. Statistical significance was determined using an unpaired, two-tailed Student’s t test, with a 95% confidence interval, after assessing the normality distribution of the data with D’Agostino–Pearson normality test.

4. Results

4.1. Localization of Dad in *Drosophila* tissues

It has been reported that Dad stably associates with Tkv receptor and thereby inhibits Tkv-induced Mad phosphorylation and nuclear translocation (Inoue, Imamura et al. 1998, Kamiya, Miyazono et al. 2008). In mammals, the localization of Dad orthologues Smad6/7 has been better characterized. Smad7 localizes in the nuclei of different type of cells (Itoh, Landström et al. 1998, Zhang, Fei et al. 2007), whereas Smad6 is observed in the nuclei and cytoplasm (Lin, Liang et al. 2003, Estrada, Retting et al. 2011). Moreover, in U-251 MG human cell line, rabbit Smad7 antibody from Sigma-Aldrich was detected in several compartments, including nucleoplasm, nucleoli fibrillar center and centrosome. However, a deep characterization of Dad localization within the cell is still not described and new tools are necessary for its study.

To further characterize the subcellular localization of Dad, we used three different strategies: Dad antibody, Dad protein-trap and UAS-GFP-Dad. The immuno-staining of control (W1118) tissues with Dad antibody revealed that endogenous Dad was present

in several compartments of the *Drosophila* cells including, nucleolus, nucleus, cytosol and membrane (Figure 4.1. A-F). In eye imaginal discs, Dad was clearly co-localized with the nucleolar protein Fibrillarin (Figure 4.1. A-C). Similarly, in salivary glands, Dad was also observed in the nucleolus (Figure 4.1. D-G). According to the intensity levels, Dad localized predominantly in the nucleolus followed by nucleus (Figure 4.1. G). In cytoplasm and plasma membrane, lower levels of Dad were observed (Figure 4.1. D-D'). Similar results were obtained with Dad protein trap in salivary glands (Figure S4.1. D-F). However, in eye imaginal discs, the signal was more diffuse and we could not accurately describe it localization pattern (Figure S4.1. A-C). Nevertheless, in wing disc the signal was clearer and Dad was sharply more intense in the nucleolus (Figure S4.1. G-L).

Next, we used a new UAS-transgenic line with Dad fused with Green Fluorescent Protein (GFP) at the N terminus to take advantage of Gal4-UAS system and manipulate Dad in different regions of *Drosophila* tissues. To induce the expression of *dad* in eye imaginal discs and salivary glands, we took advantage of two different drivers: eyeless (ey)-Gal4 and optix-Gal4. The expression of ey and optix drivers has different intensities during eye development. In imaginal eye discs, *ey* is highly expressed during the L1 and L2 instar larva, but its expression decreases during late L2- early L3 instar larva (Hazelett, Bourouis et al. 1998). On the other hand, *optix* starts its expression later in development during L2 instar larva (Ostrin, Li et al. 2006). The expression of Dad in eye imaginal discs under ey-Gal4 control

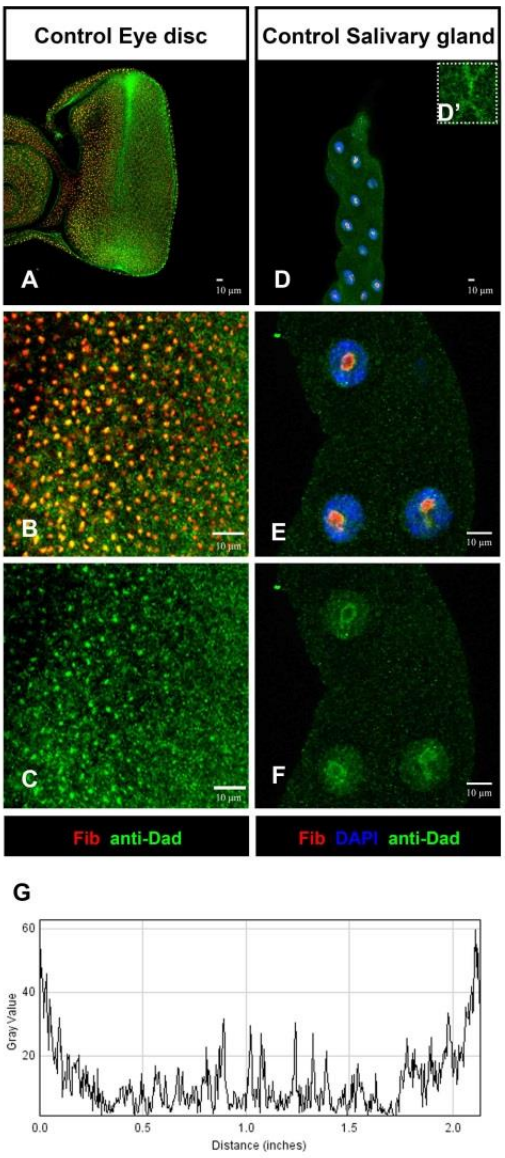


Figure 4.1. Dad localizes in the nucleolus of eye imaginal discs and salivary glands. (A-C) W118 eye imaginal discs stained with anti-Dad (green) and anti-Fibrillarin (nucleolus, red). (D-F) W118 salivary glands stained with anti-Dad, anti-Fibrillarin (nucleolus, red) and DAPI (DNA, blue). (D') Distal view of cell membrane of salivary glands. Scale bars correspond to 10 μm. (G) Intensity map of Dad between two different nucleolus of the salivary gland.

led to dramatic consequences on these discs (Figure 4.2. A-D, discussed forward) but Dad was not detected. The absence of Dad in eye imaginal discs of ey>GFP-*dad* ^{OE} at the wandering L3 stage might be a consequence of a turn-off of ey-Gal4 expression by Dpp signaling downregulation. To further investigate this hypothesis, we took advantage of another fluorescent protein, cd4-td-TOM, and we overexpressed both GFP-*dad* and *cd4*-td-TOM under the control of ey-Gal4. In a similar manner to GFP-*dad*^{OE}, the combinatorial overexpression of GFP-*dad* and *cd4*-td-TOM led to a strong decrease of *cd4*-td-TOM levels (Figure S4.2. A-D), which suggests that the absence of Dad in eye imaginal discs of ey>GFP-*dad* ^{OE} resulted from a turn-off of ey-Gal4, induced by Dad. Nevertheless, in eye imaginal discs overexpressing *dad* under control of optix-Gal4 driver, Dad was observed anteriorly to the morphogenetic furrow (MF)

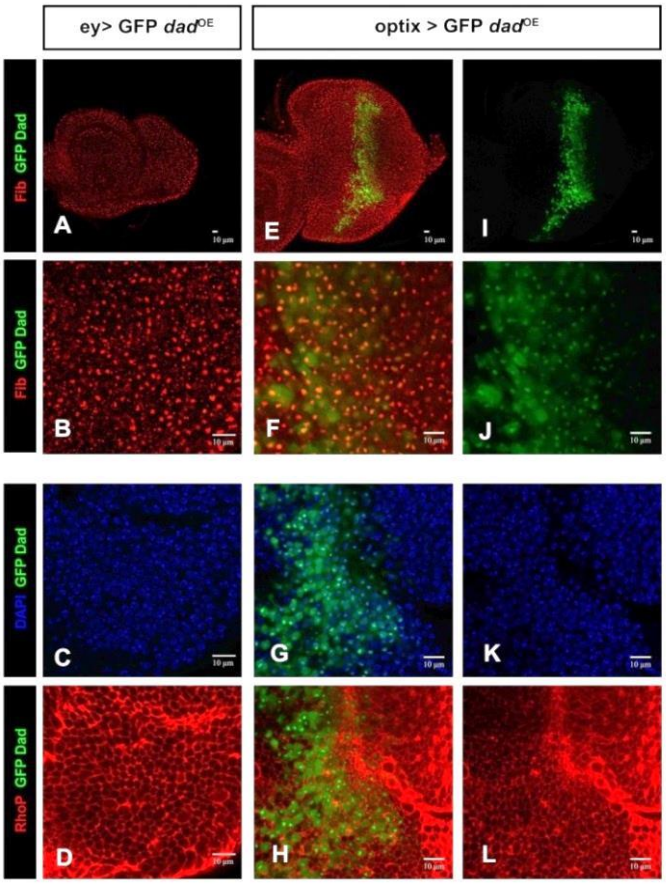


Figure 4.2. Expression of GFP-Dad in eye imaginal discs. (A-D) Overexpression of Dad under control of ey-Gal4 driver inhibits eye differentiation and GFP-Dad is not observed. (A-B) Eye imaginal discs stained with Fibrillarin (nucleolus, red) and (C-D) with DAPI (DNA, blue) and RhoP (membranes, red). (E-F, I-J) Similarly, overexpression of GFP-*dad* under control of optix-Gal4 driver inhibits eye differentiation whereas Dad is observed in and behind the morphogenetic furrow (MF). Eye imaginal discs stained with Fibrillarin (nucleolus, red). Dad is present in the different cellular compartments of the cell, including nucleolus, nucleus, cytoplasm and membrane. (G-H, K-L) Eye imaginal discs stained with DAPI (DNA, blue) and RhoP (membranes, red). Scale bars correspond to 10 μm.

(Figure 4.2. E-L). Regarding its cellular localization, Dad was observed in the nucleolus, nucleus, cytoplasm and membrane (Figure 4.2. E-F, I-J, G-H and K-L). However, the levels of Dad in the nucleolus of cells of imaginal eye discs were higher than the levels of Dad in the remaining compartments. In salivary glands overexpressing GFP-*dad*^{OE}, Dad was more intensely expressed in both nucleolus and membranes (Figure 4.3. A-C, E-G), followed by nucleus (Figure 4.3. D, F and G). In cytoplasm, Dad was observed at residual levels (Figure 4.3. F-G).

Together, these data show that Dad localizes in several compartments of *Drosophila* cells, including nucleolus, nucleus, cytosol and membrane. The ectopic expression of Dad induces an accumulation of this protein in the membranes of salivary glands.

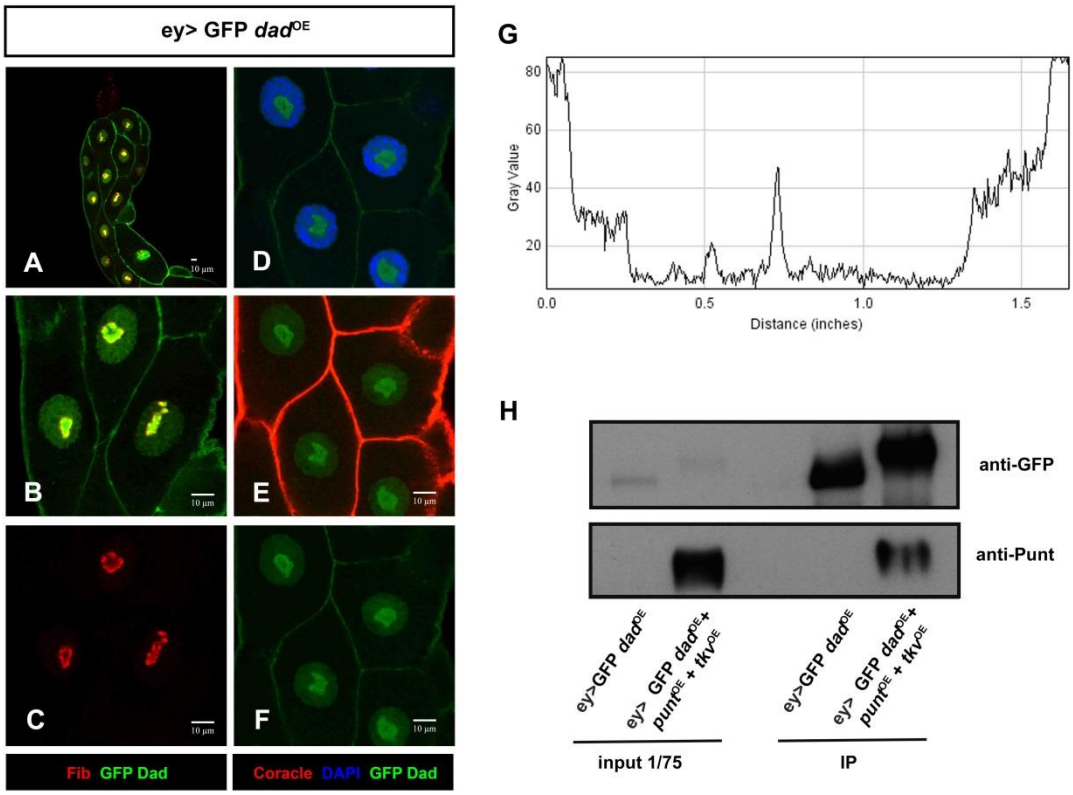


Figure 4.3. Expression of GFP-Dad in salivary glands. (A-C) Dad is present in the nucleolus of salivary glands overexpressing *dad*. Salivary glands stained with Fibrillarin (nucleolus, red). (D-F) Overexpression of *dad* in salivary glands induces an accumulation of this protein in the membrane. Salivary glands stained with DAPI (DNA, blue) and RhoP (membranes, red). Scale bars correspond to 10 μm. (G) Intensity map between two different nucleolus of ey> GFP-*dad*^{OE}. (H) Dad binds to Pun-Tkv complex. Immunoprecipitation of GFP Dad followed by Western Blot against Punt and GFP in the indicated genotypes.

4.2. Dad localization is mediated by TGFβ membrane receptors

Given that a significant portion of Dad is localized in the membranes of salivary glands overexpressing *dad,* we reasoned that membrane-associated Dad is a consequence of the binding of Dad to TGFβ receptors (Inoue, Imamura et al. 1998, Kamiya, Miyazono et al. 2008), which was corroborated by co-immunoprecipitation of Dad and Punt (Figure 4.3. H). To further investigate the effect of TGFβ membrane receptors in GFP-Dad membrane dynamics, we manipulated the expression of *punt* and *tkv* receptors and analyzed the consequences on Dad localization. GFP-Dad labeled membranes with GFP gray values near 40 (Figure 4.4. A-C). In cytosol, GFP levels decreased to

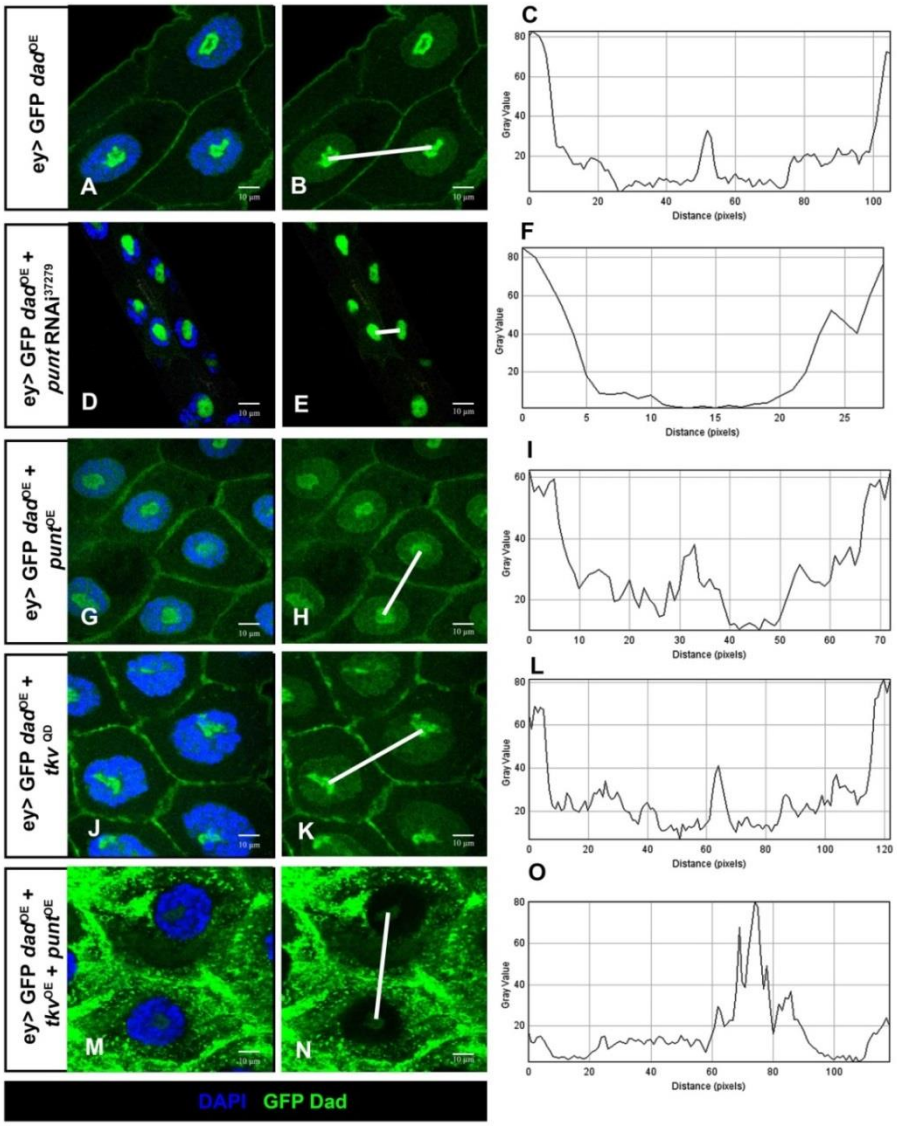


Figure 4.4. TGFβ signaling membrane receptors regulates Dad localization within the cell. (A-O) Dad localization within the cell of the indicated genotypes and the correspondent intensity map. (A-C) ey> GFP-*dad*^{OE}, (D-F) ey> GFP-*dad*^{OE}+ *punt* RNAi^{37279}, (G-I) ey> GFP-*dad*^{OE}+ *punt* ^{OE}, (J-L) ey> GFP-*dad*^{OE}+ *tkv* ^{QD} and (M-O) ey> GFP-*dad*^{OE}+ *tkv* ^{OE}+ *punt* ^{OE}. Scale bars correspond to 10 μm.

values near zero and then increased gradually from the nucleus to the nucleolus. When the type II receptor common to BMP and Activin branches, *punt*, was downregulated by RNAi, GFP-Dad localized exclusive in the nucleolus and it was absent from other compartments (Figure 4.4. D-F). Nevertheless, when *punt* was overexpressed, GFP-Dad levels slight decreased in the nucleolus and increased in the cytosol and membranes (Figure 4.4. G-I). The constitutively active version of Tkv, led to the presence of small intense punctate GFP-Dad structures in the membrane and cytosol (Figure 4.4. J-L). Finally, the co-overexpression of *punt* and *tkv* reduced GFP-Dad in the nucleolus and we were not able to detect it (Figure 4.4. M-O). However, in the membrane and cytosol, we observed a high increase of small punctuated structures (Figure 4.4. M-N).

These data suggest that knockdown of TGFβ receptors is not sufficient to block the expression of *dad*, however the produced Dad is not able to translocate to the membrane. Furthermore, the simultaneous upregulation of Punt and Tkv leads to a dramatically downregulation of Dad in the nucleus and nucleolus, which might result from an intense recruitment of Dad to the membrane.

Afterwards we characterized the dynamic of GFP-Dad when we expressed the different membrane receptors. For that we took advantage of MPM-based fluorescence recovery after photobleaching (FRAP) technique (Yao, Munson et al. 2006, Yao, Ardehali et al. 2007). This technique allows us to determine the recovery rate of fluorescent-tagged GFP-Dad in living salivary gland cells. The recovery depends on the time GFP-Dad takes to replace the photobleached protein. We tested the dynamic of the GFP-Dad in the third instar larvae bleaching membrane and we observed a rapidly recovering of GFP-Dad in the membrane bleached area (Figure 4.5. A-B). However, the immobile fraction was near 30%, representing the percentage of GFP-Dad that remains immobile in the membrane after bleaching. Interestingly, FRAP results revealed that the recovery rate of GFP-Dad in salivary glands with constitutively active Tkv was almost 58% slower than GFP-Dad control ($t_{1/2}$ of ~109 s compared with $t_{1/2}$ of ~190 s, Figure 4.5. A-B). The GFP-Dad signal reaches plateau in constitutively active Tkv salivary glands almost ~750 s after photobleaching (Figure 4.5. A-B). Based on this result, we suggest that Tkv expression have an impact on the dynamics of GFP-Dad in the membrane. Moreover, the overexpression of the type II receptor, *punt*, or the combination of both *punt* and *tkv*, presented a dramatic increase of immobile fraction, and the recovery of GFP-Dad signal was negligible (Figure 4.5. A-B). Accordingly, the upregulation of TGFβ receptors seem to increase the stabilization of Dad in the membrane.

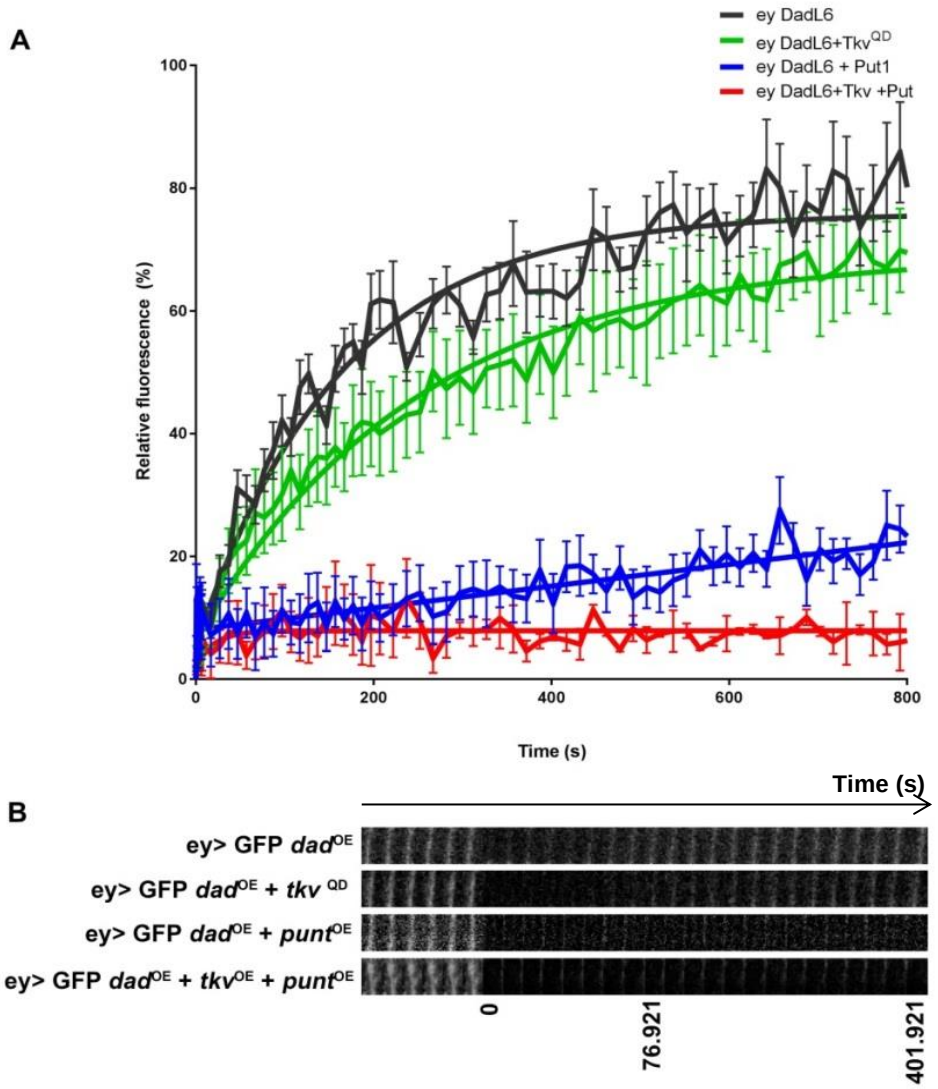


Figure 4.5.GFP-Dad dynamics on the cellular membrane. (A) Representative graph of the time of GFP-Dad recovery in the membrane after photobleach. (B) Output frames at the indicated times postbleach show how the bleach region changes with time in the different genotypes (ey> GFP-*dad*^{OE}, ey> GFP-*dad*^{OE}+ *tkv* ^{QD}, ey> GFP-*dad*^{OE}+ *punt* ^{OE} and ey> GFP-*dad*^{OE}+ *tkv* ^{OE}+ *punt* ^{OE}.

4.3. Dad inhibits Mad phosphorylation

Since Dad is the I-Smad of TGFβ signaling pathway, we expected that alterations in the expression of this protein led to dramatic consequences on cell growth and differentiation. Overexpression of Dad inhibited differentiation in imaginal eye discs, as well as in adult eyes (Figure 4.6. A-C, E-G), resembling the phenotype caused by *punt* loss-of-function (Marinho, Martins et al. 2013, Martins, Eusebio et al. 2017, Eusebio and Pereira 2018). Importantly, overexpression of *dad* caused a strong deficit in tissue

growth (Figure 4.6. P). On the other hand, the RNAi for *dad* led to a slight overgrowth of the eye imaginal disc with some ectopic differentiation (Figure 4.6. P) and the adult eye presented a higher area of differentiation (Figure 4.6. A and I). The presence of ectopic differentiation (Figure 4.6. J-K) in ey>*dad* RNAi^{110644} and the stronger inhibition of differentiation in ey>*dad*^{OE} eye discs (Figure 4.6. F-G) suggested that Dad plays an important role in retinal differentiation. In eye imaginal disc, Mad activation is necessary

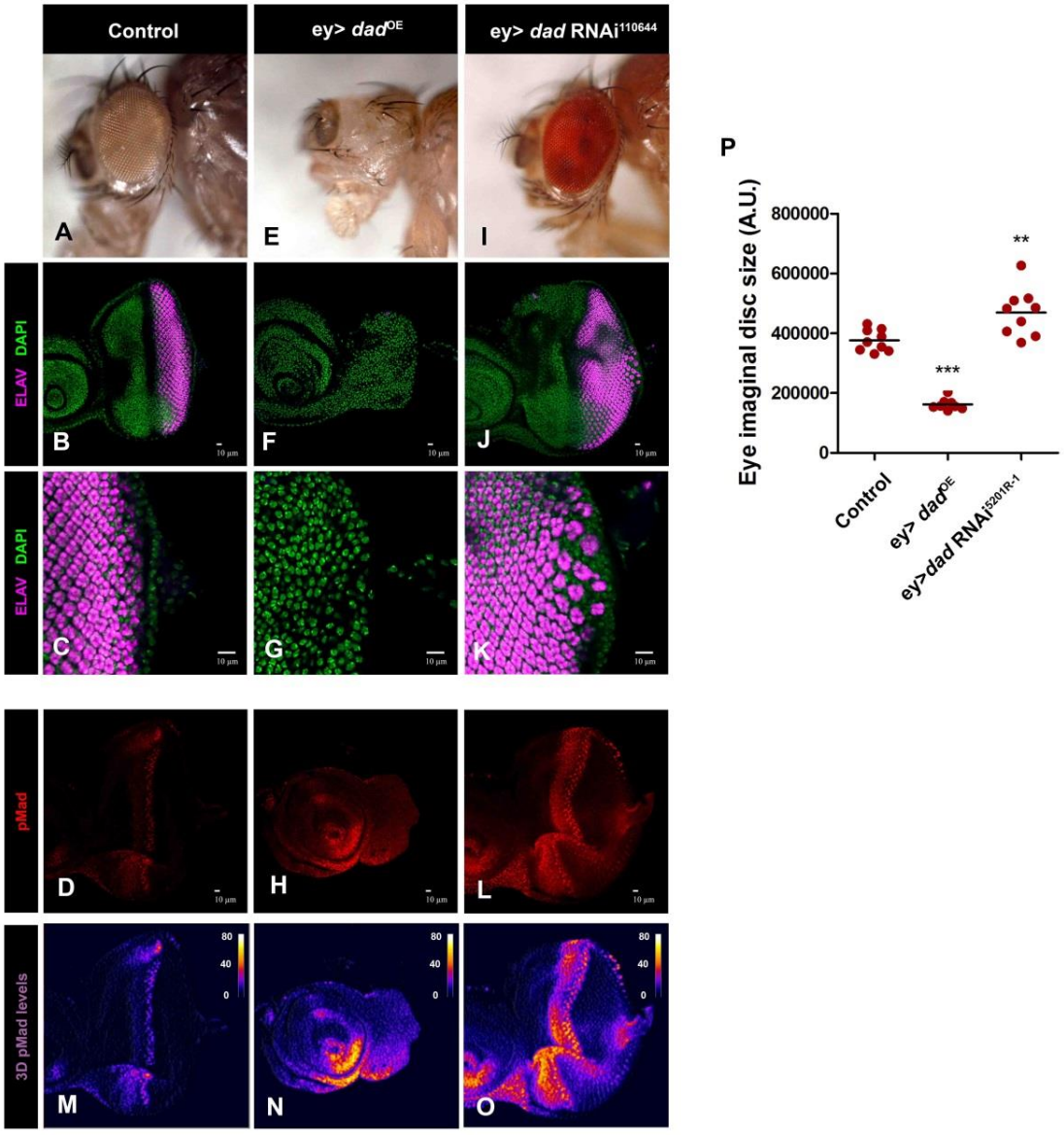


Figure 4.6. Dad regulates eye differentiation. (A-L) Downregulation of Dpp signaling through the overexpression of *dad* blocks photoreceptor differentiation whereas the downregulation of *dad* induces ectopic differentiation in *Drosophila* eyes. (A, E, I) Adult phenotypes of the indicated genotypes. (B-D) Control (ey>*LacZ*) eye imaginal discs. (F-H) Overexpression of *dad* inhibits eye differentiation and Mad activation. (J-L) Downregulation of Dad by RNAi stimulates eye differentiation and Mad activation. Eye imaginal discs of the indicated genotypes stained with DAPI (DNA, green), anti-ELAV (photoreceptors, magenta) and pMad (red). Scale bars correspond to 10 μm. (M-O) 3D histograms of pMad patterns in eye discs of the indicated genotypes. (I) Quantification of eye imaginal discs size of the indicated genotypes, **, P<0.01 and ***, P<1^10^{-4}.

to switch the progenitor cell state into the precursor state allowing the initiation and progression of retinal differentiation (Bessa, Gebelein et al. 2002, Lopes and Casares 2010). Therefore, the over-differentiation observed in ey>*dad* RNAi^{110644} might result from the increase of Mad activation. To test this hypothesis, we used an antibody that recognizes the phosphorylated form of *Drosophila* Mad (pMad) in *dad* overexpression and *dad* RNAi eye discs. Control eye imaginal discs (Figure 4.6. D and M) exhibited a sharp and intense pMad band close to the MF and a broader less intense in the anterior domain (Vrailas and Moses 2006, Firth, Bhattacharya et al. 2010). As predictable, in eye imaginal discs overexpressing *dad*, pMad was reduced to residual levels (Figure 4.6. H and N, (Ogiso, Tsuneizumi et al. 2011, Eusebio and Pereira 2018)). Similarly to the control, ey>*dad* RNAi^{110644} imaginal eye discs exhibited a sharp and intense pMad band close to the MF and a broader less intense in the anterior domain (Figure 4.6. L and O). However, in these discs an additional pMad band was observed close to the posterior boarder of the disc (Figure 4.6. L and O). In wing discs overexpressing *dad*, we observed a decrease of pMad levels (Figure S4.3. A-F). This downregulation of Mad activation was associated with a decrease of the adult wing size (Figure S4.3. G-J). Thus, our results corroborate the previous assumptions indicating Dad as a negative regulator of Dpp-BMP2/4 signaling pathway (Tsuneizumi, Nakayama et al. 1997, Kamiya, Miyazono et al. 2008, Eusebio and Pereira 2018).

4.4. The N-terminal domain of Dad is required for an efficient inhibition of Mad phosphorylation

We next examined which domains of Dad are responsible for inhibition of Mad activation and consequently inhibition of TGFβ signaling pathway. For that purpose, we truncated the first 113 a.a. of Dad protein, because this region is rich in its proline content (19 prolines) (Figure 4.7. P). The enriched proline regions are known to be associated with several transient intermolecular interactions, including signal transduction, cell-cell communication and cytoskeletal organization (Williamson 1994, Ball, Kühne et al. 2005). Moreover, the N-terminal domains of Smad 6 and Smad7 have been described as very important for their inhibitory functions on TGFβ signaling pathway (Itoh, Landström et al. 1998, Hanyu, Ishidou et al. 2001, Lin, Liang et al. 2003). As previously described, in eye imaginal discs overexpressing *dad* under control of ey-Gal4 driver, pMad was reduced to residual levels (Figure 4.6. H and N) (Eusebio and Pereira 2018). Similarly, the overexpression of *dad* under control of optix-Gal4 driver also led to a strong downregulation of Mad activation (Figure 4.7. F-H). This inhibition of Mad activation seems to be a direct effect of the presence of Dad close

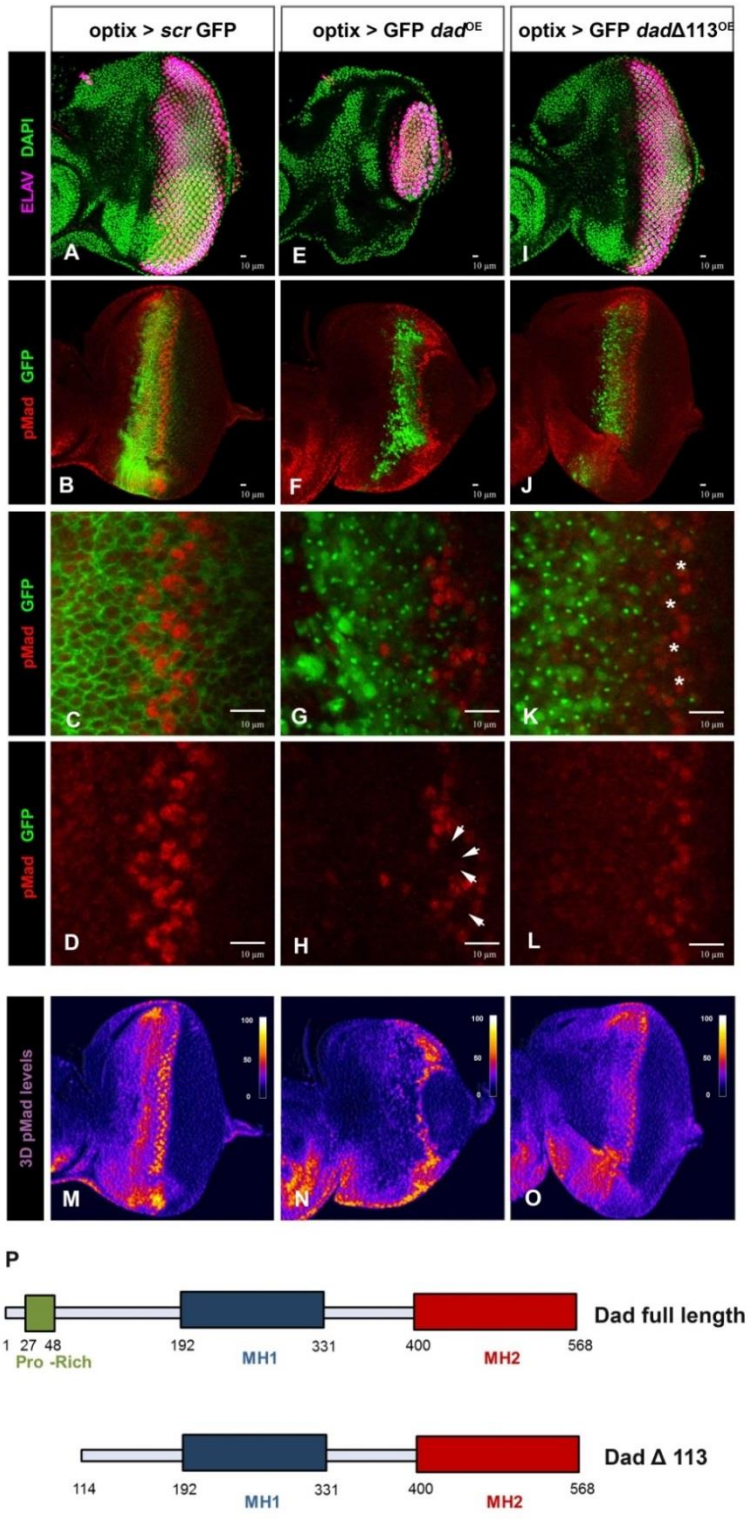


Figure 4.7. N-terminal domain of Dad is required for an efficient inhibition of Mad activation. Eye imaginal discs of control (optix>*src*GFP) (A), optix> GFP*dad*^{OE} (E) and optix> GFP*dadΔ113*^{OE} stained with DAPI (DNA, green) and anti-ELAV (photoreceptors, magenta). (B-D) Control, (F-H) optix> GFP*dad*^{OE} and (J-L) optix> GFP*dadΔ113*^{OE} stained with pMad (red). Arrows represent cells expressing GFP-*dad*^{OE} in the absence of pMad staining. Asterisks represent cells expressing GFP- *dad*^{OE} in the presence of pMad staining. Scale bars correspond to 10 μm. (M-O) 3D histograms of pMad patterns in the eye discs of the indicated genotypes. (P) Schematic representation of Dad full length and Dad Δ113.

and within the MF, since a strong downregulation of pMad expression was observed in cells expressing *dad* (Figure 4.7. F-H and N). Remarkably, cells with Dad exclusively in the nucleolus were also able to inhibit pMad (Figure 4.7. G-H). However, overexpression of the truncated *dad*^{OE} Δ113 version only partially inhibited Mad activation in and anterior to MF (Figure 4.7. J-L and O), despite the expression leves of full and truncated *dad* being similar. Consequently, photoreceptor cell differentiation was only moderately affected. Curiously, in salivary glands overexpressing the truncated *dad*^{OE} Δ113 version, we observed a delocalization of Dad protein (Figure S4.4.). In particular, we observed a decrease of Dad levels in the membrane and an increase in cytoplasm. This suggests that Dad N-terminal domain may code a peptide sequence that is necessary for Dad-membrane association.

Together, these results suggest that the first 113 a.a. are required for efficient inhibition of Mad phosphorylation and Dad localization in the membrane.

4.5. An upregulation of TGFβ signaling induces Dad phosphorylation inhibiting its function

Next we asked if Dad is able to inhibit TGFβ signaling when we strongly stimulated this signaling. For that we combinatorial overexpressed *punt* and *tkv* and analyzed the resulted phenotype. In imaginal eye discs, the overexpression of *punt* and *tkv* led to a strong increase of photoreceptors size and alterations in retinal patterning (Figure 4.8. A-B). As expected the combinatorial overexpression of *punt* and *tkv* induced a strong upregulation of pMad, with increased intensity and extensive broadening anterior and posterior to the MF when compared with control eye discs (Figure 4.8. D-I and M-N). Remarkably, the overexpression of Dad in a Punt and Tkv background was sufficient to downregulate Mad activation (Figure 4.8. J-L and O) and consequently rescued the alterations in retinal patterning induced by Punt and Tkv (Figure 4.8. A-C). Curiously, in salivary glands the combinatorial expression of both TGFβ signaling receptors led to several alterations in Actin structure of salivary glands, including the increase of the density of Actin filaments and the formation of multiple intercellular lumens (Figure 4.9. A-F). However, these defects were not rescued by *dad* overexpression (Figure 4.9. G-I). Thus, we asked if some post-translational modification was responsible for this loss-of-function of Dad.

Phosphorylation is an important post-translational modification that controls the function, localization, and binding specificity of target proteins. This post-translational modification involves the coupling and addition of a phosphoryl group (PO3-) to the target protein, possibly changing the structure of a protein by altering interactions with

neighboring amino acids. Additionally, the phosphorylation of a protein can cause a mobility shift of the target protein during SDS-PAGE. Taking advantage of Western Blot analysis, we detected a mobility shift of GFP-Dad protein in a *punt* and *tkv* overexpression background comparing to the control GFP-Dad (Figure 4.9. J). Thus, we asked if the overexpression of tkv^{QD} would be sufficient to induce the GFP-Dad shift observed when we overexpressed *punt* and *tkv* receptors (Figure 4.9. J). Tkv^{QD} has an

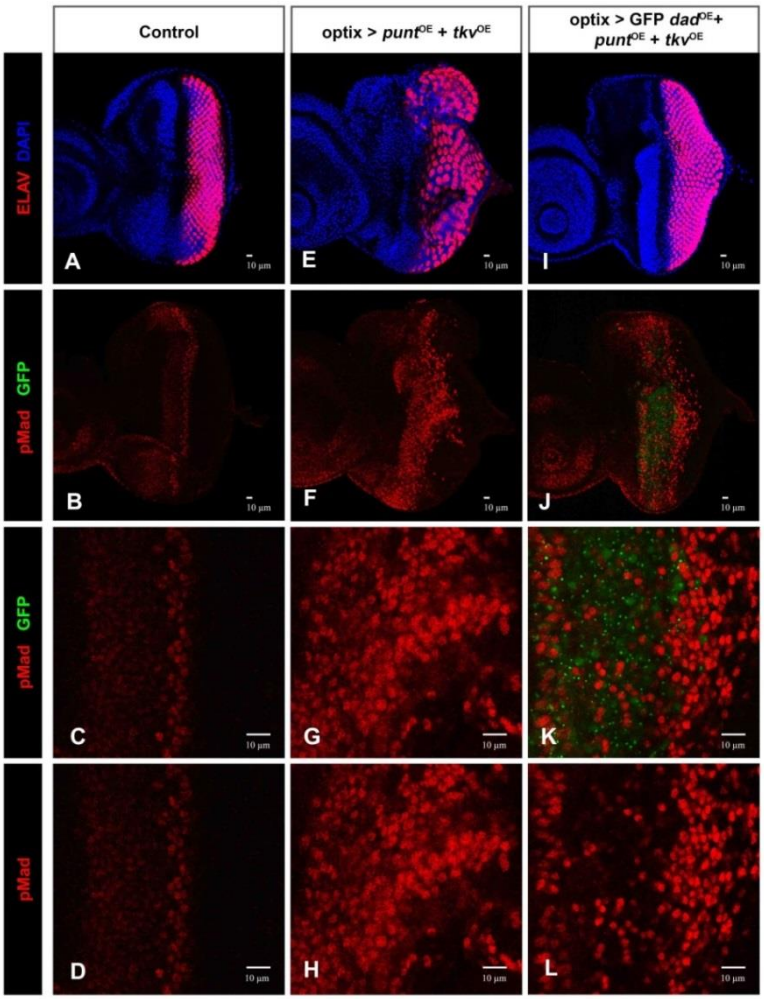


Figure 4.8. Dad recues the TGFβ-induced phenotype in eye imaginal disc. (A-L) Eye imaginal discs of the indicated genotypes stained with DAPI (DNA, blue), anti-ELAV (photoreceptors, magenta) and pMad (red). Scale bars correspond to 10 µm.

amino acid change in the 253 position of GS domain and acts as a constitutive active type I receptor (Wieser, Wrana et al. 1995, Nellen, Burke et al. 1996). However, this TGFβ signaling activation was unable to induce GFP-Dad shift (Figure 4.9. J). These results suggest that both BMP and Activin branches might be required to induce GFP-Dad shift. To determine if this GFP-Dad shift is an effect of Dad phosphorylation, we treated GFP-Dad control and GFP-Dad in a combinatorial *punt* and *tkv* overexpression background samples with Lambda Protein Phosphatase (Figure 4.9. K). This

phosphatase is able to release phosphate groups from phosphorylated serine, threonine and tyrosine residues in proteins. After the treatment of GFP-Dad in a combinatorial Punt and Tkv background, GFP-Dad increased its mobility and achieved similar molecular weights observed in GFP-Dad control (Figure 4.9. K). Thus, the simultaneous overexpression of *punt* and *tkv* appear to be sufficient to induce Dad phosphorylation blocking its inhibitory function on the TGFβ signaling pathway.

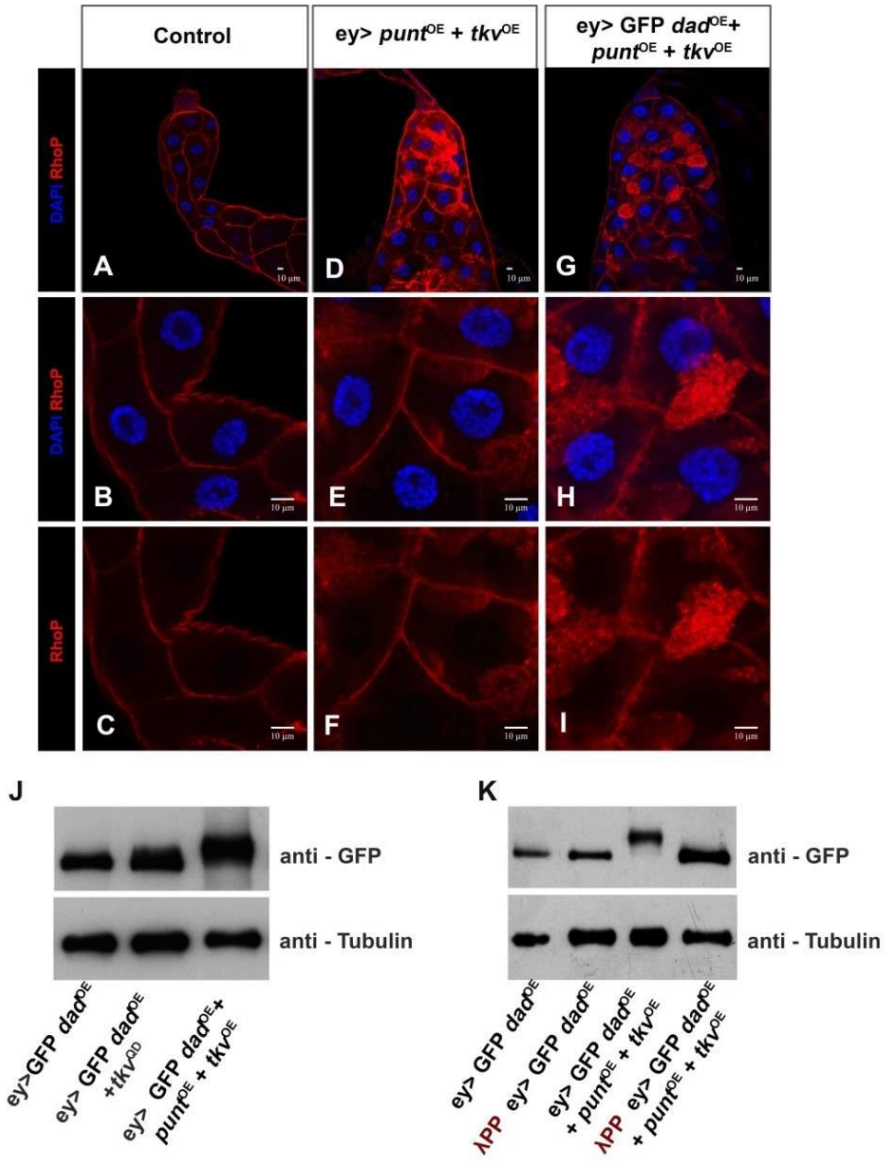


Figure 4.9. TGFβ signaling induces Dad phosphorylation. (A-C) Control (ey>*LacZ*) salivary gland. (D-F) Ectopic co-expression of *punt* and *tkv* induces defects in the salivary glands. (G-I) Ectopic expression of Dad does not recuperate Pun/Tkv-induced salivary gland defects. Salivary glands of the indicated genotypes were stained with DAPI (DNA, blue) and RhoP (membranes, red). Scale bars correspond to 10 μm. (J) Western blot of the indicated genotypes. The co-expression of *punt* and *tkv* causes a shift of Dad to a lower migrating band. (K) Western blot of the indicated genotypes. ey>GFP- *dad*^{OE} and ey>GFP-*dad*^{OE}+ *punt*^{OE} + *tkv*^{OE} were treated with lambda phosphatase (λPP). Treated GFP-Dad in a combinatorial *punt* and *tkv* overexpression background increases its mobility and achieved similar molecular weights observed in GFP-Dad control.

Furthermore, we also investigated whether the GFP-Dad shift induced by phosphorylation is a consequence of the addition of phosphoryl groups in the N-terminal domain of Dad. For that we overexpressed GFP-*dad* Δ113 in a *punt* and *tkv* overexpression background. The truncated version of Dad was not able to rescue the defects induced by *punt* and *tkv* overexpression in salivary glands (Figure 4.10. A-I). Moreover, a mobility shift of GFP-Dad Δ113 protein was observed in *punt* and *tkv* overexpression background when compared to the control GFP-*dad* Δ113 (Figure 4.10. J). This shift was not observed after a treatment with Lambda Protein Phosphatase, which suggests that the N-terminal domain of Dad is not required for Dad phosphorylation.

Next, we tried to identify possible phosphorylated sites in Dad sequence. Dad has seven serines or threonines with a proline in the +1 position, constituting the minimal consensus for phosphorylation by mitogen-activated protein kinases (Jacobs, Glossip et al. 1999, Sharrocks, Yang et al. 2000, Biondi and Nebreda 2003) or cyclin-dependent kinases (Kitagawa, Higashi et al. 1996, Moses, Hériché et al. 2007). From those, Ser333 and Ser488 and its neighboring residues are conserved from *Drosophila* Dad to human I-Smads (Figure 4.11. A-B). To determine precisely the sites of Dad phosphorylation, in salivary glands, we chose a mass spectrometry-based approach (Figure S4.5.). The MALDI-TOF analysis of salivary glands was able to identify 50% (279/568 aa) of Dad protein sequence. From those peptides, we identified three possible phosphorylation sites: Ser154, Thr335 and Ser539 (Figure S4.5. A-B); and a phosphopeptide that contain four possible threonine/serine sites: Thr275, Thr277, Thr284 and Ser286 (Figure S4.5. A-B). Ser539 and the surrounding residues are conserved from flies to humans, including the SIK motif containing Ser535 already described as target of phosphorylation by protein kinase X in Smad6 (Glesne and Huberman 2006). Ser154 and Thr335 are not conserved in human I-Smad, however they are followed by a proline (Figure 4.11. B and Figure S4.5. A-B). Particularly, Thr335 is found next to the SP motif of Ser333, suggesting a possible target of phosphorylation (Figure 4.11. B). Nevertheless, Thr275, Thr277, Thr284 and Ser286 are not conserved in human I-Smad or close to a phosphorylation motif. Thus, we believe that Ser333, Thr335, Ser488, Ser535 and Ser539 are potential Dad residues target of phosphorylation.

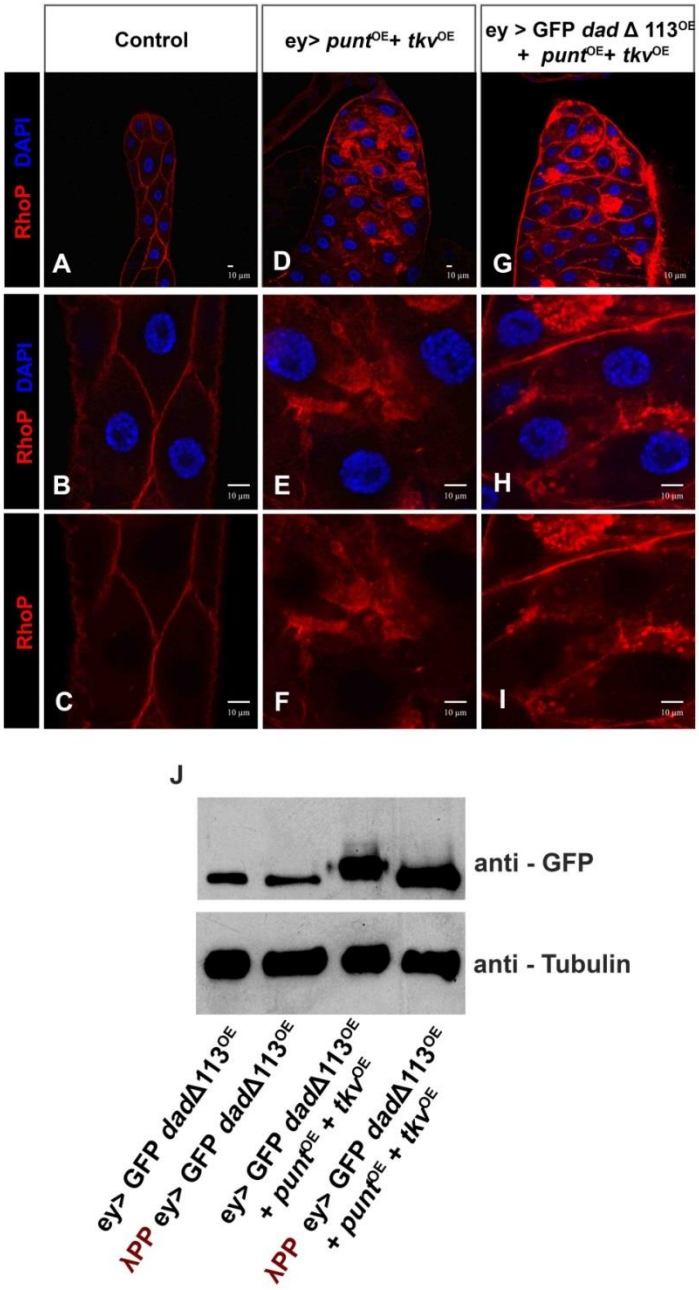
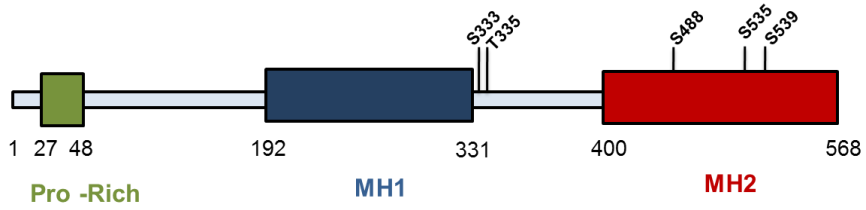


Figure 4.10. N-terminal domain of Dad is not required for Dad phosphorylation. (A-C) Control (ey>*LacZ*) salivary gland. (D-F) Ectopic co-expression of *punt* and *tkv* induces defects in salivary glands. (G-I) Ectopic expression of *dad*Δ113 does not recuperate Pun/Tkv-induced salivary gland defects. Salivary glands of the indicated genotypes were stained with DAPI (DNA, blue) and RhoP (membranes, red). Scale bars correspond to 10 μm. (J) Western blot of the indicated genotypes. ey>GFP -*dadΔ113*^{OE} and ey>GFP-*dad Δ113*^{OE}+ *punt*^{OE} + *tkv*^{OE} were treated with lambda phosphatase (λPP). Treated GFP-Dad Δ113 in a combinatorial Punt and Tkv overexpression background increases its mobility and achieved similar molecular weights observed in GFP-Dad control.

A



B

Expected S/T	Motif	Conserved	Predicted Kinase by NetPhos	MS identification	Identified in Smad6 or Smad7
S^{333}	TESPTP	+	Cdk5	-	-
T^{335}	SPTPP	-	Cdk5, GSK3	+	-
S^{488}	VDSPTL	+	P38MAPK, Cdk5, GSK3	-	-
S^{535}	YFSIKIS	+	unsp	-	+
S^{539}	KISFGK	+	PKC	+	-

Figure 4.11. Expected phospho-motifs. (A) Schematic representation of Dad protein and potential residues target of phosphorylation. (B) Table with the potential Dad residues target of phosphorylation supplemented with neighbors residues, conservation with Human I-Smads, predicted kinases by NetPhos (http://www.cbs.dtu.dk/services/NetPhos/), identification by Mass Spectrometry analysis and phosphorylated T/S in Smad6 or Smad7.

5. Discussion

Dad is a member of the SMAD family responsible for antagonizing TGFβ signaling (Tsuneizumi, Nakayama et al. 1997). Transcription of *dad* is positively regulated by Dpp (Tsuneizumi, Nakayama et al. 1997), however Dad is able to regulate its own expression by directly regulating TGFβ signaling, in a negative feedback loop (Tsuneizumi, Nakayama et al. 1997, Kamiya, Miyazono et al. 2008). Dad was initially found to associate with Tkv to inhibit Tkv-induced phosphorylation of Mad (Tsuneizumi, Nakayama et al. 1997). In addition, Dad also inhibits Sax, another BMP-specific type I receptor that transmits signal through Mad, but not the Activin type I receptor Babo, which transmits signals through Smad2 (Kamiya et al., 2008). In our study, we demonstrated for the first time that Dad inhibits TGFβ signaling in the nucleolus. The ectopic expression of *dad* in imaginal eye discs revealed that the presence of Dad in the nucleolus was sufficient to inhibit Mad activation in the MF. This TGFβ signaling downregulation by Dad led to undifferentiated and small eye imaginal discs. The Dad N-terminal domain was required for the regulation of Dad localization and function. In

particular, Dad N-terminal domain was necessary for an efficient Dad-membrane association in salivary glands and also for an efficient Mad inhibition in eye imaginal discs. Finally, in salivary glands, a strong upregulation of TGFβ signaling was sufficient to induce Dad phosphorylation blocking its inhibitory function on the TGFβ signaling pathway.

5.1. Subcellular localization of Dad

In mammals, the subcellular localization of I-Smads is differentially regulated between Smad6 and Smad7. Smad7 has been reported to be present in the nucleus of transfected COS1, COS7 and Mv1Lu cells (Itoh, Landström et al. 1998, Hanyu, Ishidou et al. 2001, Zhang, Fei et al. 2007), while it was predominantly located in the cytoplasm of HepG2 and R mutant Mv1Lu cells (Itoh, Landström et al. 1998, Hanyu, Ishidou et al. 2001). Smad6 has been described to be located in both the nucleus and the cytoplasm (Hanyu, Ishidou et al. 2001, Lin, Liang et al. 2003). In *Drosophila*, only one I-Smad, Dad, has been identified. In our study, Dad was localized in the nucleolus of control eye imaginal discs. Similarly, in eye imaginal discs overexpressing Dad, high levels of this protein were detected in the nucleolus. Interestingly, we observed that the presence of Dad in the nucleolus was sufficient to inhibit TGFβ signaling. The overexpression of *dad* in eye imaginal discs inhibited eye growth and differentiation. In control salivary glands, Dad was also observed in the nucleolus. Accordingly to the intensity levels, Dad was more intense in the nucleolus, followed by nucleus. In the plasma membrane and cytosol of a control salivary gland, Dad levels were very low. However, in gland cells overexpressing *dad*, this protein accumulated in the membrane, presenting similar levels to the nucleolus. These findings are in agreement with earlier reports that characterized Dad as an Inhibitory Smad that acts at type I receptors level (Tsuneizumi, Nakayama et al. 1997, Kamiya, Miyazono et al. 2008). The downregulation of TGFβ signaling by RNAi for type II receptor *punt* was able to strongly downregulate the expression of *dad* within the cell, with the exception of nucleolus. On the other hand, the strong upregulation of TGFβ signaling by overexpression of both *punt* and *tkv* receptors induced nucleolar and nuclear export of Dad, which was also observed for Smad7 in COS1 cells (Itoh, Landström et al. 1998). Moreover, *punt* and *tkv* receptors induced a stabilization of Dad in the membrane, in contrast to the transient localization that we observed when we overexpressed Dad in a control background. Together these results suggest that TGFβ signaling receptors regulate Dad localization and dynamics.

5.2. N-terminal domain of Dad is required for an efficient inhibition of Mad phosphorylation

Intermolecular interactions mediated by proline-rich motifs are involved in regulation of several important signaling cascades. The proline-rich regions are known to preferentially adopt a polyproline II helical conformation, a fundamental secondary structure that plays a role in several transient intermolecular interactions such as signal transduction, cell-cell communication and cytoskeletal organization (Williamson 1994, Ball, Kühne et al. 2005). The N-terminal of Dad is rich in its proline content, which suggests that domain might be important for Dad functions. For that reason, we truncated the first 113 a.a of Dad and we observed a loss of the inhibitory capacity of Dad in TGFβ signaling. In eye imaginal discs, the overexpression of the truncated *dad*^{OE} Δ113 version only partially inhibited Mad activation in and anterior to MF, which contrasted with the strong inhibition induced by Dad full length. This inefficient Mad inhibition led to smooth consequences in eye differentiation. Accordingly, the eye differentiation was only moderately affected. In salivary glands, the overexpression of *dad*^{OE} Δ113 version resulted in a delocalization of Dad from the membrane to the cytoplasm, which suggests that the N-terminal domain is necessary for Dad-membrane association. Similarly, the mammalian N-domain of Smad7 is important for an efficient inhibition of TGFβ signaling and also determines its subcellular localization (Itoh, Landström et al. 1998, Hanyu, Ishidou et al. 2001).These findings suggest that Dad N-terminal domain might be a peptide sequence that is necessary for Dad-membrane association and Mad regulation.

5.3. TGFβ signaling induces Dad phosphorylation

In vertebrates, I-Smads undergo several post-translational modifications, including ubiquitination, acetylation, phosphorylation and methylation (Pulaski, Landström et al. 2001, Simonsson, Heldin et al. 2005, Glesne and Huberman 2006, Kume, Haneda et al. 2007, Seong, Jung et al. 2010, Xu, Wang et al. 2013). In *Drosophila,* only two different post-translational modifications have been described to modify Dad function: arginine methylation and palmitoylation. The methylation of Dad by Dart1 inhibits the function of Dad as inhibitory Smad of BMP signaling in *Drosophila* wing development (Xu, Wang et al. 2013). On the other hand, Dad is also palmitoylated by palmitoyltransferase dHIP14 and this modification is critical for membrane-function of Dad (Li, Li et al. 2017). Remarkably, we found that a strong upregulation of TGFβ signaling was able to induce Dad phosphorylation and block its inhibitory function. This phosphorylation was only induced by the overexpression of both *tkv* and *punt*.

Intriguingly, the upregulation of Tkv^{QD} (a mutant receptor with a single amino acid change of Q253 to aspartic acid (Nellen, Burke et al. 1996)) was not sufficient to induce Dad phosphorylation. The main difference between Tkv^{QD} and the co-expression of Punt and Tkv in the activation of TGFβ signaling is the nature of regulation. Tkv^{QD} is a constitutive active version of this receptor; consequently, it induces a continuous upregulation of the pathway. On the other hand, the co-expression of Punt and Tkv resembles a normal activation of TGFβ signaling. Consequently, the co-expression of Punt and Tkv induces a physiological regulation of TGFβ signaling, which might be required for Dad phosphorylation. Moreover, Punt is a type II receptor common to BMP and Activin branches. Thus, the phosphorylation of Dad by TGFβ signaling might require the activation of the both branches of this signaling, BMP and Activin.

We showed that N-terminal domain of Dad is important for an efficient inhibition of TGFβ signaling and also determines its subcellular localization. Thus, we next asked whether Dad phosphorylation occurs in the N-terminal domain of this protein. However, the truncated N-terminal domain version of Dad was also phosphorylated when TGFβ signaling was upregulated, which suggested that N-terminal domain of Dad is not required for Dad phosphorylation. Nevertheless, the function of the N-terminal domain of Dad might be regulated by both arginine methylation and palmitoylation. We know that Dad is methylated by Dart1 in both 293T and Drosophila Kc cells, but the arginine site target of Dart1 methylation is still unknown (Xu, Wang et al. 2013). Smad6 is methylated by PRMT1 on arginines 74 and 81 located on the N-terminal domain of this I-Smad. Despite the low conservation between the N-terminal domain of Dad and Smad6, the N-terminal domain of Dad might be target of arginine methylation, which could be necessary for the regulation of its function. On the other hand, Dad is palmitoylated in the C-terminal Cys556 site, which is conserved in vertebrate Smad6/7 (Li, Li et al. 2017). The N-terminal domain of Dad has no conserved cysteines with vertebrate Smad6/7. However, Dad might have a nonconserved cysteine site with Smad6/7 target of palmitoylation and critical for its function.

The phosphorylation of Dad might be a direct consequence of TGFβ signaling activation or from the activation of other kinases induced by TGFβ signaling. Remarkably, we identified possible kinase target motifs in Dad, including motifs for P38MAPK, Cdk5, GSK3 and PKC. Thus, it would be very interestingly to determine which kinase is responsible for Dad phosphorylation.

In summary, our findings extended our knowledge on the molecular mechanisms underlying the negative regulation of TGFβ signaling by Dad. In addition to the interference with R-Smad phosphorylation and association with type I receptors

inhibiting Mad activation, Dad can exert its inhibitory effect directly in the nucleolus. Moreover, the N-terminal domain of Dad is required for an efficient inhibition of Mad phosphorylation and Dad localization in the membrane. Curiously, TGFβ signaling may regulate Dad activity through phosphorylation. Phosphorylated Dad is not able to exert its inhibitory activity on TGFβ signaling.

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7. References

Ball, L. J., R. Kühne, J. Schneider-Mergener and H. Oschkinat (2005). "Recognition of Proline-Rich Motifs by Protein–Protein-Interaction Domains." Angewandte Chemie International Edition **44**(19): 2852-2869.

Bessa, J., B. Gebelein, F. Pichaud, F. Casares and R. S. Mann (2002). "Combinatorial control of *Drosophila* eye development by Eyeless, Homothorax, and Teashirt." Genes & Development **16**(18): 2415-2427.

Biondi, R. M. and A. R. Nebreda (2003). "Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions." Biochemical Journal **372**(Pt 1): 1-13.

Estrada, K. D., K. N. Retting, A. M. Chin and K. M. Lyons (2011). "Smad6 is essential to limit BMP signaling during cartilage development." Journal of Bone and Mineral Research **26**(10): 2498-2510.

Eusebio, N. and P. S. Pereira (2018). "An eye-targeted double-RNAi screen reveals negative roles for the Archipelago ubiquitin ligase and CtBP in *Drosophila* Dpp-BMP2/4 signaling." under review.

Feng, X.-H. and R. Derynck (2005). "Specificity and versatility in TGF-β signaling through Smads." Annual Review of Cell and Developmental Biology **21**(1): 659-693.

Ferreirinha, P., A. Correia, M. Teixeira-Coelho, H. Osório, L. Teixeira, A. Rocha and M. Vilanova (2016). "Mucosal immunization confers long-term protection against intragastrically established Neospora caninum infection." Vaccine **34**(50): 6250-6258.

Firth, L. C., A. Bhattacharya and N. E. Baker (2010). "Cell cycle arrest by a gradient of Dpp signaling during *Drosophila* eye development." BMC Developmental Biology **10**(1): 28.

Glesne, D. and E. Huberman (2006). "Smad6 is a protein kinase X phosphorylation substrate and is required for HL-60 cell differentiation." Oncogene **25**: 4086.

Hanyu, A., Y. Ishidou, T. Ebisawa, T. Shimanuki, T. Imamura and K. Miyazono (2001). "The N domain of Smad7 is essential for specific inhibition of transforming growth factor-β signaling." The Journal of Cell Biology **155**(6): 1017-1028.

Hazelett, D. J., M. Bourouis, U. Walldorf and J. E. Treisman (1998). "decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc." Development **125**(18): 3741-3751.

Inoue, H., T. Imamura, Y. Ishidou, M. Takase, Y. Udagawa, Y. Oka, K. Tsuneizumi, T. Tabata, K. Miyazono and M. Kawabata (1998). "Interplay of Signal Mediators of Decapentaplegic (Dpp): Molecular Characterization of Mothers against dpp, Medea, and Daughters against dpp." Molecular Biology of the Cell **9**(8): 2145-2156.

Itoh, S., M. Landström, A. Hermansson, F. Itoh, C.-H. Heldin, N.-E. Heldin and P. ten Dijke (1998). "Transforming Growth Factor β1 Induces Nuclear Export of Inhibitory Smad7." Journal of Biological Chemistry **273**(44): 29195-29201.

Jacobs, D., D. Glossip, H. Xing, A. J. Muslin and K. Kornfeld (1999). "Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase." Genes & Development **13**(2): 163-175.

Kamiya, Y., K. Miyazono and K. Miyazawa (2008). "Specificity of the inhibitory effects of Dad on TGF-β family type I receptors, Thickveins, Saxophone, and Baboon in *Drosophila*." FEBS Letters **582**(17): 2496-2500.

Kitagawa, M., H. Higashi, H. K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, S. Nishimura and Y. Taya (1996). "The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2." The EMBO Journal **15**(24): 7060-7069.

Kume, S., M. Haneda, K. Kanasaki, T. Sugimoto, S.-i. Araki, K. Isshiki, M. Isono, T. Uzu, L. Guarente, A. Kashiwagi and D. Koya (2007). "SIRT1 Inhibits Transforming Growth Factor β-Induced Apoptosis in Glomerular Mesangial Cells via Smad7 Deacetylation." Journal of Biological Chemistry **282**(1): 151-158.

Li, W., W. Li, L. Zou, S. Ji, C. Li, K. Liu, G. Zhang, Q. Sun, F. Xiao and D. Chen (2017). "Membrane targeting of inhibitory Smads through palmitoylation controls TGF-β/BMP signaling." Proceedings of the National Academy of Sciences.

Lin, X., Y.-Y. Liang, B. Sun, M. Liang, Y. Shi, F. C. Brunicardi, Y. Shi and X.-H. Feng (2003). "Smad6 Recruits Transcription Corepressor CtBP To Repress Bone Morphogenetic Protein-Induced Transcription." Molecular and Cellular Biology **23**(24): 9081-9093.

Lopes, C. S. and F. Casares (2010). "*hth* maintains the pool of eye progenitors and its downregulation by Dpp and Hh couples retinal fate acquisition with cell cycle exit." Developmental Biology **339**(1): 78-88.

Marinho, J., T. Martins, M. Neto, F. Casares and P. S. Pereira (2013). "The nucleolar protein Viriato/Nol12 is required for the growth and differentiation progression activities of the Dpp pathway during *Drosophila* eye development." Developmental Biology **377**(1): 154-165.

Martins, T., N. Eusebio, A. Correia, J. Marinho, F. Casares and P. S. Pereira (2017). "TGFβ/Activin signalling is required for ribosome biogenesis and cell growth in *Drosophila* salivary glands." Open Biology **7**(1).

Massagué, J., J. Seoane and D. Wotton (2005). "Smad transcription factors." Genes & Development **19**(23): 2783-2810.

Miyazono, K., Y. Kamiya and K. Miyazawa (2008). "SUMO amplifies TGF-β signalling." Nat Cell Biol **10**(6): 635-637.

Miyazono, K., Y. Kamiya and M. Morikawa (2010). "Bone morphogenetic protein receptors and signal transduction." Journal of Biochemistry **147**(1): 35-51.

Moses, A. M., J.-K. Hériché and R. Durbin (2007). "Clustering of phosphorylation site recognition motifs can be exploited to predict the targets of cyclin-dependent kinase." Genome Biology **8**(2): R23-R23.

Nellen, D., R. Burke, G. Struhl and K. Basler (1996). "Direct and Long-Range Action of a DPP Morphogen Gradient." Cell **85**(3): 357-368.

Ogiso, Y., K. Tsuneizumi, N. Masuda, M. Sato and T. Tabata (2011). "Robustness of the Dpp morphogen activity gradient depends on negative feedback regulation by the inhibitory Smad, Dad." Dev Growth Differ **53**(5): 668-678.

Ostrin, E. J., Y. Li, K. Hoffman, J. Liu, K. Wang, L. Zhang, G. Mardon and R. Chen (2006). "Genome-wide identification of direct targetsof the *Drosophila* retinal determinationprotein Eyeless." Genome Research **16**(4): 466-476.

Pulaski, L., M. Landström, C.-H. Heldin and S. Souchelnytskyi (2001). "Phosphorylation of Smad7 at Ser-249 Does Not Interfere with Its Inhibitory Role in Transforming Growth Factor-β-dependent Signaling but Affects Smad7-dependent Transcriptional Activation." Journal of Biological Chemistry **276**(17): 14344-14349.

Seong, H.-A., H. Jung and H. Ha (2010). "Murine protein serine/threonine kinase 38 stimulates TGF-β signaling in a kinase-dependent manner via direct phosphorylation of Smad proteins." Journal of Biological Chemistry.

Sharrocks, A. D., S.-H. Yang and A. Galanis (2000). "Docking domains and substrate-specificity determination for MAP kinases." Trends in Biochemical Sciences **25**(9): 448-453.

Simonsson, M., C.-H. Heldin, J. Ericsson and E. Grönroos (2005). "The Balance between Acetylation and Deacetylation Controls Smad7 Stability." Journal of Biological Chemistry **280**(23): 21797-21803.

Tsuneizumi, K., T. Nakayama, Y. Kamoshida, T. B. Kornberg, J. L. Christian and T. Tabata (1997). "*Daughters against dpp* modulates dpp organizing activity in *Drosophila* wing development." Nature **389**(6651): 627-631.

Vrailas, A. D. and K. Moses (2006). "*smoothened*, *thickveins* and the genetic control of cell cycle and cell fate in the developing *Drosophila* eye." Mechanisms of Development **123**(2): 151-165.

Wieser, R., J. L. Wrana and J. Massagué (1995). "GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex." The EMBO Journal **14**(10): 2199-2208.

Williamson, M. P. (1994). "The structure and function of proline-rich regions in proteins." Biochemical Journal **297**(2): 249-260.

Xu, J., A. H. Wang, J. Oses-Prieto, K. Makhijani, Y. Katsuno, M. Pei, L. Yan, Y. G. Zheng, A. Burlingame, K. Brückner and R. Derynck (2013). "Arginine Methylation Initiates BMP-Induced Smad Signaling." Molecular Cell **51**(1): 5-19.

Yao, J., M. B. Ardehali, C. J. Fecko, W. W. Webb and J. T. Lis (2007). "Intranuclear Distribution and Local Dynamics of RNA Polymerase II during Transcription Activation." Molecular Cell **28**(6): 978-990.

Yao, J., K. M. Munson, W. W. Webb and J. T. Lis (2006). "Dynamics of heat shock factor association with native gene loci in living cells." Nature **442**: 1050.

Zhang, S., T. Fei, L. Zhang, R. Zhang, F. Chen, Y. Ning, Y. Han, X.-H. Feng, A. Meng and Y.-G. Chen (2007). "Smad7 Antagonizes Transforming Growth Factor β Signaling in the Nucleus by Interfering with Functional Smad-DNA Complex Formation." Molecular and Cellular Biology **27**(12): 4488-4499.

8. Supplementary Information

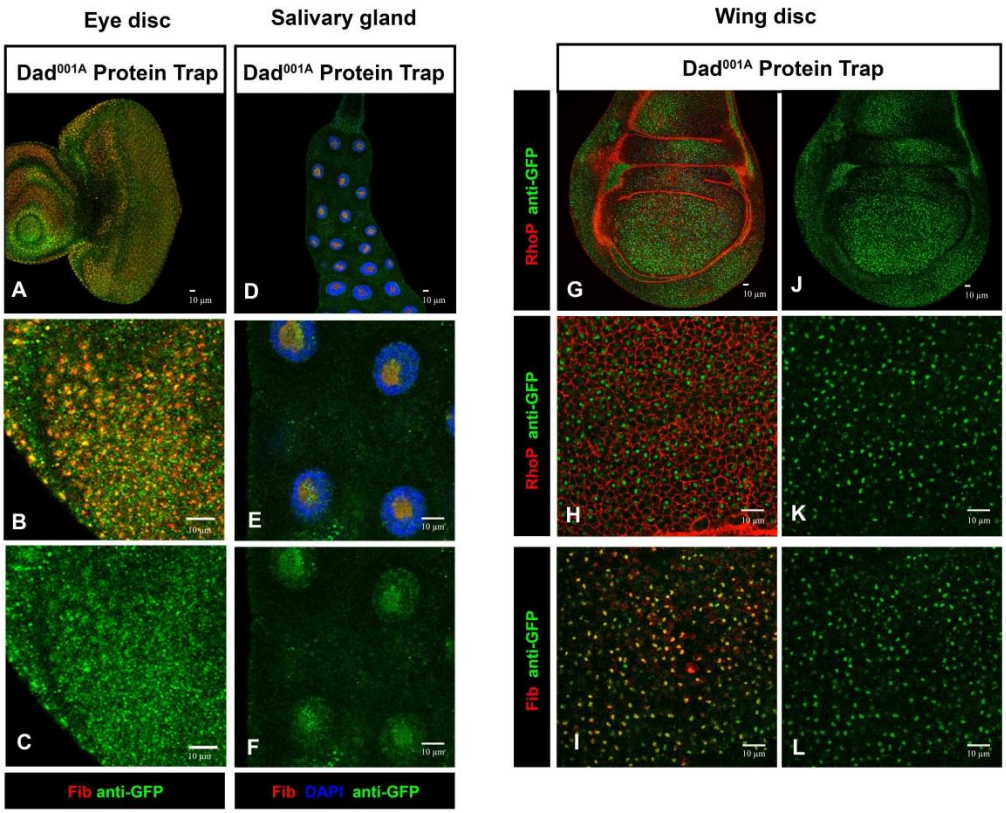


Figure S4.1. Dad localization in imaginal discs and salivary glands using a protein trap. (A-C) Dad protein trap eye imaginal discs and (D-F) Dad protein trap salivary glands stained with anti-Fibrillarin (nucleolus, red), anti-GFP (Dad, green) and DAPI (DNA, blue). (G-H, J-K) Dad protein trap wing discs stained with RhoP (membrane, red) and anti-GFP (Dad, green). (I and L) Additional staining of Dad protein trap wing discs with RhoP (membrane, red) and anti-GFP (Dad, green). Scale bars correspond to 10 μm.

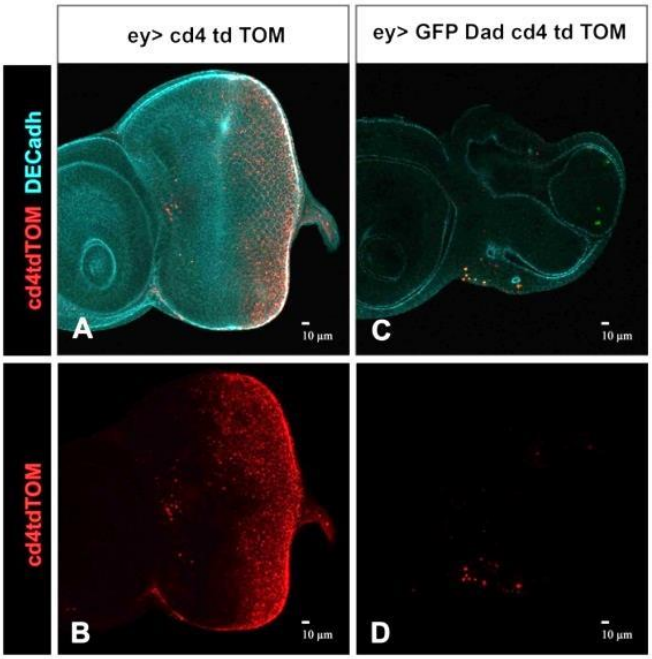


Figure S4.2. Dad turn-off ey-Gal4 during eye development. (A-B) Expression of *cd4*tdTOM in imaginal eye discs. (C-D) Expression of *cd4*tdTOM in imaginal eye discs in a GFP-*dad* overexpression background. Imaginal eye discs were stained with E-Cadh (membrane, cyan). The expression of *cd4*tdTom can be observed in red. Scale bars correspond to 10 µm.

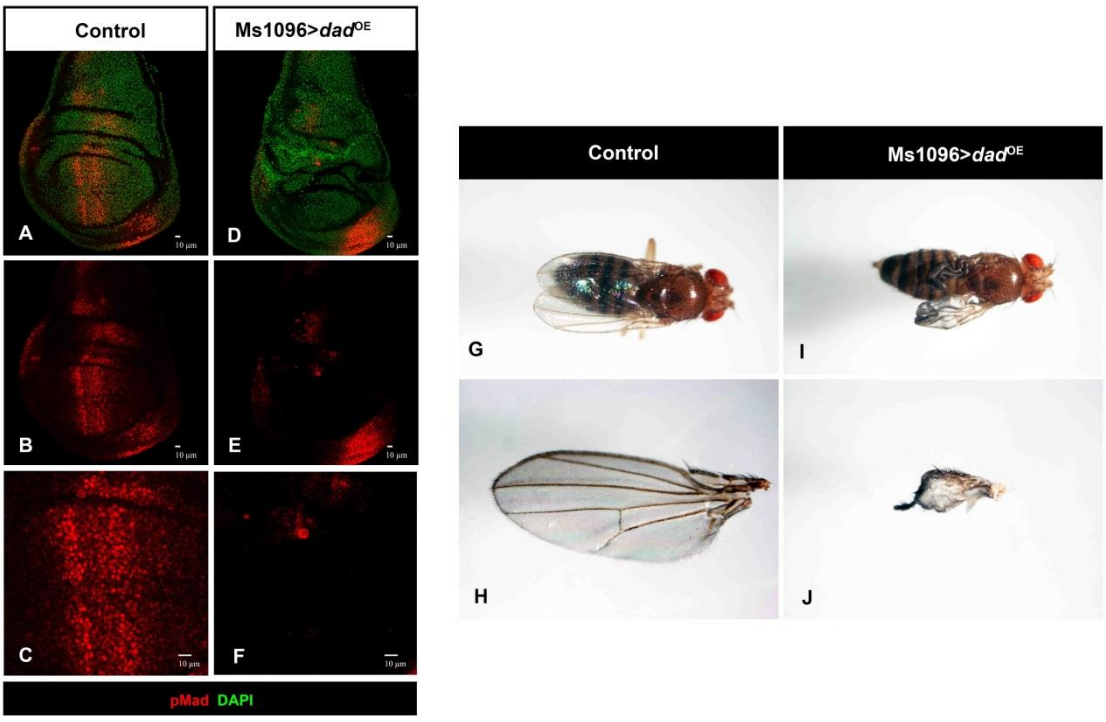


Figure S4.3. Dad inhibits Mad phosphorylation in wing discs. (A-C) Control (Ms1096> *LacZ*) wing discs. (D-F) Overexpression of *dad* under Ms1096 control inhibits Mad activation. Wing discs of the indicated genotypes stained with DAPI (DNA, green) and pMad (red). Scale bars correspond to 10 µm. (G-H) Adult wings of control (Ms1096> *LacZ*) and (I-J) Ms1096> dad^{OE} .

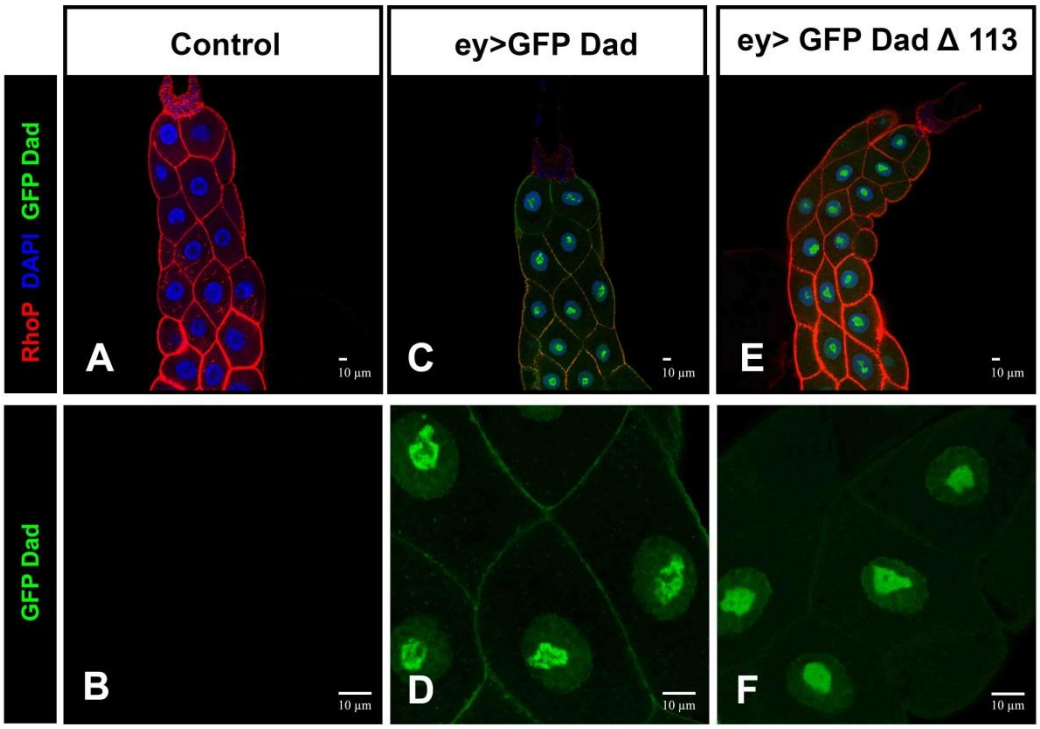
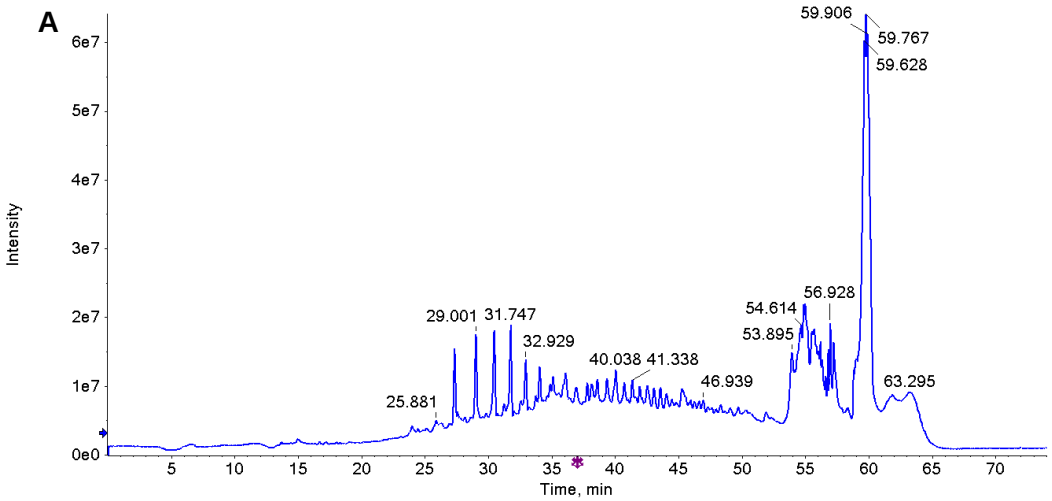


Figure S4.4 Dad N-terminal domain is necessary for an efficient Dad-membrane association. (A-C) Control (ey> W1118) salivary gland. (D-F) Ectopic expression of *dad* is associated with an accumulation of Dad in the membrane. (E-F) Ectopic expression of *dad*Δ113 inhibits the localization of Dad in the membrane. Salivary glands of the indicated genotypes were stained with DAPI (DNA, blue), RhoP (membranes, red) and pMad (grey).

A



B

Peptide sequence	Purpose phosphorylated S/T
(R)ILHQPETESPTPPYQR(S)	T^{335}
(K)ISFGKGWGR(D)	S^{539}
(R)SPDQGQVQPVDRR(M)	S^{154}
(K)TQTVWEPHVTASRLFFWR(E)	T^{275}/ T^{277}/ T^{284}/ S^{286}

Figure S4.5. Mass spectrometry analysis. (A) An example of mass spectrum of a Dad in a *punt* and *tkv* overexpression background, representing the distribution of ions by intensity. (B) Table indicating the phosphopeptides identified by mass spectrometry.

CHAPTER 5

TGFβ signaling cooperates with Bnl in the regulation of cell polarity and epithelial integrity

Contents

Deregulation of TGFβ signaling compromises epithelial integrity in the *Drosophila* salivary gland

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1. Abstract

Loss of cell polarity and Actin stress fibers are frequently linked with cancer progression. TGFβ signaling has been associated with the regulation of cell shape and cytoskeletal organization in *Drosophila*. However, its role in epithelial-mesenchymal transition remains unclear. In this study, using *Drosophila* salivary glands as an *in vivo* model system, we showed that upregulation of TGFβ signaling compromised cell polarity and epithelial integrity. The upregulation of *punt* and *tkv* receptors induced mesenchymal features in epithelial cells of salivary glands. These epithelial alterations resulted from an increase of Actin stress fibers and Mmp1 expression. The downregulation of *bnl* partially rescued TGFβ-induced phenotype by inhibiting Actin stress fibers and Mmp1 activity. Thus, Bnl and TGFβ cooperate to efficiently induce multiple biochemical changes that enable epithelial cells to assume mesenchymal cell features. FGFs, the human homolog of *Drosophila* Bnl, also cooperate with TGFβ signaling to enhance EMT responses, suggesting that FGFs inactivation may be a potential target for treatment in human cancers.

2. Introduction

Cell dissemination and invasion are critical steps for tumor metastasis and involves multiple processes, including decreased cell-cell junction, increase motility and invasive properties (Yilmaz and Christofori 2010). These characteristics allow cell detachment from the primary tissue and invasion of neighboring tissues by collective or individual cell migration. This cell behavior results from epithelial–mesenchymal transition (EMT), a process in which polarized epithelial cells lose their epithelial features and acquire the motile and invasive characteristics of mesenchymal cells (Nisticò, Bissell et al. 2012). EMT initiates with the loss of apical-basal polarity and dissolution of cell-cell adhesion (Thiery and Sleeman 2006, Kalluri and Weinberg 2009). Epithelial markers such as E-cadherin and Claudins are downregulated and mesenchymal markers such as Fibronectin and Metalloproteinases (MMPs) are upregulated. Additionally, the Actin cytoskeleton is intensely reorganized and stress fibers are formed. Concomitant with the loss of apical-basal polarity and alterations in epithelial markers, MMPs are actively expressed and secreted inducing invasive properties on these newborn mesenchymal cells.

In vertebrates, EMT can be stimulated and controlled through several signaling pathways, including TGFβ, FGF, Notch and Wnt (Moustakas and Heldin 2007). From those, TGFβ signaling pathway has received special attention due to its incredible

ability to induce EMT during normal development as well as during oncogenic events (Thiery and Sleeman 2006, Katsuno, Lamouille et al. 2013). The induction of EMT by TGFβ can be done through Smad and non-Smad signaling pathways (Xu, Lamouille et al. 2009, Moustakas and Heldin 2012). The canonical TGFβ/Smad signaling directly regulates the expression of different EMT-inducing transcription factors, Snail/Slug, Twist and ZEB1/2. These transcriptional factors inhibit the expression of epithelial protein markers stimulating mesenchymal characteristics. TGFβ also triggers other non-Smad dependent signaling pathways, such as JNK and ERK MAPK, which contribute for the increasing of Actin reorganization, cell motility and invasion (Zhang 2017).

In *Drosophila melanogaster,* TGFβ signaling has also been described as a regulator of epithelial architecture. In wing imaginal epithelium, BMP-like ligand Decapentaplegic loss of function clones extrude from the cell layer as viable cysts displaying strong abnormalities in cell shape and cytoskeletal organization (Gibson and Perrimon 2005, Widmann and Dahmann 2009). In *Drosophila* follicle epithelium, TGFβ mutants present E-cadherin molecules on the apical membrane domain and this signaling is necessary for efficient temporal adherens junctions remodeling in these cells (Brigaud, Duteyrat et al. 2015). TGFβ signaling also regulates the formation of Actin filaments and the localization of activated Myosin II, suggesting that internal forces are generated and promote cytoskeleton rearrangement (Brigaud, Duteyrat et al. 2015). Additionally, TGFβ signaling modulates basement membrane stiffness around the cells undergoing the cuboidal-to-squamous transition (Chlasta, Milani et al. 2017).

In this study, we show that TGFβ signaling cooperates with the FGF receptor Bnl in the regulation of cell polarity and epithelial integrity. Additionally, we demonstrate that one potential mechanism by which *bnl* knockdown prevents mesenchymal behavior is through the inhibition of Actin stress fibers and Mmp1 activity. Together, these results shed light on the importance of TGFβ signaling and Bnl/FGF signaling in mesenchymal-induced features.

3. Material and Methods

3.1. Fly husbandry

All crosses were raised at 25°C under standard conditions. The following stocks (described in FlyBase, unless stated otherwise) were used: ey-Gal4, UAS-lacZ, w1118, UAS-*myc* (a gift from Filipe Josué), UAS-*myc* RNAi (VDRC #2948), UAS-*punt* (a gift from Konrad Basler), UAS-*tkv* (Tom Bunch lab), UAS-tkv^{QD} (#36536) and UAS-*bnl*

RNAi (VDRC #5730). The knockdown of genes by RNAi was validated by more than one line.

Mitotic recombination was induced using the FLP/FRT method. Clones overexpressing *punt* and *tkv* receptors or control clones, were induced by heat shock (60 min at 37°C) at 48 ± 24 hours after egg laying (AEL) in larvae of the genotype yw hsflp/+; act>y+>Gal4, UAS-GFP/UAS-*punt*; UAS *tkv* and yw hsflp/+; act>y+>Gal4, UAS-GFP/+; UAS-LacZ/+.

3.2. Immunostaining

Salivary glands and imaginal discs were dissected in cold Phosphate Buffer Saline (PBS) and fixed in 3.7% formaldehyde/PBS for 20 minutes. Immunostaining was performed using standard protocols. Primary antibodies used were: rat anti-Elav 1:100 (7E8A10 DSHB), rabbit anti-pMad (P-Smad1/5, Ser463/465) antibody at 1:100 (9516, Cell Signaling), guinea pig anti-Mad at 1:100 (Newfeld, Mehra et al. 1997), mouse anti-Fibrillarin at 1:500 (Abcam ab4566), rat anti-*Drosophila* E-cadherin at 1:100, mouse anti-Armadillo at 1:100 (DSHB N2 7A1), mouse anti-integrin βPS IgG2b at 1:5 (DSHB CF.6G11), mouse anti-αPS1 IgG1 at 1:5 (DSHB DK.1A4), rabbit anti-αPS3 at 1:300 (Shigeo Hayashi), rabbit anti-P-Myosin Light Chain 2 (Ser19) at 1:50 (Cell Signaling 3671), mouse anti-pJNK antibody at 1:100 (9255, Cell Signaling), mouse anti-Mmp1 antibody at 1:25 (1:1:1 of 14A3D2, 3A6B4 and 5H7B11 all from DSHB), and rabbit anti-pERK antibody (Phospho-p44/42 MAPK) at 1:200 (4370, Cell Signaling). To stain for cellular limits phalloidin conjugated with rhodamine was used at a dilution of 1:1000. BODIPY (Thermo Fisher Scientific) was used to stain lipids. Appropriate Alexa Fluor conjugated secondary antibodies used were from Molecular Probes.

Images were obtained with the Leica SP5 confocal system and processed with Adobe Photoshop CS6.

3.3. Transmission electron microscopy

Dissected third-instar salivary glands were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min and post-fixed with 4% osmium tetroxide. After washing, salivary glands were incubated with 0.5% uranyl acetate (30 min) and further dehydrated through a graded ethanol series (70% for 10 min, 90% for 10 min and four changes of 100%). Salivary glands were then soaked in propylene oxide for 10 min and then in a mixture (1 : 1) of propylene oxide and Epon resin (TAAB Laboratories) for 30 min. This mixture was then replaced by 100% Epon resin for 24 h. Finally, fresh Epon replaced the Epon and polymerization took place at 60°C for 48 h. Ultrathin sections

were obtained using an ultramicrotome, collected in copper grids and then double contrasted with uranyl acetate and lead citrate. In total, at least 15 independent cells of six independent salivary glands were analyzed for each genotype. Micrographs were taken using a TEM Jeol JEM-1400, with Orius SC 1000 digital camera (80 kV).

3.4. Intensity measurements and statistics

The total protein intensity was determined using a fixed ROI square and the mean intensity of each salivary gland was measured using ImageJ. For each genotype, more than 4 independent salivary glands were used. Statistical analysis and generation of the graphical output was done using the GraphPad Prism 5.0. Statistical significance was determined using an unpaired, two-tailed Student's t-test, with a 95% confidence interval, after assessing the normality distribution of the data with D'Agostino-Pearson normality test.

4. Results

4.1. Upregulation of TGFβ signaling promotes alterations in epithelial integrity

Previous studies showed that wing cell clones deprived of Dpp/BMP present abnormalities in cell shape and cytoskeletal organization (Gibson and Perrimon 2005). During *Drosophila* oogenesis, the activation of the TGFβ pathway induces epithelial cell flattening and also regulates the formation of Actin filaments and the localization of activated Myosin II (Brigaud, Duteyrat et al. 2015). Accordingly, TGFβ signaling seems to play important roles in cell shape and cytoskeletal organization, but it remains unclear how the TGFβ signaling regulates these mechanisms. To gain insight into this question, we first tested whether simultaneous overexpression of type II and type I TGFβ receptors, *punt* and *tkv* respectively, also affects epithelial cell organization. For that we took advantage of *Drosophila* salivary glands, a pair of elongated tubes composed by secretory and duct cells. Secretory cells are columnar epithelial cells which secrete high levels of protein. Duct cells are cuboidal epithelial cells surrounding an inner lumen, which are connected to the larval mouth. These simple tubular organs are made of polyploid cells. Thus, the salivary glands growth is not dependent of cell division but simply controlled by the volume of individual cells (Smith and Orr-Weaver 1991, Edgar and Orr-Weaver 2001). Consequently, the alterations that occur during development happen within and between pre-existing cells, which simplifies its analysis. The overexpression of *punt* and *tkv* receptors was induced in salivary glands using ey-GAL4 (Figure 5.1. E-H, M-P and S1 D-F) and the upregulation of TGFβ signaling was demonstrated by the strong upregulation of the TGFβ activated R-Smad,

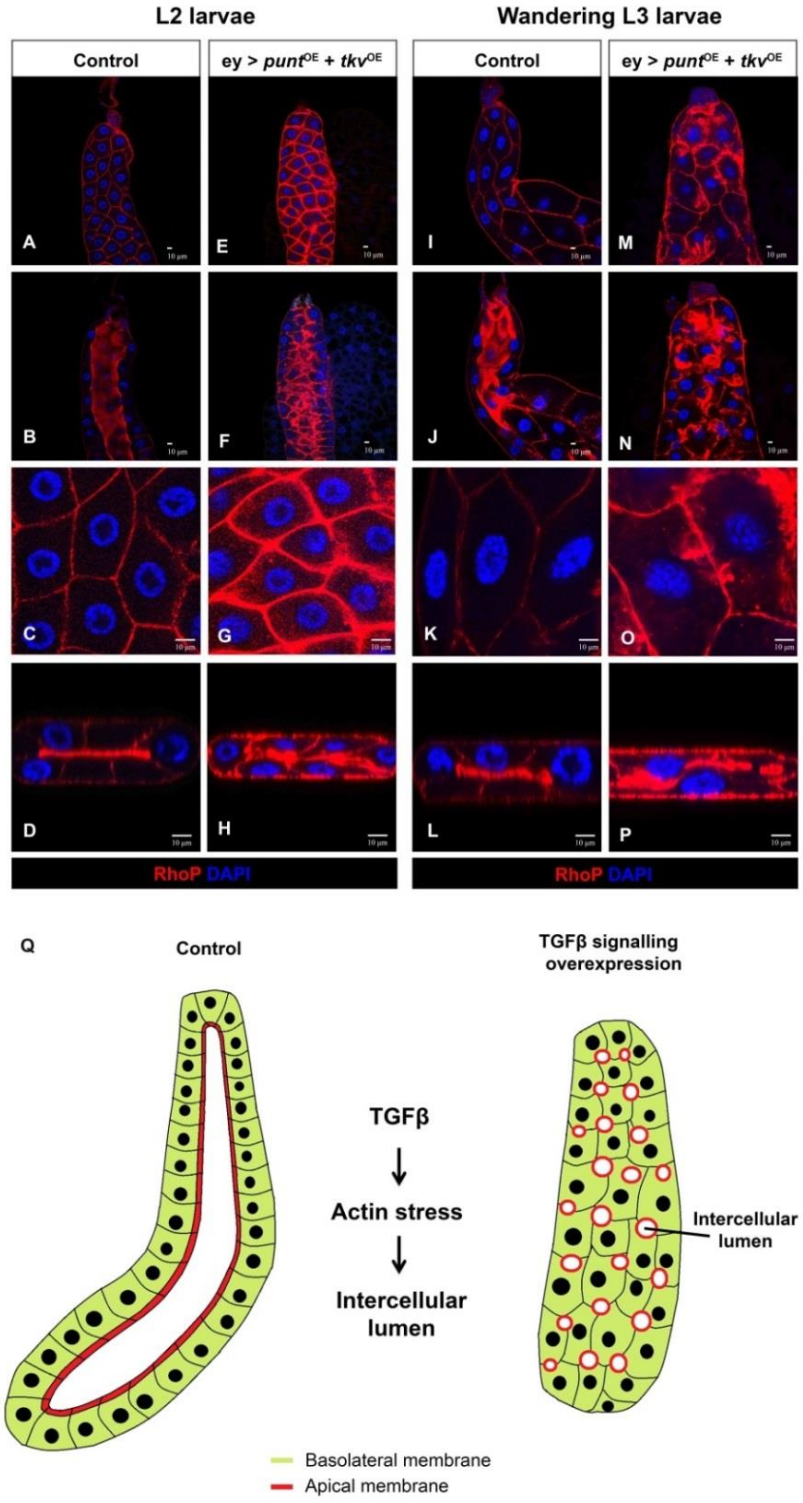

Figure 5.1. TGFβ signaling regulates lumen shape and size during salivary gland migration in *Drosophila*. (A-P) Overexpression of TGFβ signaling causes Actin stress and formation of multiple intercellular lumens in *Drosophila* salivary glands. (A) Basal and (B) apical planes of control L2 salivary glands. (C) Magnification of the basal plane of control (ey>*LacZ*) L2 salivary glands. (D) Transversal section of control L2 salivary glands. (E) Basal and (F) apical planes of L2 salivary glands overexpressing TGFβ signaling. (G) Magnification of the basal plane of L2 salivary glands overexpressing TGFβ signaling. (H) Transversal section of L2 salivary glands overexpressing TGFβ signaling. (I) Basal

and (J) apical planes of control (ey>*LacZ*) L3 salivary glands. (K) Magnification of the basal plane of control L3 salivary glands. (L) Transversal section of control L3 salivary glands. (M) Basal and (N) apical planes of L3 salivary glands overexpressing TGFβ signaling. (O) Magnification of the basal plane of L3 salivary glands overexpressing TGFβ signaling. (P) Transversal section of L3 salivary glands overexpressing TGFβ signaling. Salivary glands stained with DAPI (DNA, blue) and RhoP (membranes, red). Scale bars correspond to 10 μm. (Q) Diagrams depicting a lateral view of the salivary glands. Overexpression of TGFβ signaling promotes internalization of Actin filaments forming intercellular lumens. Diagrams are not drawn to scale. Adherens junctions are not represented in the diagram.

Mad (Figure S5.1. A-H). The overexpression of *punt* and *tkv* induced several alterations in cell shape and morphology of L3 salivary glands. We observed an increase of the density of Actin filaments and the formation of numerous Actin-based membrane protrusions (Figure 5.1. M-P). These protrusions usually result from pushing forces that are applied on the cell surface to displace the forward cell (Blanchoin, Boujemaa-Paterski et al. 2014). Additionally, overall Actin reorganization was observed. This Actin dynamics is the first step that precedes cell movement of motile cells (Xu, Lamouille et al. 2009, Brigaud, Duteyrat et al. 2015).

In addition to the previously described defects in the individual salivary gland cells, an integral lumen was not formed in salivary glands overexpressing *punt* and *tkv* receptors. Instead, several intercellular lumens containing Actin filaments were observed (Figure 5.1. N, P and Q), in contrast to the single continuous lumen of wild-type salivary glands (Figure 5.1. J, L and Q). These intercellular lumens were also observed with Pak1 activation (Pirraglia, Walters et al. 2010), a protein that regulates cytoskeletal remodeling and it is necessary for the TGFβ1 induced human prostate cancer (Al-Azayzih, Gao et al. 2015). To understand how gland organization changes over the course of gland development, we analyzed early salivary glands of wild-type and we compared with TGFβ gain-of-function siblings. In L2 salivary glands the integrity of the lumen was also affected but not so severely as in L3 salivary glands (Figure 5.1. B, F, D, H, J, N, L and P). This suggests that intercellular lumen formation in TGFβ signaling overexpression glands was preceded by a progressive loss of integrity of the single central lumen during larval development. Next, we examined whether TGFβ signaling upregulation in *Drosophila* imaginal discs produced similar effects in Actin cytoskeleton dynamics. In both eye and wing discs, *punt* and *tkv* overexpressing clones exhibited defects in the ability to establish or maintain the pseudostratified columnar cell shape (Figure 5.2. A-L and S5.2. A-H). Particularly, in imaginal eye discs, clones induced in the posterior margins of the discs resulted in abnormal cell shape and cytoskeletal organization leading to cell protrusions. Moreover, cell clones in the middle of the discs exhibited strong alterations in

cytoskeletal organization and in some cases they seemed to form a new cell mass tissue but without signs of epithelial extrusion.

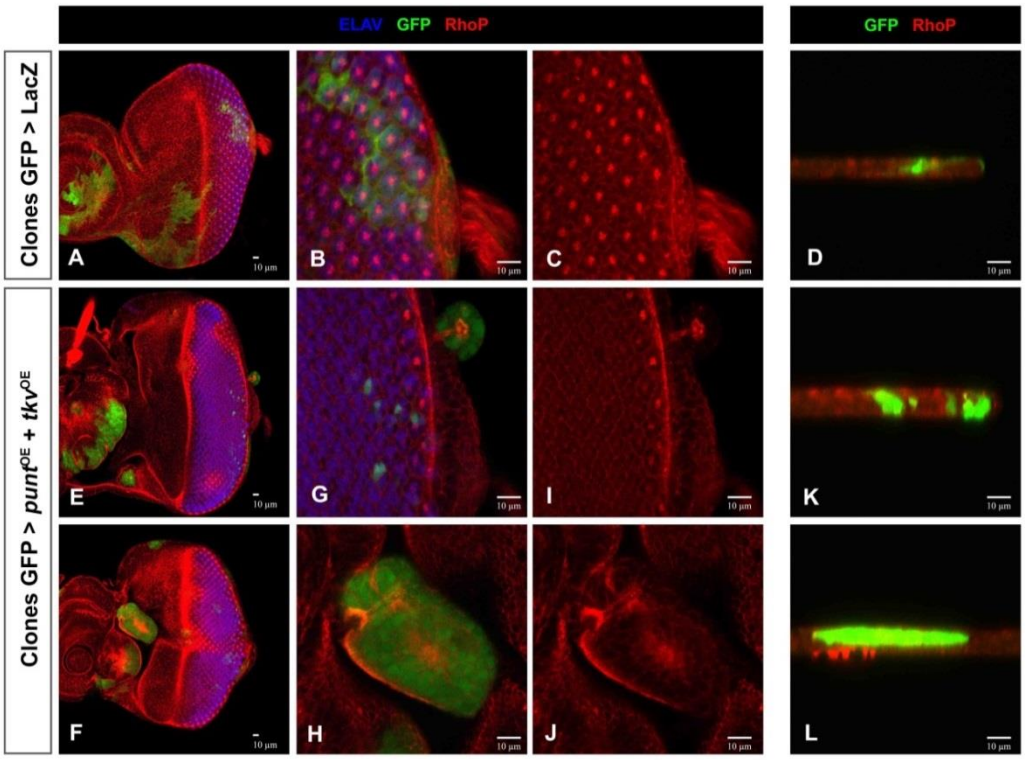

Figure 5.2. Defective morphogenesis induced by clones overexpressing TGFβ signaling in imaginal eye discs. (A-L) Clones overexpressing *punt* and *tkv* exhibited defects in the ability to establish or maintain the pseudostratified columnar cell shape. (A) Control clones with GFP expression in imaginal eye disc. (B-C) Magnification and (D) transversal section of control clones in imaginal eye disc. (E-F) Combinatorial overexpression of *punt* and *tkv* clones in imaginal eye discs. (G-J) Magnification and (K-L) transversal section of clones overexpressing *punt* and *tkv* receptors in imaginal eye discs. Imaginal eye discs stained with ELAV (photoreceptors, blue) and RhoP (membranes, red). Scale bars correspond to 10 µm**.**

4.2. TGFβ signaling controls cell polarity

It is already known that polarity proteins and the Actin cytoskeleton organization are important in polarity maintenance (Doerflinger, Benton et al. 2006, Leibfried, Müller et al. 2013). In humans, the assembly of the Cadherin–Catenin complex controls Actin cytoskeleton organization and the development of molecular bridges between neighboring epithelial cells (Drees, Pokutta et al. 2005, Yamada, Pokutta et al. 2005). To determine whether TGFβ signaling overexpression remodels *Drosophila* apico-basal polarity, we analyzed Armadillo (Arm) and E-Cadherin (E-Cadh) localization in control and salivary glands overexpressing *punt* and *tkv* receptors (Figure 5.3. A-V). In contrast to control epithelial cells, where Arm and E-Cadh are localized in the basolateral membrane (Figure 5.3. A-B, G-H, O and Q), in *punt* and *tkv* overexpression glands (Figure 5.3. D-E, J-K, P and R), a strong delocalization of Arm and E-Cadh was

observed from the basolateral membrane to the cytoplasm and apical domain. Moreover, measurements of the total antibody intensity demonstrated that gland cells overexpressing *punt* and *tkv* receptors contained more Arm and E-Cadh than control cells (Figure 5.3. U and V). Therefore, TGFβ signaling is required to maintain basolateral junction proteins at adhesive membranes, which are required for junctional assembly. Moreover, Actin levels were increased in gland cells overexpressing *punt* and *tkv* (Figure 5.3. F, L, S-T and Figure 5.7.M), which indicates that TGFβ signaling specifically regulates Actin dynamics. The mislocalization of Arm and E-Cadh in gland cells overexpressing *punt* and *tkv* suggests that Arm and E-Cadh localization might be responsible for the observed defects in the epithelial architecture. E-Cadh is an essential component of the intercellular adhesion and its lost from cell-cell contacts has been associated with detachment-induced phenotype (Perl, Wilgenbus et al. 1998). Thus, we asked whether the localization of other adhesion proteins is altered in TGFβ overexpression gland cells.

Among the adhesion proteins, Integrins have been described as αβ transmembrane receptors that link the cytoskeleton of the cells to the extracellular matrix. Importantly, Integrins have an important function on the strong stabilization of attachments between cells and their downregulation is associated to a dramatic reduction in the rate of the migration (Martin-Bermudo, Alvarez-Garcia et al. 1999, Hood and Cheresh 2002). *Drosophila* has 5 α integrin subunits (αPS1-5) and 2 β integrin subunits (βPS and βv). From those, we found out if αPS1, αPS3 and βPS Integrins are altered in gland cells overexpressing *punt* and *tkv* receptors. These three different Integrins are described as important factors for *Drosophila* cell migration (Martin-Bermudo, Alvarez-Garcia et al. 1999, Comber, Huelsmann et al. 2013, Tavares, Pereira et al. 2015). In control salivary glands, αPS1 (Figure S5.3. A-B) and αPS3 (Figure S5.3. C) were more intense in the plasma membrane but residual levels of these proteins were also observed in the cytoplasm. βPS (Figure S5.3. D) were detected only in the plasma membrane. Curiously, in gland cells overexpressing *punt* and *tkv*, an intensification of αPS1 (Figure S5.3. E-F and I) and αPS3 (Figure S5.3. G and J) were observed in the cytoplasm and plasma membrane (Figure S5.3. I-J). Moreover, βPS levels were maintained in the plasma membrane of gland cells overexpressing *punt* and *tkv*, but an upregulation of βPS levels in the cytoplasm was observed (Figure S5.3. H, K and K).

Ultrastructural TEM analysis of salivary gland cells overexpressing *punt* and *tkv* confirmed the cell detachment in this genotype (Figure 5.4. A-F). In gland cells overexpressing TGFβ signaling, we observed several intercellular spaces between plasma membranes (Figure 5.4. B-B'), which contrasts with the stable membrane

attachment observed in control gland cells (Figure 5.4. A-A'). This detachment was also observed in fat body (another polyploid tissue) by confocal microscopy (Figure S5.4. A-D). Further, TEM analysis of salivary glands showed an accumulation of lipid droplets in the cytoplasm (Figure 5.4. C-D), which was also observed with the intrinsically lipophilic Bodipy staining at confocal microscopy (Figure 5.4. E-I). Interestingly, high lipid droplet content has been associated with several types of cancer and seems to play crucial roles in the cancer progression (Koizume and Miyagi 2016, Tirinato, Pagliari et al. 2017).

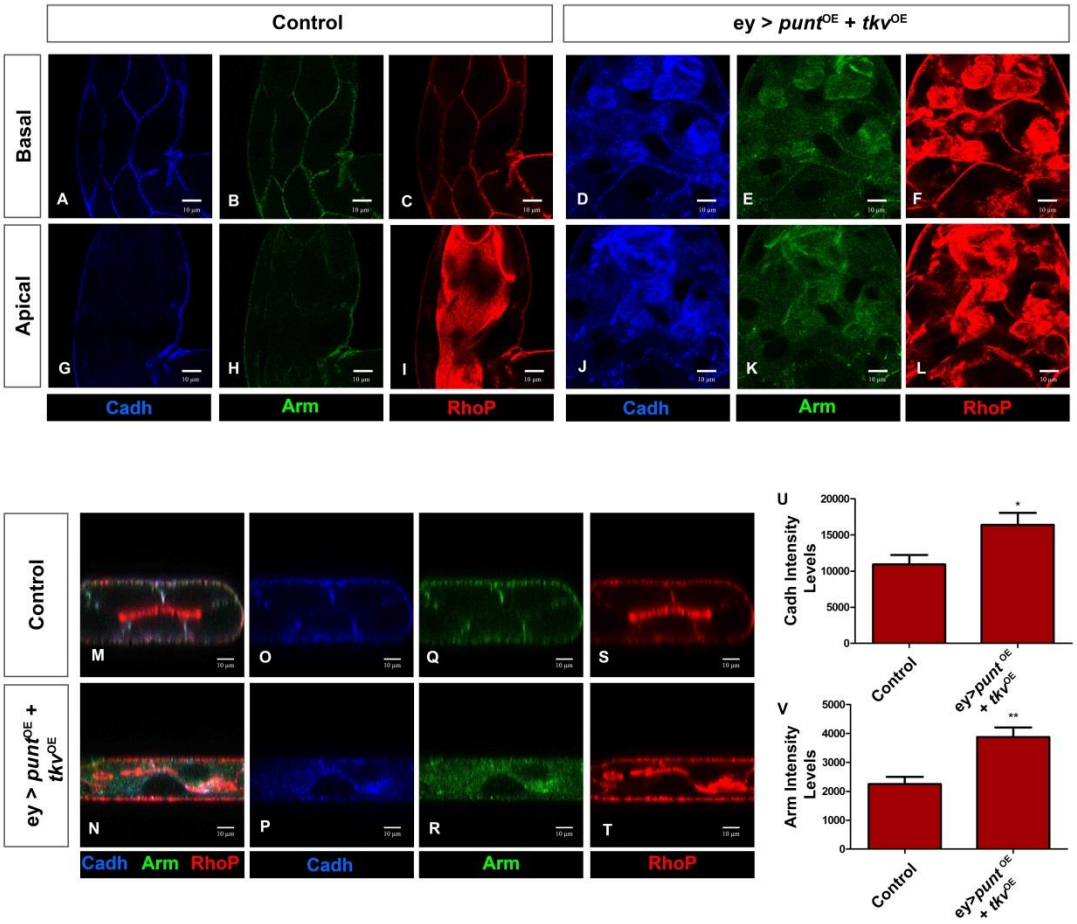

Figure 5.3. TGFβ signaling regulates cell polarity and actin dynamics. (A-T) Cell glands overexpressing *punt* and *tkv* induce delocalization of Arm and E-Cadh. Basal (A-C), and apical planes (G-I) of control (ey>*LacZ*) salivary glands. Basal (D-F) and apical planes (J-L) of gland cells overexpressing *punt* and *tkv*. (M-T) Transversal sections of the indicated genotypes. Salivary glands stained with E-Cadherin (blue), Armadillo (green) and RhoP (red). Scale bars correspond to 10 µm**.** (U-V) Quantification of total protein intensity levels of the indicated genotypes, n=5; *, P<0.05; ** and P<0.01.

Taking together, the strongly upregulation of TGFβ signaling induced severe alterations in gland epithelia, including the loss of cell–cell adhesion and apical–basal polarity. This progressive loss of epithelial characteristics and gain of mesenchymal features is typically associated with EMT.

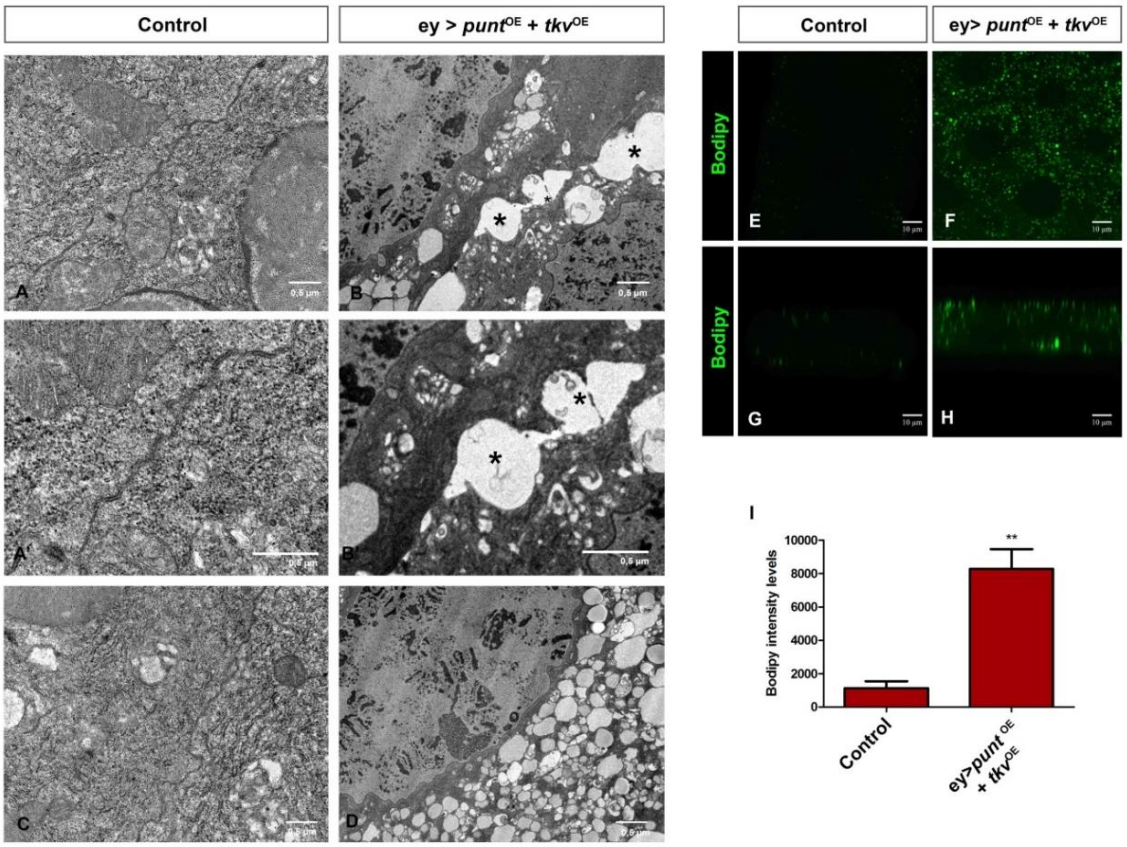

Figure 5.4. The upregulation of TGFβ signaling induces cell detachment. (A-D) Transmission electron micrographs of control (ey>*LacZ*) salivary glands (A) and salivary glands overexpressing *punt* and *tkv* receptors (B). Higher magnifications of gland cells overexpressing *punt* and *tkv* receptors (B') reveal intercellular spaces that are not find in control glands (A'). Asterisks points to intercellular spaces. Cell glands overexpressing *punt* and *tkv* receptors (D) present higher lipid content than control gland cells (C). Scale bars correspond to 0.5 µm. Magnifications of basal domain and transversal sections reveal higher lipid content in gland cells overexpressing *punt* and *tkv* receptors (F and H) compared to the control (ey>*LacZ*) gland cells (E and G). Salivary glands stained with bodipy (lipids, green). Scale bars correspond to 10 µm**.** Quantification of total protein intensity levels of the indicated genotypes, n=5; ** P<0.01.

4.3. TGFβ signaling induces ERK1/2 pathway and Mmp1 expression

ERK/MAPK and Jun N-terminal kinase (JNK) signaling pathways are important mediators of Actin dynamics. In mammals, ERK MAP Kinase regulates Actin organization and cell motility (Han, Kosako et al. 2007, Mendoza, Vilela et al. 2015) and it is required for TGFβ1-induced EMT (Xie, Law et al. 2004). JNK also plays important roles in Actin modulation and during *Drosophila* embryogenesis it controls Actin polymerization and cell adhesion (Homsy, Jasper et al. 2006). Since TGFβ modulates architecture and dynamics of Actin filaments, JNK and/or Erk pathways might be involved in these epithelial alterations. To test this hypothesis, we monitored pJNK and pERK1/2 protein expression in control and gland cells overexpressing *punt* and *tkv* receptors. Control and gland cells overexpressing TGFβ signaling displayed similar total pJNK protein levels (Figure 5.5. D, I and M). In gland cells overexpressing *punt* and *tkv* receptors, the protein localization of pERK1/2 remained unchanged (Figure 5.5. A-B and F-G). Nevertheless, pERK1/2 protein expression was severely increased in the cytoplasm and membranes of gland cells overexpressing *punt* and *tkv* when compared with control gland cells (Figure 5.5. K). Therefore, ERK/MAPK signaling may cooperate with TGFβ signaling in the regulation of cell polarity and epithelial integrity.

In vertebrates, several reports point to the regulation of MMPs by the ERK/MAPK pathway (Brauchle, Glück et al. 2000, Huntington, Shields et al. 2004, Deroanne, Hamelryckx et al. 2005). MMP1 plays an essential role in the degradation of extracellular matrix components (Kessenbrock, Plaks et al. 2010) and is consequently important for cell dissociation and progression of *Drosophila* tumors (Uhlirova and Bohmann 2006, Beaucher, Hersperger et al. 2007, Jiang, Scott et al. 2011). Accordingly, we observed a dramatically increased expression of MMP1 in gland cells overexpressing *punt* and *tkv* compared with the control ones (Figure 5.5. A, C, F, H and L). Moreover, non-muscle phosphorylated Myosin II (pMyosin II) that plays a crucial role in cytoskeleton, tissue organization and cell invasion (Vicente-Manzanares, Ma et al. 2009, Jiang, Scott et al. 2011) was increased in glands overexpressing *punt* and *tkv* receptors (Figure 5.5. E, J and N). This result suggests that TGFβ signaling positively regulates the hyperphosphorylation of myosin II and Erk1/2 signaling.

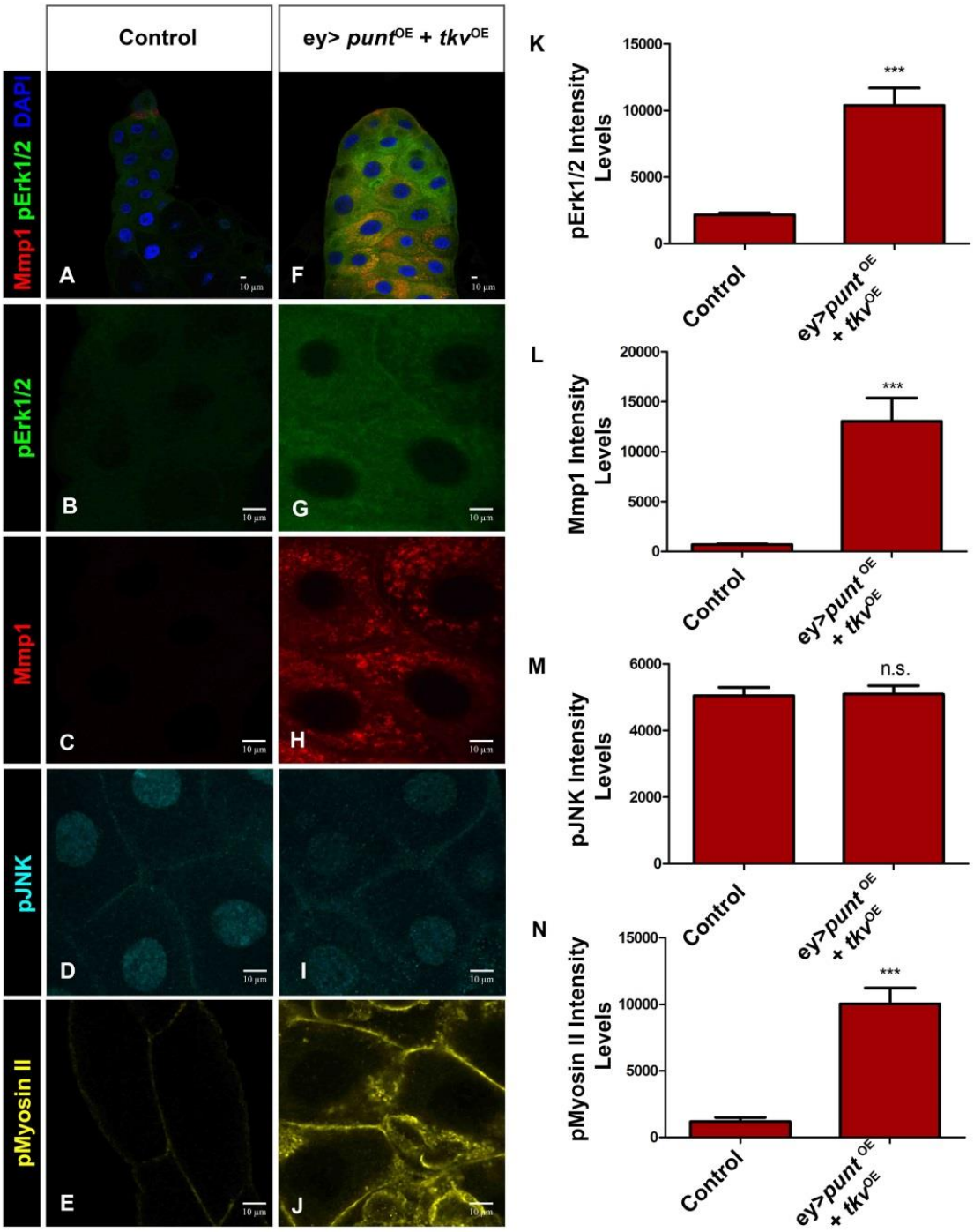

Figure 5.5. Overexpression of TGFβ signaling leads to increased pErk1/2 and Mmp1 expression. (A-J) The overexpression of *punt* and *tkv* receptors in gland cells induces an upregulation of pErk1/2, Mmp1 and pMyosin II. Higher magnifications of control (ey>*LacZ*) (B-E) and gland cells overexpressing *punt* and *tkv* receptors (G-J). Salivary glands stained with DAPI (DNA, blue), pJNK (cyan). Mmp1 (red) and pMyosin II (yellow). Scale bars correspond to 10 µm. (K-N) Quantification of total protein intensity levels of the indicated genotypes, n=5, n.s. means no statistical difference between samples; ***, P<1^10^{-4}.

4.4. Bnl is required for TGFβ-induced phenotype

FGF signaling and Myc have been involved in multiple developmental processes, including cell growth and shape, proliferation and migration. FGFs, the homologues of *Drosophila* Bnl, act as EMT inducers in development (Sauka-Spengler and Bronner-Fraser 2008) and in tumor microenvironment they can cooperate with TGFβ signaling to enhance EMT responses (Shirakihara, Horiguchi et al. 2011). Particularly, the activation of FGF signaling leads to both cell proliferation and migration (Boilly, Vercoutter-Edouart et al. 2000). Conversely, *Drosophila* eye discs deprived of FGF/Bnl signaling present increased glial cell migration accompanied by activation of Mmp1 (Tavares, Correia et al. 2017). Similarly, downregulation of *dMyc* also induces cell migration on these tissues. Nevertheless, no clear interaction has been observed between *bnl* and *dMyc*, suggesting that distinct mechanisms are involved in their regulation of cell migration (Tavares, Correia et al. 2017). Moreover, Myc is also an important growth factor that promotes cell growth and proliferation, as well as tumorigenesis (Ruggero 2009). To determine whether *bnl* and *dMyc* remodels *Drosophila* TGFβ-induced responses in salivary glands, we manipulated dMyc and Bnl levels in a TGFβ-induced background. In gland cells, *bnl* and *dMyc* induced distinct changes in the context of TGFβ-induced phenotype. The downregulation of *dMyc* inhibited salivary gland growth but the Actin stress phenotype persisted (Figure 5.6 E-F). Therefore, dMyc seems to cooperate with TGFβ signaling promoting cell growth but it is not required for the induction of mesenchymal features in epithelial cells. Then, we asked whether the downregulation of Bnl would be sufficient to inhibit the mesenchymal features induced by TGFβ signaling, in epithelial cells. For that we expressed RNAi for *bnl* in a TGFβ-induced background (Figure 5.7. G-L). The RNAi for *bnl* was able to restore Arm and E-Cadh levels in the basolateral membrane (Figure 5.7. D-I and N-O). Moreover, Bnl seemed to inhibit Arm accumulation in the apical domain of salivary glands. The downregulation of *bnl* restored the density of Actin filaments and the several alterations in the Actin structure (Figure 5.7. A-C and M). Additionally, RNAi for *bnl* was able to induce an apical constriction of salivary glands (Figure S5.5. E-H) and the single lumen of salivary glands was strongly restored (Figure 5.7. J-L). Furthermore, we tried to determine how *bnl* was regulating the induction of mesenchymal features in epithelial cells. For that we analyzed the expression of pErk1/2 and Mmp1 expression in the combinatorial expression of *bnl* RNAi and *punt* and *tkv* receptors (Figure 5.8. A-K). We observed that salivary glands co-expressing RNAi for *bnl* in cells overexpressing *punt* and *tkv* receptors maintain similar levels of pErk1/2 compared to the gland cells overexpressing *punt* and *tkv*

receptors (Figure 5.8. G-I and K). However, the expression of Mmp1 was strongly downregulated (Figure 5.8. D-F and J).

This suggests that Bnl is acting upstream of Mmp1 which is critical for the cell mesenchymal behavior induced by TGFβ signaling. In addition, we found that epithelial organization, including Actin defects were rescued by *bnl* downregulation (Figure 5.8. A-I), suggesting that knockdown of *bnl* may play an important role mediating epithelial integrity. Overall, these results show that TGFβ signaling requires the function of Bnl for an efficient induction of mesenchymal features in epithelial cells, possibly regulating both Mmp1 expression and Actin dynamics.

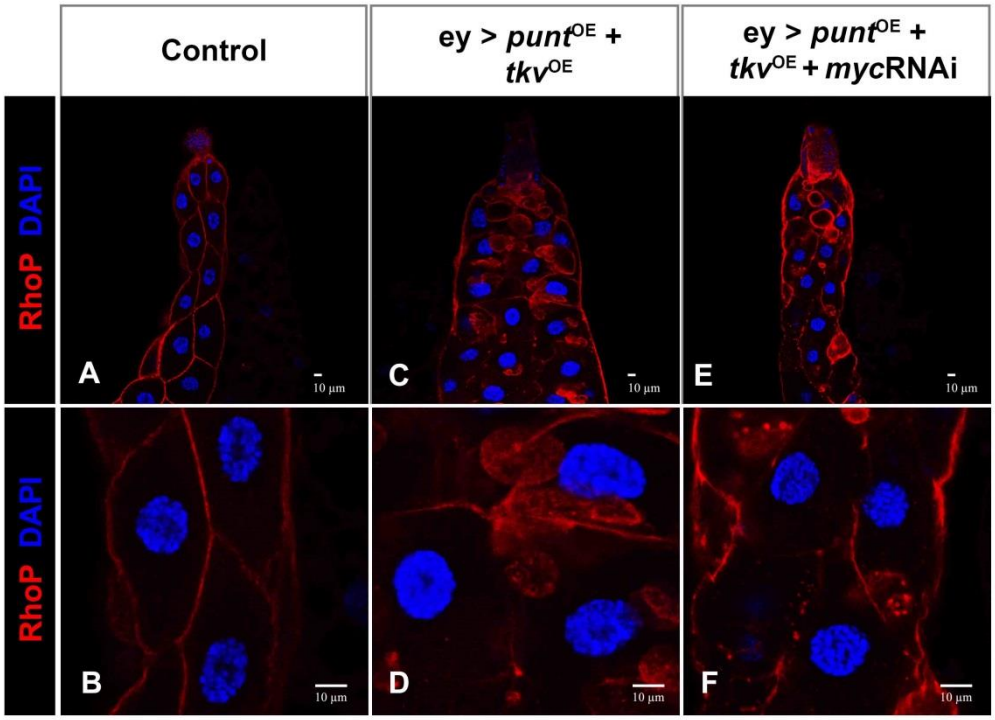

Figure 5.6. dMyc regulates cell growth but not actin stress. (A-B) Control (ey>*LacZ*) salivary gland. (C-D) Ectopic co-expression of *punt* and *tkv* induces defects in salivary glands. (E-F) RNAi for *dMyc* inhibits cell growth in a Punt/Tkv-overexpression background. Salivary glands of the indicated genotypes were stained with DAPI (DNA, blue) and RhoP (membranes, red). Scale bars correspond to 10 µm.

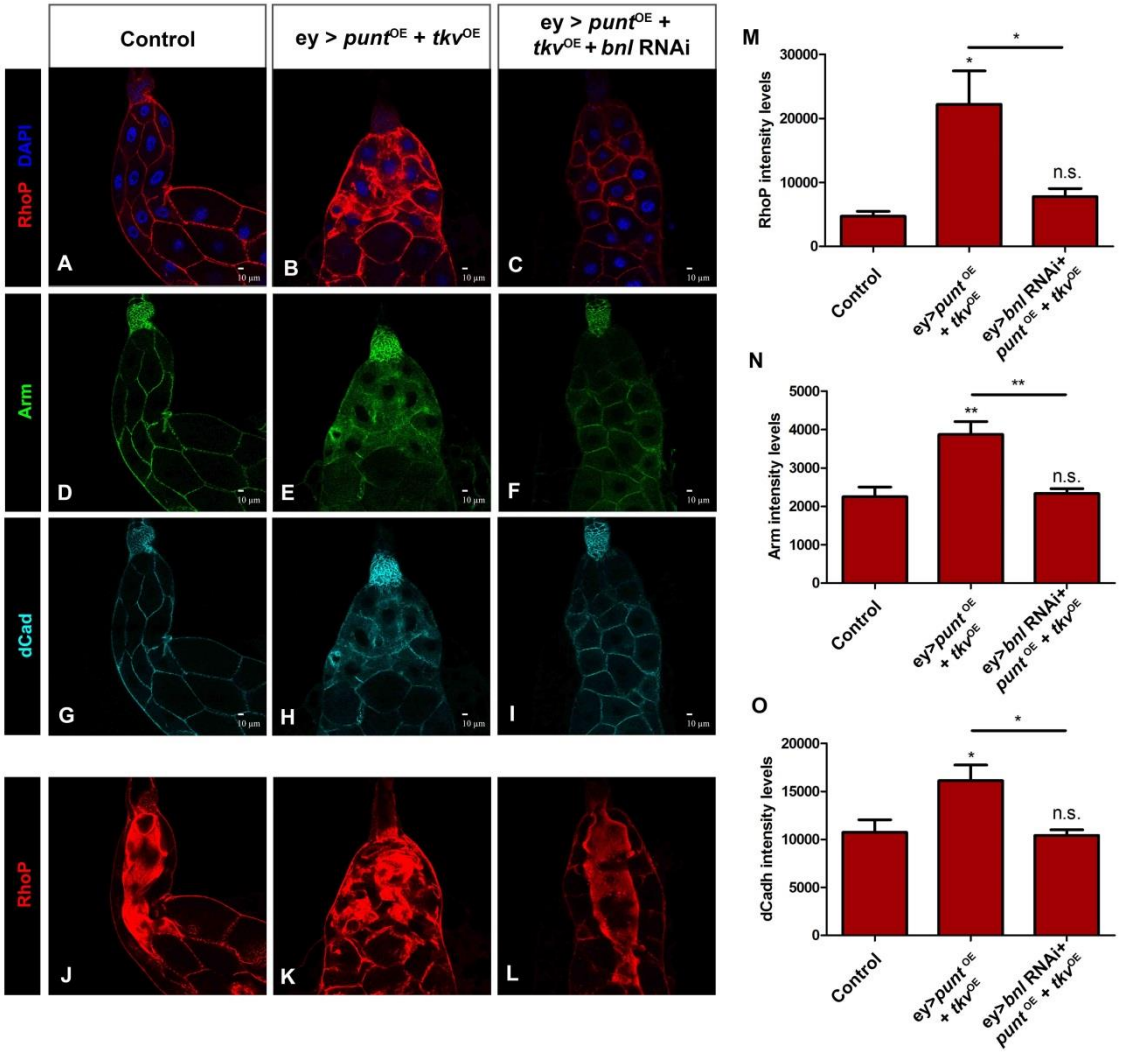

Figure 5.7. Downregulation of *bnl* partially restores cell polarity and epithelial integrity. (A–I) The co-expression of RNAi for *bnl* in cells overexpressing *punt* and *tkv* receptors suppresses the defects in the localization of E-Cadh and Arm and rescues epithelial integrity. Basal (A-I) and apical planes (J-L) of the indicated genotypes. Salivary glands stained with E-Cadherin (cyan), Armadillo (green), RhoP (red) and DAPI (DNA, blue). Scale bars correspond to 10 µm**.** Quantification of total protein intensity levels of the indicated genotypes, n>4, n.s. means no statistical difference between samples; *, P<0.05; **, P<0.01 and ***, P<1^10^{-4}.

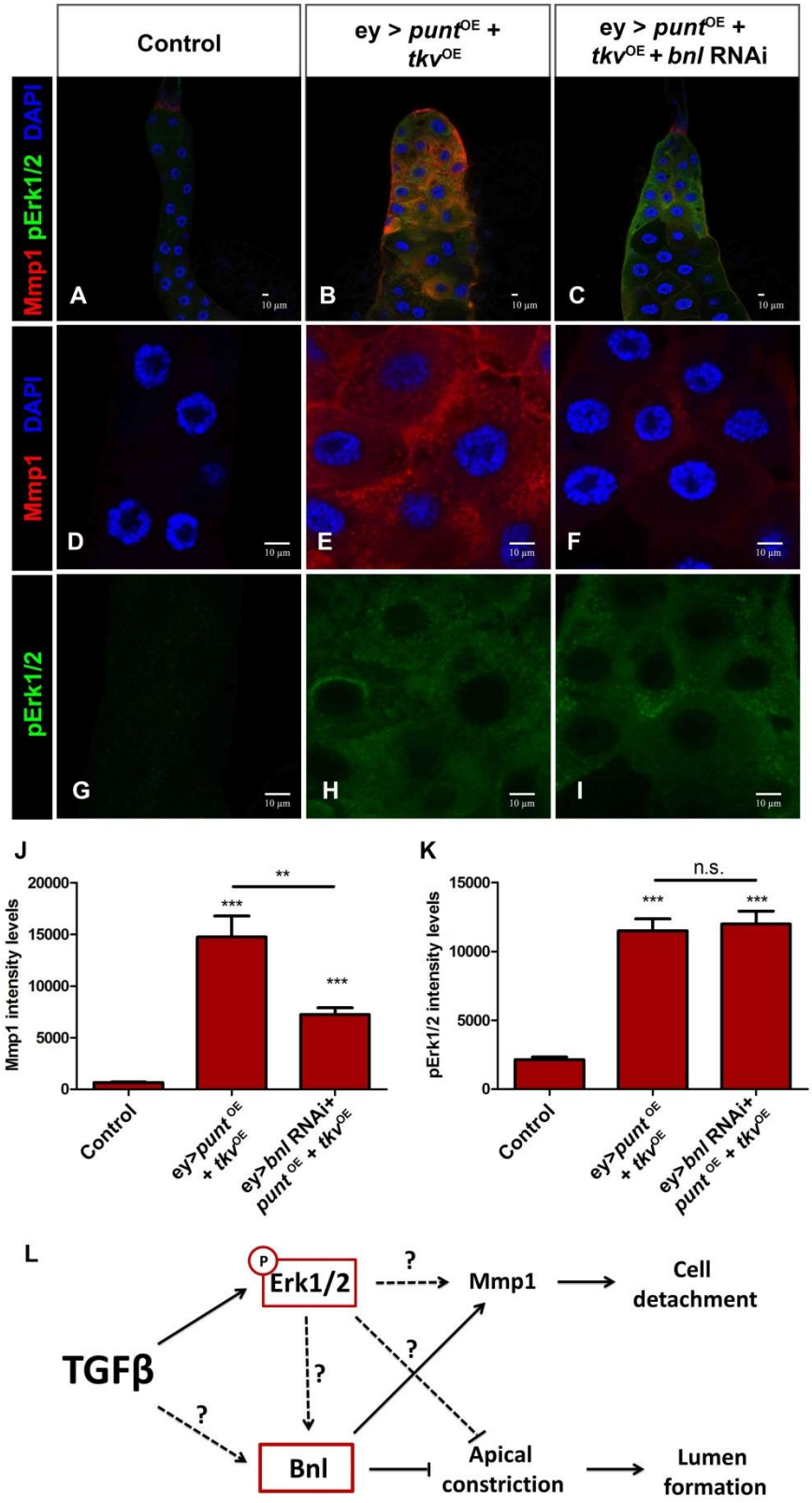

Figure 5.8. TGFβ signaling cooperates with Bnl in the regulation of apical constriction and Mmp1 expression.
(A–I) The co-expression of RNAi for *bnl* in cells overexpressing *punt* and *tkv* receptors suppresses the cell mesenchymal phenotype caused by upregulation of TGFβ signaling. Note that activation of pErk1/2 (green) is not suppressed by *bnl* RNAi (H compared to I). Nevertheless, the expression of RNAi for *bnl* in cells overexpressing *punt*

and *tkv* receptors strongly downregulated Mmp1 levels. Salivary glands stained with Mmp1 (red), pErk1/2 (green) and DAPI (DNA, blue). Scale bars correspond to 10 μm**.** (J-K) Quantification of total protein intensity levels of the indicated genotypes, n=5, n.s. means no statistical difference between samples; **, P<0.01 and ***, P<1^10^{-4}. (L) A model for TGFβ signaling as a regulator of Mmp1 expression and lumen formation in *Drosophila.* TGFβ signaling functions as positive regulator of the activation of Erk signaling. The activation of Erk signaling may regulate directly or indirectly Mmp1 expression and consequently cell detachment. Moreover, Erk signaling may also regulate apical constriction, which is required for lumen formation. Bnl works downstream of TGFβ signaling and regulates apical constriction and lumen formation. Additionally, Bnl also promotes Mmp1 secretion, which is important for cell detachment.

5. Discussion

TGFβ signaling is involved in several cellular processes in both the adult organism as well as during the developing embryo, including differentiation, growth, proliferation, migration, and adhesion (Rahimi and Leof 2007, Massague 2012, Katsuno, Lamouille et al. 2013). In vertebrates, the inappropriate expression of TGFβ signaling is strongly associated with EMT (Moustakas and Heldin 2007). TGFβ signaling controls the expression of several genes involved in the regulation of EMT (Zavadil and Böttinger 2005, Horiguchi, Shirakihara et al. 2009) and it has a dual function in tumor microenvironment. At early stages of tumor development, TGFβ signaling is a tumor suppressor and a growth inhibitor whereas in advanced stages of cancers, TGFβ signaling stimulates tumor growth and progression, promoting EMT (Massague 2012, Katsuno, Lamouille et al. 2013). During EMT, epithelial integrity is strongly affected, with Actin cytoskeleton remodeling and focal adhesion formation. The importance of TGFβ signaling in the regulation of epithelial morphology has been already reported in several vertebrate models (Moustakas and Heldin 2007, Kalluri and Weinberg 2009, Moustakas and Heldin 2012, Katsuno, Lamouille et al. 2013, Chlasta, Milani et al. 2017). However, the mechanism by which TGFβ signaling regulates the integrity of epithelia has not been deeply studied. In here, we investigated if an upregulation of TGFβ signaling in *Drosophila* salivary gland trigger Actin-cell responses during development. Indeed, *Drosophila* cells sense the increase of TGFβ signaling and lose epithelial integrity. The induced epithelial reorganization lead to the formation of multiple lumen accompanied by a mislocalization of β-catenin Arm and E-Cadh. We believe that this mislocalization of Arm and E-Cadh lead to a disruption of cell polarity and a fail of cell adhesion. Moreover, we observed an accumulation of E-Cadh and Arm in the cytoplasm, a pattern that has been observed in most invasive tumors (Nawrocki, Polette et al. 1998). Additionally other adhesion proteins such as Integrins were also affected. Accordingly, striking defects in cell-cell adhesion were detected. Rather than forming an organized

epithelium, cells strongly expressing TGFβ signaling seem to round up and disassociate, forming a layer with mesenchymal features.

The activation of Erk signaling is essential in TGFβ induced-EMT (Xie, Law et al. 2004) and activating Ras mutations are common to a large number of cancers (Wan, Garnett et al. 2004). Increased Erk kinase signaling enhances TGFβ-induced EMT, characterized by cell morphological changes, disassembly of adherens junctions and cell motility (Zavadil et al. 2001; Xie et al. 2004). In particular, Erk signaling amplifies TGFβ-induced responses, enabling the downregulation of E-cadherin, upregulation of N-cadherin and MMP expression (Grände, Franzen et al. 2002, Uttamsingh, Bao et al. 2007). Therefore, we investigated if the mesenchymal features induced by TGFβ signaling in *Drosophila* epithelial gland cells are associated with upregulation of Erk signaling. Indeed, gland cells overexpressing *punt* and *tkv* receptors increased pErk1/2 and MMP1 expression, which might contribute for basement membrane degradation.

In mammals, FGFs are key mediators of EMT and are frequently associated with bad prognosis in several cancers (Acevedo, Gangula et al. 2007). FGF signaling has also been described as playing important roles in the regulation of mesenchyme induction (Ciruna, Schwartz et al. 1997), as well as stimulating EMT in epithelial cells by increasing the expression of important mesenchymal genes that regulate cell adhesion and motility (Strutz, Zeisberg et al. 2002). FGF signaling also plays an important role in Actin cytoskeleton reorganization through crosstalk between Rho GTPases and PI3K, promoting the mesenchymal cell phenotype linked to cell motility (Lee and Kay 2006). Since TGFβ signaling is often correlated with malignancy of several cancers (Massagué 2008), the expression and effects of FGFs during cancer progression may be controlled by TGFβ signaling that is autonomously secreted from cancer cells. Therefore, inhibition of FGF signaling may soften cancer prognosis through the inhibition of TGFβ-mediated EMT, and accordingly may be a powerful therapeutic target. Thus, we decided to test the importance of Bnl/FGF signaling for TGFβ signaling induced mesenchymal cell phenotype. Our results confirmed that Bnl/FGF signaling is necessary for the induction of mesenchymal features in epithelial cells mediated by TGFβ signaling. Importantly, *bnl* deprived cells partially rescued the TGFβ-induced mesenchymal phenotype, inhibiting Mmp1 expression and Actin stress. Conversely, downregulation of *bnl* substantially recuperated β-catenin Arm and E-Cadh positioning in the basolateral membrane, consistent with *bnl* functioning as a mesenchymal cell phenotype inducer. Moreover, the downregulation of *bnl* induced apical constriction, which was required for a correct lumen formation. Finally, we propose a novel model, in which *bnl* functions

downstream of TGFβ signaling as an essential positive regulator of TGFβ-induced phenotype, controlling Mmp1 expression and apical constriction (see model in Figure 5.8. L). However, the molecular mechanisms linking Bnl and TGFβ signaling have not been identified.

Our results show that overactivation of TGFβ signaling is able to induce mesenchymal cell features in epithelial cells. Moreover, evidence is provided that TGFβ signaling controls the activities of Erk signaling and the regulatory light chain of non-muscle Myosin II. Misregulation of Erk signaling and the regulatory light chain of non-muscle Myosin II activity is associated with alteration on apical-basal cell polarity and upregulation of metalloproteinases. Finally, it was demonstrated that a decrease in Bnl/FGF signaling partially prevents TGFβ-induced phenotype by restoring cell polarity and downregulating Mmp1 activity. Thus, Bnl and TGFβ cooperate to efficiently induce mesenchymal features in epithelial cells.

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7. References

Acevedo, V. D., R. D. Gangula, K. W. Freeman, R. Li, Y. Zhang, F. Wang, G. E. Ayala, L. E. Peterson, M. Ittmann and D. M. Spencer (2007). "Inducible FGFR-1 Activation Leads to Irreversible Prostate Adenocarcinoma and an Epithelial-to-Mesenchymal Transition." Cancer Cell **12**(6): 559-571.

Al-Azayzih, A., F. Gao and P. R. Somanath (2015). "P21 activated kinase-1 mediates transforming growth factor β1-induced prostate cancer cell epithelial to mesenchymal transition." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1853**(5): 1229-1239.

Beaucher, M., E. Hersperger, A. Page-McCaw and A. Shearn (2007). "Metastatic ability of *Drosophila* tumors depends on MMP activity." Developmental Biology **303**(2): 625-634.

Blanchoin, L., R. Boujemaa-Paterski, C. Sykes and J. Plastino (2014). "Actin Dynamics, Architecture, and Mechanics in Cell Motility." Physiological Reviews **94**(1): 235-263.

Boilly, B., A. S. Vercoutter-Edouart, H. Hondermarck, V. Nurcombe and X. Le Bourhis (2000). "FGF signals for cell proliferation and migration through different pathways." Cytokine & Growth Factor Reviews **11**(4): 295-302.

Brauchle, M., D. Glück, F. Di Padova, J. Han and H. Gram (2000). "Independent Role of p38 and ERK1/2 Mitogen-Activated Kinases in the Upregulation of Matrix Metalloproteinase-1." Experimental Cell Research **258**(1): 135-144.

Brigaud, I., J.-L. Duteyrat, J. Chlasta, S. Le Bail, J.-L. Couderc and M. Grammont (2015). "Transforming Growth Factor β/activin signalling induces epithelial cell flattening during *Drosophila* oogenesis." Biology Open **4**(3): 345-354.

Chlasta, J., P. Milani, G. Runel, J.-L. Duteyrat, L. Arias, L.-A. Lamiré, A. Boudaoud and M. Grammont (2017). "Variations in basement membrane mechanics are linked to epithelial morphogenesis." Development **144**(23): 4350-4362.

Ciruna, B. G., L. Schwartz, K. Harpal, T. P. Yamaguchi and J. Rossant (1997). "Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak." Development **124**(14): 2829-2841.

Comber, K., S. Huelsmann, I. Evans, B. J. Sánchez-Sánchez, A. Chalmers, R. Reuter, W. Wood and M. D. Martín-Bermudo (2013). "A dual role for the βPS integrin *myospheroid* in mediating *Drosophila* embryonic macrophage migration." Journal of Cell Science **126**(15): 3475-3484.

Deroanne, C. F., D. Hamelryckx, T. T. G. Ho, C. A. Lambert, P. Catroux, C. M. Lapière and B. V. Nusgens (2005). "Cdc42 downregulates MMP-1 expression by inhibiting the ERK1/2 pathway." Journal of Cell Science **118**(6): 1173-1183.

Doerflinger, H., R. Benton, I. L. Torres, M. F. Zwart and D. St Johnston (2006). "*Drosophila* Anterior-Posterior Polarity Requires Actin-Dependent PAR-1 Recruitment to the Oocyte Posterior." Current Biology **16**(11): 1090-1095.

Drees, F., S. Pokutta, S. Yamada, W. J. Nelson and W. I. Weis (2005). "α-Catenin Is a Molecular Switch that Binds E-Cadherin-β-Catenin and Regulates Actin-Filament Assembly." Cell **123**(5): 903-915.

Edgar, B. A. and T. L. Orr-Weaver (2001). "Endoreplication Cell Cycles: More for Less." Cell **105**(3): 297-306.

Gibson, M. C. and N. Perrimon (2005). "Extrusion and Death of DPP/BMP-Compromised Epithelial Cells in the Developing *Drosophila* Wing." Science **307**(5716): 1785-1789.

Grände, M., Å. Franzen, J.-O. Karlsson, L. E. Ericson, N.-E. Heldin and M. Nilsson (2002). "Transforming growth factor-β and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes." Journal of Cell Science **115**(22): 4227-4236.

Han, M.-Y., H. Kosako, T. Watanabe and S. Hattori (2007). "Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase Regulates Actin Organization and Cell Motility by Phosphorylating the Actin Cross-Linking Protein EPLIN." Molecular and Cellular Biology **27**(23): 8190-8204.

Homsy, J. G., H. Jasper, X. G. Peralta, H. Wu, D. P. Kiehart and D. Bohmann (2006). "JNK signaling coordinates integrin and actin functions during *Drosophila* embryogenesis." Developmental Dynamics **235**(2): 427-434.

Hood, J. D. and D. A. Cheresh (2002). "Role of integrins in cell invasion and migration." Nature Reviews Cancer **2**: 91.

Horiguchi, K., T. Shirakihara, A. Nakano, T. Imamura, K. Miyazono and M. Saitoh (2009). "Role of Ras Signaling in the Induction of Snail by Transforming Growth Factor-β." Journal of Biological Chemistry **284**(1): 245-253.

Huntington, J. T., J. M. Shields, C. J. Der, C. A. Wyatt, U. Benbow, C. L. Slingluff and C. E. Brinckerhoff (2004). "Overexpression of Collagenase 1 (MMP-1) Is Mediated by the ERK Pathway in Invasive Melanoma Cells: ROLE OF BRAF MUTATION AND FIBROBLAST GROWTH FACTOR SIGNALING." Journal of Biological Chemistry **279**(32): 33168-33176.

Jiang, Y., K. L. Scott, S.-J. Kwak, R. Chen and G. Mardon (2011). "Sds22/PP1 Links Epithelial Integrity and Tumor Suppression via Regulation of Myosin II and JNK Signaling." Oncogene **30**(29): 3248-3260.

Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." The Journal of Clinical Investigation **119**(6): 1420-1428.

Katsuno, Y., S. Lamouille and R. Derynck (2013). "TGF-β signaling and epithelial–mesenchymal transition in cancer progression." Current Opinion in Oncology **25**(1): 76-84.

Kessenbrock, K., V. Plaks and Z. Werb (2010). "Matrix Metalloproteinases: Regulators of the Tumor Microenvironment." Cell **141**(1): 52-67.

Koizume, S. and Y. Miyagi (2016). "Lipid Droplets: A Key Cellular Organelle Associated with Cancer Cell Survival under Normoxia and Hypoxia." International Journal of Molecular Sciences **17**(9): 1430.

Lee, J. G. and E. P. Kay (2006). "Cross-talk among Rho GTPases Acting Downstream of PI 3-Kinase Induces Mesenchymal Transformation of Corneal Endothelial Cells Mediated by FGF-2." Investigative Ophthalmology & Visual Science **47**(6): 2358-2368.

Leibfried, A., S. Müller and A. Ephrussi (2013). "A Cdc42-regulated actin cytoskeleton mediates *Drosophila* oocyte polarization." Development **140**(2): 362-371.

Martin-Bermudo, M. D., I. Alvarez-Garcia and N. H. Brown (1999). "Migration of the *Drosophila* primordial midgut cells requires coordination of diverse PS integrin functions." Development **126**(22): 5161-5169.

Martins, T., N. Eusebio, A. Correia, J. Marinho, F. Casares and P. S. Pereira (2017). "TGFβ/Activin signalling is required for ribosome biogenesis and cell growth in *Drosophila* salivary glands." Open Biology **7**(1).

Massague, J. (2012). "TGFβ signalling in context." Nat Rev Mol Cell Biol **13**(10): 616-630.

Massagué, J. (2008). "TGFβ in Cancer." Cell **134**(2): 215-230.

Mendoza, M. C., M. Vilela, J. E. Juarez, J. Blenis and G. Danuser (2015). "ERK reinforces actin polymerization to power persistent edge protrusion during motility." Science signaling **8**(377): ra47-ra47.

Moustakas, A. and C.-H. Heldin (2007). "Signaling networks guiding epithelial–mesenchymal transitions during embryogenesis and cancer progression." Cancer Science **98**(10): 1512-1520.

Moustakas, A. and C.-H. Heldin (2012). "Induction of epithelial–mesenchymal transition by transforming growth factor β." Seminars in Cancer Biology **22**(5): 446-454.

Nawrocki, B., M. Polette, J. Van Hengel, J.-M. Tournier, F. Van Roy and P. Birembaut (1998). "Cytoplasmic Redistribution of E-Cadherin-Catenin Adhesion Complex Is Associated with Down-Regulated Tyrosine Phosphorylation of E-Cadherin in Human Bronchopulmonary Carcinomas." The American Journal of Pathology **153**(5): 1521-1530.

Newfeld, S. J., A. Mehra, M. A. Singer, J. L. Wrana, L. Attisano and W. M. Gelbart (1997). "Mothers against dpp participates in a DDP/TGF-beta responsive serine-threonine kinase signal transduction cascade." Development **124**(16): 3167-3176.

Nisticò, P., M. J. Bissell and D. C. Radisky (2012). "Epithelial-Mesenchymal Transition: General Principles and Pathological Relevance with Special Emphasis on the Role of Matrix Metalloproteinases." Cold Spring Harbor Perspectives in Biology **4**(2).

Perl, A.-K., P. Wilgenbus, U. Dahl, H. Semb and G. Christofori (1998). "A causal role for E-cadherin in the transition from adenoma to carcinoma." Nature **392**: 190.

Pirraglia, C., J. Walters and M. M. Myat (2010). "Pak1 control of E-cadherin endocytosis regulates salivary gland lumen size and shape." Development (Cambridge, England) **137**(24): 4177-4189.

Rahimi, R. A. and E. B. Leof (2007). "TGF-β signaling: A tale of two responses." Journal of Cellular Biochemistry **102**(3): 593-608.

Ruggero, D. (2009). "The Role of Myc-Induced Protein Synthesis in Cancer." Cancer Research **69**(23): 8839-8843.

Sauka-Spengler, T. and M. Bronner-Fraser (2008). "A gene regulatory network orchestrates neural crest formation." Nature Reviews Molecular Cell Biology **9**: 557.

Shirakihara, T., K. Horiguchi, K. Miyazawa, S. Ehata, T. Shibata, I. Morita, K. Miyazono and M. Saitoh (2011). "TGF-β regulates isoform switching of FGF receptors and epithelial–mesenchymal transition." The EMBO Journal **30**(4): 783-795.

Smith, A. V. and T. L. Orr-Weaver (1991). "The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny." Development **112**(4): 997-1008.

Strutz, F., M. Zeisberg, F. N. Ziyadeh, C.-Q. Yang, R. Kalluri, G. A. Müller, E. G. Neilson, A. Renziehausen and Z. Sisic (2002). "Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation." Kidney International **61**(5): 1714-1728.

Tavares, L., A. Correia, M. A. Santos, J. B. Relvas and P. S. Pereira (2017). "dMyc is required in retinal progenitors to prevent JNK-mediated retinal glial activation." PLoS Genetics **13**(3): e1006647.

Tavares, L., E. Pereira, A. Correia, M. A. Santos, N. Amaral, T. Martins, J. B. Relvas and P. S. Pereira (2015). "*Drosophila* PS2 and PS3 integrins play distinct roles in retinal photoreceptors–glia interactions." Glia **63**(7): 1155-1165.

Thiery, J. P. and J. P. Sleeman (2006). "Complex networks orchestrate epithelial–mesenchymal transitions." Nature Reviews Molecular Cell Biology **7**: 131.

Tirinato, L., F. Pagliari, T. Limongi, M. Marini, A. Falqui, J. Seco, P. Candeloro, C. Liberale and E. Di Fabrizio (2017). "An Overview of Lipid Droplets in Cancer and Cancer Stem Cells." Stem Cells International **2017**: 1656053.

Uhlirova, M. and D. Bohmann (2006). "JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in *Drosophila*." The EMBO Journal **25**(22): 5294-5304.

Uttamsingh, S., X. Bao, K. T. Nguyen, M. Bhanot, J. Gong, J. L. K. Chan, F. Liu, T. T. Chu and L. H. Wang (2007). "Synergistic effect between EGF and TGF-β1 in inducing oncogenic properties of intestinal epithelial cells." Oncogene **27**: 2626.

Vicente-Manzanares, M., X. Ma, R. S. Adelstein and A. R. Horwitz (2009). "Non-muscle myosin II takes centre stage in cell adhesion and migration." Nature reviews. Molecular cell biology **10**(11): 778-790.

Wan, P. T. C., M. J. Garnett, S. M. Roe, S. Lee, D. Niculescu-Duvaz, V. M. Good, C. G. Project, C. M. Jones, C. J. Marshall, C. J. Springer, D. Barford and R. Marais (2004). "Mechanism of Activation of the RAF-ERK Signaling Pathway by Oncogenic Mutations of B-RAF." Cell **116**(6): 855-867.

Widmann, T. J. and C. Dahmann (2009). "Dpp signaling promotes the cuboidal-to-columnar shape transition of *Drosophila* wing disc epithelia by regulating Rho1." Journal of Cell Science **122**(9): 1362-1373.

Xie, L., B. K. Law, A. M. Chytil, K. A. Brown, M. E. Aakre and H. L. Moses (2004). "Activation of the Erk Pathway Is Required for TGF-β1-Induced EMT In Vitro." Neoplasia (New York, N.Y.) **6**(5): 603-610.

Xu, J., S. Lamouille and R. Derynck (2009). "TGF-β-induced epithelial to mesenchymal transition." Cell research **19**(2): 156-172.

Yamada, S., S. Pokutta, F. Drees, W. I. Weis and W. J. Nelson (2005). "Deconstructing the Cadherin-Catenin-Actin Complex." Cell **123**(5): 889-901.

Yilmaz, M. and G. Christofori (2010). "Mechanisms of Motility in Metastasizing Cells." Molecular Cancer Research **8**(5): 629-642.

Zavadil, J. and E. P. Böttinger (2005). "TGF-β and epithelial-to-mesenchymal transitions." Oncogene **24**: 5764.

Zhang, Y. E. (2017). "Non-Smad Signaling Pathways of the TGF-β Family." Cold Spring Harbor Perspectives in Biology **9**(2).

8. Supplementary Figures

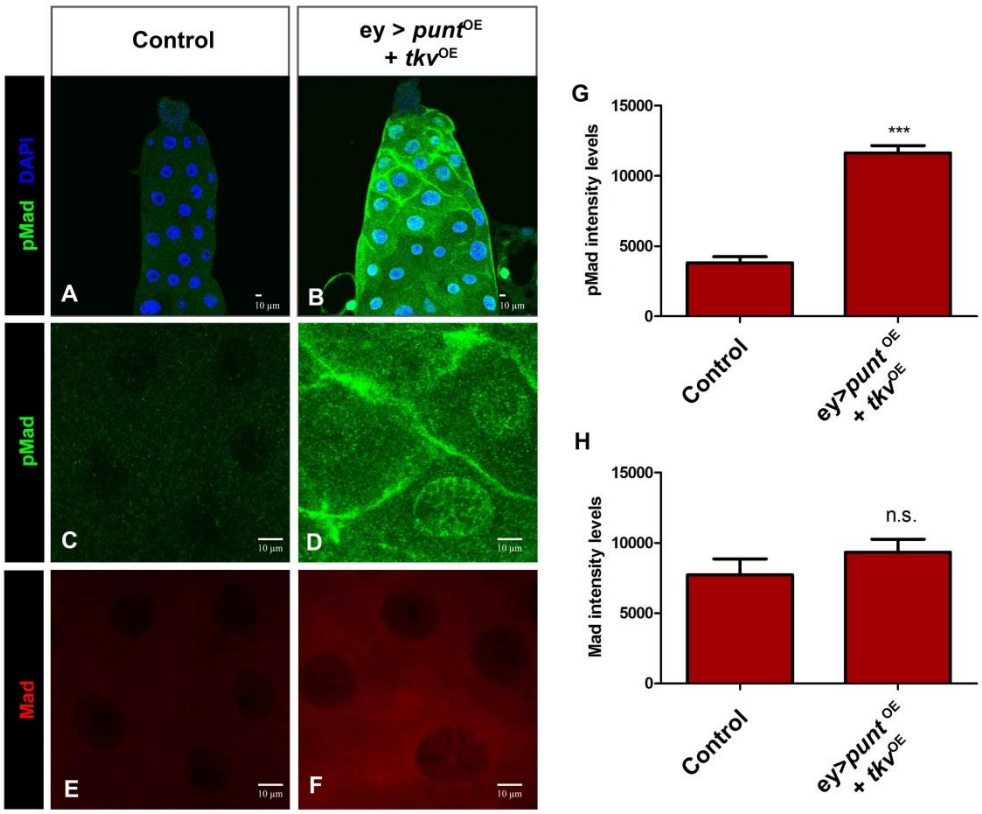

Figure S5.1. Overexpression of TGFβ signaling induces an accumulation of Mad and pMad in gland cells. (A, C and E) Basal plane of control (ey>*LacZ*) salivary gland. (C and E) Magnification of the basal plane of control salivary gland. (B, D and F) Basal plane of salivary gland overexpressing *punt* and *tkv*. (D and F) Magnification of the basal plane of salivary gland overexpressing *punt* and *tkv*. Salivary glands stained with DAPI (DNA, blue), pMad (green) and Mad (red). Scale bars correspond to 10 μm. Quantification of total protein intensity levels of the indicated genotypes, n=5, n.s. means no statistical difference between samples; ***, $P<1\text{^}10^{-4}$.

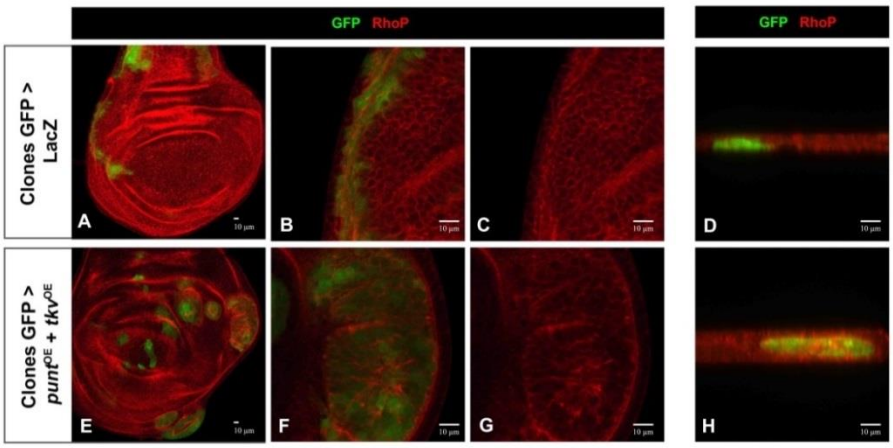

Figure S5.2. Defective morphogenesis in TGFβ clones of imaginal wing discs. (A-H) Clones overexpressing *punt* and *tkv* exhibit defects in the ability to establish or maintain the pseudostratified columnar cell shape. (A) Control clones with GFP expression in imaginal eye disc. (B-C) Magnification and (D) transversal section of control clones in imaginal

wing disc. (E) Combinatorial overexpression of *punt* and *tkv* clones in the wing disc. (F-G) Magnification and (H) transversal section of clones overexpressing *punt* and *tkv* in the wing disc. Wing discs stained with RhoP (membranes, red). Scale bars correspond to 10 µm.

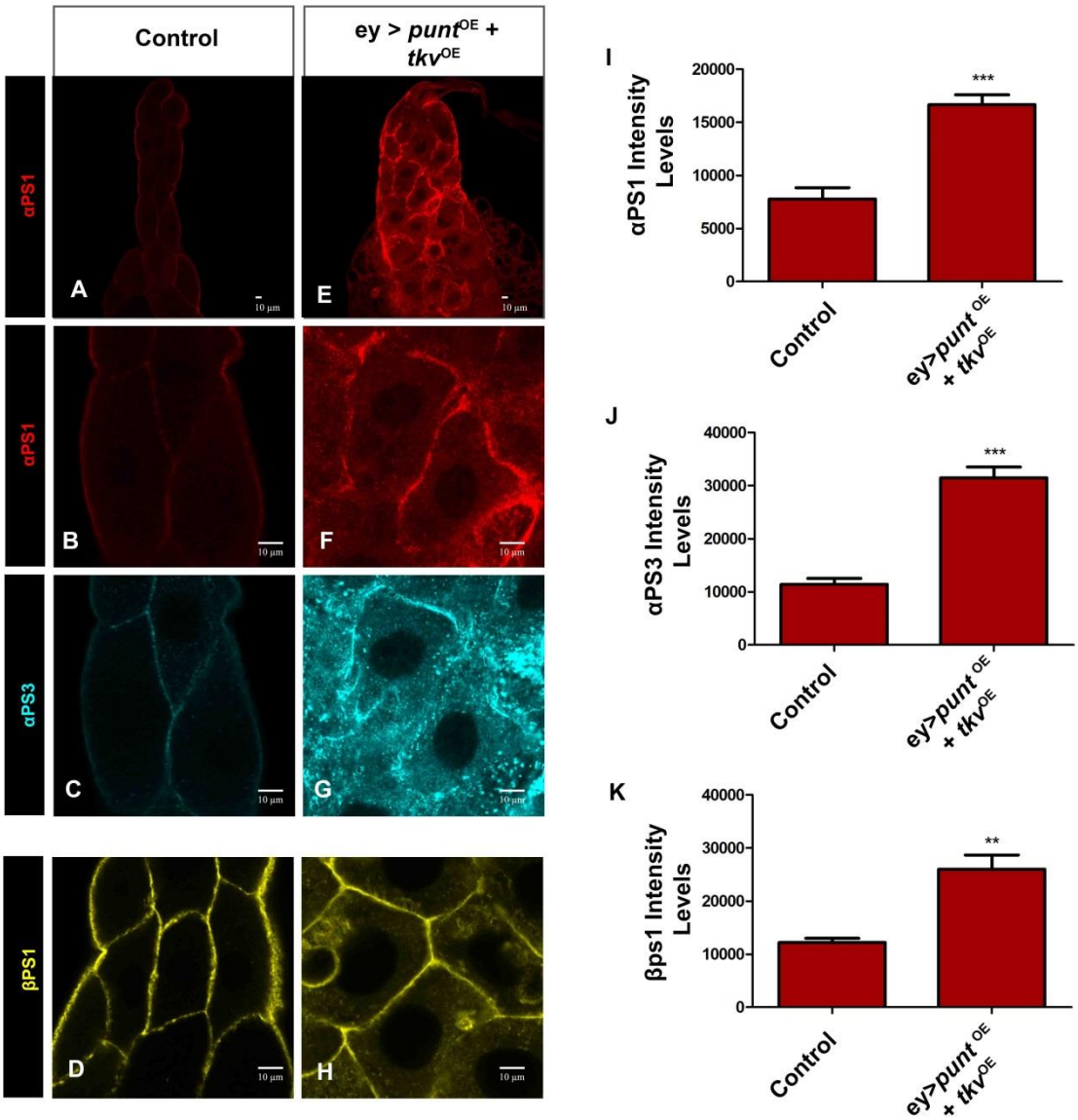

Figure S5.3. The levels and localization of αPS1, αPS3 and βPS Integrins are altered in gland cells overexpressing TGFβ signaling. (A-D) Basal plane of control (ey>*LacZ*) salivary gland. (B-D) Magnification of the basal plane of control salivary gland. (E-H) Basal plane of salivary gland overexpressing *punt* and *tkv*. (F-H) Magnification of the basal plane of salivary gland overexpressing *punt* and *tkv*. Salivary glands stained with αPS1 (red), αPS3 (cyan) and βPS (yellow). Scale bars correspond to 10 µm. (I-K) Quantification of total protein intensity levels of the indicated genotypes, n=5, **, P<0.01 and ***, P<1^10^{-4}.

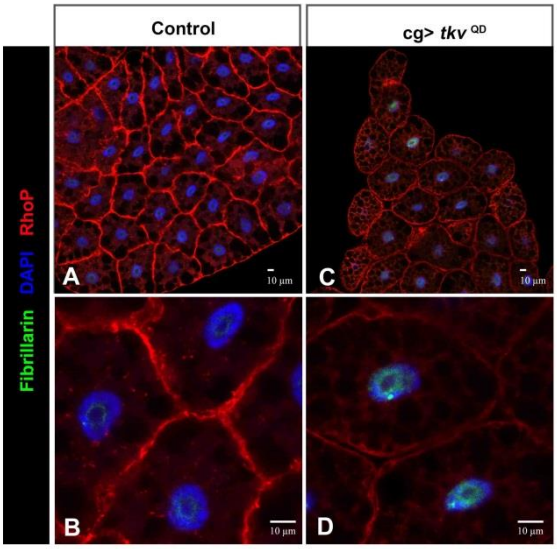

Figure S5.4. Cell detachment is observed in fat body overexpressing TGFβ signaling. (A-B) Control (ey>*LacZ*) fat body cells. (C-D) Fat body cells overexpressing the constitutive active *tkv* receptor. Fat body cells stained with DAPI (DNA, blue), Fibrillarin (nucleolus, green) and RhoP (membranes, red). Scale bars correspond to 10 µm.

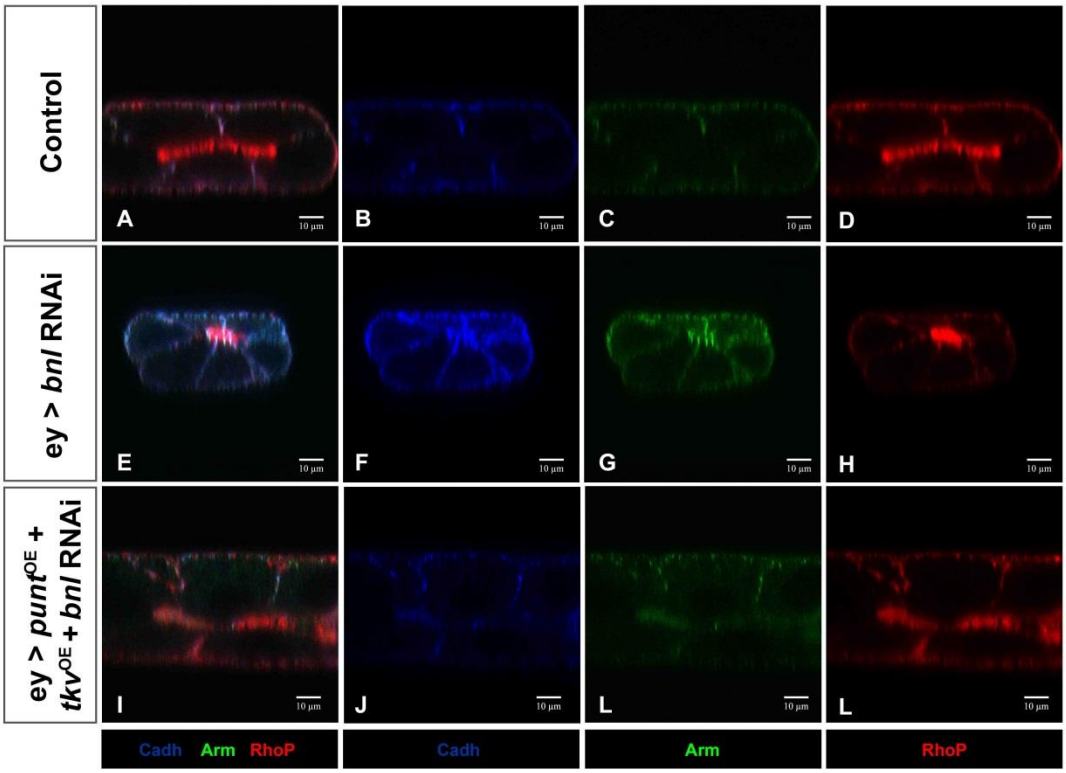

Figure S5.5. Downregulation of *bnl* induces apical constriction.
Transversal sections of (A-D) control (ey>W1118) cell glands, (E-F) cell glands expressing RNAi for *bnl* and (I-L) cell glands expressing RNAi for *bnl* and overexpressing *punt* and *tkv*. Salivary glands stained with E-Cadherin (blue), Armadillo (green) and RhoP (red). Scale bars correspond to 10 µm.

CHAPTER 6

General Discussion

Contents

In this chapter, we critically examine our findings in the light of the previous state of the art as outlined in the background, and make assumptions as to what has been learnt in our work. Additionally, we propose some directions that our work can take to improve our knowledge in this field.

The transition of unicellular to multicellular organisms, as well as, the determination of specific cell functions, complex tissues and specialized organs need the institution of cell-cell communication mechanisms. The behavior of a given cell in a multicellular organism is a very complex task that requires a constant regulation. The development of a single cell into a multicellular organism is the consequence of an amazingly high number of events, including proliferation, cell growth and differentiation. The regulation of these mechanisms comprises a complex network of signaling pathways that maintain the correct balance between positive and negative growth signals, ensuring that the right size and differentiation of tissues are achieved. In this thesis, we have aimed to better understand the contribution of TGFβ signaling in basic cell properties of *Drosophila melanogaster*, including growth, differentiation and polarity.

1. How does TGFβ signaling regulate cell growth in *Drosophila*?

TGFβ signaling plays multiple roles during animal development through the regulation of cellular growth, proliferation, differentiation and survival (Massague 2012). TGFβ signaling inhibits the growth of several cell types, such as epithelial cells, endothelial cells and hematopoietic cells but also induces growth of other cell types, including mesenchymal cells (Roberts and Sporn 1993, Roberts 1998). The growth function of TGFβ signaling has been studied in a diversity of in vitro cultured cell systems, but the role of TGFβ signaling in cell growth is not well understood. Previously, the Activin type I receptor, Baboon, and the R-Smad, Smad2, were shown to be specifically required for cellular proliferation and overall growth of the wing imaginal disc (Hevia and de Celis 2013). Interestingly, the TGFβ/Activin branch was not found to affect any specific transition of the cell cycle or to cause extensive apoptosis in the wing disc (Hevia and de Celis 2013). Our results from Chapter 2 showed that in *Drosophila* salivary glands, TGFβ signaling also played an important role in cell growth. Furthermore, in imaginal discs TGFβ signaling regulated both cell growth and differentiation. Cells deprived of type II receptor, Punt, or R-Smad, Smad2, expression presented a small size. The decrease of cell size has been accompanied by nucleolar changes. Moreover, an accumulation of unprocessed intermediate pre-rRNA transcripts, and defects in ribosome biogenesis with a significant decrease in 18S rRNA and very significant effects on the nuclear localization of ribosomal proteins were also observed in cells deprived of TGFβ signaling. This suggests that TGFβ signaling is involved in the regulation of ribosome biogenesis. TGFβ signaling may also control the

transcription of targets with direct enzymatic roles in pre-RNA processing or ribosome biogenesis and nuclear export. The 5'-3' RNA exonuclease Vito is a potential target to fulfil that role (Marinho, Casares et al. 2011). Vito knockdown in developing tissues induces the accumulation of atypical pre-RNA intermediates. Additionally, the budding yeast Vito homologue Rrp17p has a functional role in the late processing of pre-rRNA and nuclear export of pre-60S ribosomal subunits (Oeffinger, Zenklusen et al. 2009). Deregulation of *vito* expression is associated with a decrease of tissue growth in salivary glands (Marinho, Casares et al. 2011). This reduction in growth seems to result from the lack of the machinery required for the production of efficient proteins necessary for growth. Interestingly, a stronger growth reduction was observed when we downregulated TGFβ signaling, which suggests that other genes important for rRNA processing, may be regulated by this signaling. Along with Vito, other genes have been associated with the regulation of rRNA processing events and, consequently, regulate tissue growth. It is the case of *Nop60B,* a gene of *Drosophila* that encodes an essential nucleolar protein. Loss-of-function mutations in the *Drosophila Nop60B* lead to a strong body size reduction. Particularly, in the wing imaginal disc, the downregulation of *Nop60B* inhibits the size and decreases cells number (Giordano, Peluso et al. 1999, Tortoriello, de Celis et al. 2010). Furthermore, downregulation of the mouse *dyskerin* gene (the mammalian homologue of the *Drosophila* Nop60B) halts rRNA processing preventing ribosome biogenesis and consequently inhibiting cell proliferation in mouse hepatocytes (Ge, Rudnick et al. 2010). Nucleostemin (NS) is another nucleolar protein that plays an important role in the processing of pre-rRNA. In *Drosophila* and mammals, NS affects large ribosomal subunit biogenesis (Romanova, Grand et al. 2009, Rosby, Cui et al. 2009). Moreover, NS is also an important regulator of cell proliferation, through the induction of cell cycle arrest via the p53 pathway when misexpressed (Ma and Pederson 2008). The study of the regulation of the expression of these and other nucleolar proteins by TGFβ signaling might be very useful to understand how this signaling regulates rRNA processing and stimulates ribosome biogenesis. For this purpose RNA sequencing would be of great value to analyze the alterations in the transcriptome after TGFβ signaling downregulation. Moreover, it is known that *Drosophila* canonical TGFβ signaling can be induced by two different branches, Activin and BMP. Nevertheless, both branches of TGFβ signaling can physically interact downstream of ligand-receptor binding and particularly, the active receptor Baboon, is able to induce wing growth through the phosphorylation of the BMP R-Smad Mad (Gesualdi and Haerry 2007). Therefore, it would be interesting to study if the upregulation of one of the two TGFβ signaling branches can compensate

the downregulation of the other and rescue the phenotype induced by downregulation of *mad* or *smad2.*

2. How does TGFβ signaling regulate eye differentiation in *Drosophila*?

Dpp-BMP2/4 signaling plays an essential role in *Drosophila* eye development. In the eye disc, Dpp starts to be expressed in the second larval stage together with Hh through the posterior margin of the disc. At this point, Dpp is at a quiescent stage due to the inhibitory activity of Wg (Hazelett, Bourouis et al. 1998), which avoids a premature differentiation onset. When Notch, Eyg and Upd (Domínguez and Celis 1998, Chao, Tsai et al. 2004) expression is increased, the Wg and Dpp domains are physical separate and the antagonizing effect of Wg upon Dpp is unloaded (Kenyon, Ranade et al. 2003). This separation is further stimulated by *wg* repression through the JAK/STAT signaling (Tsai and Sun 2004, Ekas, Baeg et al. 2006). Therefore, the antagonizing effect of Wg upon Dpp is unloaded (Kenyon, Ranade et al. 2003) and retinal differentiation starts with the expression of the unblocked *dpp* within the morphogenetic furrow (MF), an epithelial indentation that advances from the posterior margin to the anterior region of the eye imaginal disc (Ready, Hanson et al. 1976). Despite several signaling pathways and transcription regulators having been described as necessary for eye growth and differentiation, we still have an incomplete knowledge of the regulation and functions of the Dpp-BMP2/4 during eye differentiation. In chapter 3 we have taken advantage of an eye-targeted combinatorial screen to analyze the contribution of new Dpp-BMP2/4 interactors for eye differentiation. The downregulation of type II receptor, *punt*, in eye imaginal discs inhibited eye differentiation and the majority of these flies ecloded without both retinas or exhibiting a very small retinal area. Nevertheless, this eye differentiation inhibition was restored through the downregulation of several genes involved in eye development, cell cycle, transcription, or translation. Regarding the collection of genes that recued the phenotype induced by *punt* RNAi, only four were classified as strong regulators of Dpp pathway: *dad*, *brk*, *ago* and *CtBP*. The individual RNAi for these four genes in a *punt* RNAi background was able to rescue the strong eye phenotype induced by *punt* knockdown. The eyes of flies with knockdown for each of the four Punt-interactors in a *punt* RNAi background presented a large area of photoreceptors with a similar control eye organization, which suggest that those Punt-interactors are Dpp-BMP2/4 negative regulators. Our four best candidate genes (*CtBP, ago, brk* and *dad*) for Punt-interactors comprise a group with

very different molecular functions, suggesting that *punt* activity is responsible for more than one functional output. These genes have previously associated to cell growth, cell differentiation, or linked to Dpp-BMP2/4 signaling in other cellular contexts. *brk* and *dad* have been described as negative regulators of TGFβ signaling. Brk is a negative repressor of Dpp, which competes with activated Mad, blocking the stimulation of Dpp target genes (Bray 1999, Campbell and Tomlinson 1999, Jaźwińska, Kirov et al. 1999, Saller and Bienz 2001); and Dad regulates TGFβ signaling by preventing Mad activation, through the competition with R-Smads for receptors and thereby inhibiting Tkv-induced Mad phosphorylation; or competing with Co-Smad interactions which blocks hetero-oligomerization and nuclear translocation of Mad (Tsuneizumi, Nakayama et al. 1997, Kamiya, Miyazono et al. 2008). In chapter 3, we demonstrated that CtBP negatively regulates Dpp signaling by modulating pMad levels, which is essential for a normal eye differentiation. CtBP may exert its function together with other molecules. In *Drosophila,* CtBP can be recruited by Brk and enhances the repression activity of Brk-dependent Gro in the *dpp* promoter (Hasson, Müller et al. 2001). Conversely, in mammalian cells CtBP interacts with Smad6 to repress BMP-dependent transcription (negative input), a nuclear Smad6 role that is independent of its binding to receptors (Lin, Liang et al. 2003). The interaction between CtBP and I-Smad may be conserved in *Drosophila*, as the Dpp repression by Dad requires the function of CtBP. In the future, it would be interesting to determine if CtBP interacts with Brk and Dad, improving the repressor activity of these proteins and inhibiting Mad activation in imaginal eye disc. Otherwise, another negative regulator that we found in our screen was Ago. Ago is a protein that regulates cell growth by ubiquitination of different proteins that stimulates the growth, including Myc and CycE (Moberg, Bell et al. 2001, Li, Anderson et al. 2013). Therefore, the downregulation of Ago during eye development might be critical for the clearance of the negative repression of Dpp induced by Wg. Thus, when we do RNAi for *ago*, the eye disc received signals from Myc and other growth factors to growth and the antagonizing effect of Wg upon Dpp is unloaded (Kenyon, Ranade et al. 2003). Thus, retinal differentiation starts with the expression of the unblocked *dpp* within the MF. The exact mechanism how Ago regulates TGFβ signaling activity has not been explored yet. Does Ago only inhibit eye differentiation by degradation of growth factors or does Ago also induce the degradation of Wg? This mechanism still remains unknown. Exploring the domain of interaction on Ago and Wg could be another dimension where this project could be investigated.

3. How does Dad regulate TGFβ signaling?

TGFβ signaling has three different known types of Smads, R-Smads, Co-Smad and I-Smad. R-Smads and Co-Smad are very similar in its structure and both are important for TGFβ signal propagation and activation of several target genes. Conversely, I-Smad Dad is a direct target of TGFβ signaling (Tsuneizumi et al. 1997) that regulates the robustness of the Dpp signaling gradient (Ogiso et al. 2011) by blocking Tkv-mediated phosphorylation of Mad and hetero-oligomerization and nuclear translocation of Mad (Tsuneizumi, Nakayama et al. 1997, Inoue, Imamura et al. 1998, Kamiya, Miyazono et al. 2008). In chapter 4, we demonstrated for the first time that Dad inhibits TGFβ signaling in the nucleolus. The ectopic expression of Dad in imaginal eye disc revealed that the presence of Dad in the nucleolus is sufficient to inhibit Mad activation in the MF and prevent eye growth and differentiation. Our results also showed that ectopic expression of Dad in salivary gland led to a recruitment of Dad to the membrane. In the membrane Dad interacts with Put/Tkv complex, which is required for an efficient inhibition of receptor-meditated phosphorylation of Mad. Indeed, these findings are in agreement with earlier reports that characterized Dad as an inhibitory Smad that acts at type I receptors level (Tsuneizumi, Nakayama et al. 1997, Kamiya, Miyazono et al. 2008). The transient presence of Dad in the membrane was regulated by the N-terminal domain of Dad. In vertebrates, the N-domain of the I-Smads is very important for their inhibitory functions (Itoh, Landström et al. 1998, Hanyu, Ishidou et al. 2001, Lin, Liang et al. 2003), including for the recruitment of the E2 ubiquitin-conjugating enzyme UbcH7 (Ogunjimi, Briant et al. 2005) and for the enabling of the interaction between I-Smad and type I receptors (Hanyu, Ishidou et al. 2001, Nakayama, Berg et al. 2001).

Dad activity is regulated by different post-translational modifications, including methylation and palmyolation (Xu, Wang et al. 2013, Li, Li et al. 2017). Remarkably, in chapter 4, we identified for the first time that a strong upregulation of TGFβ signaling was able to induce Dad phosphorylation, to stabilize Dad in the membrane and to block its inhibitory function. This phosphorylation was not observed when we expressed the constitutive active Tkv, which suggested that the phosphorylation of Dad by TGFβ signaling might require the activation of both branches of this signaling, BMP and Activin. Thus, it would be interesting to determine if we activate independently the type II and I BMP or Activin receptors also induce Dad phosphorylation.

The N-terminal domain of Dad is rich in its proline content which is known to play an important role in several transient intermolecular interactions such as signal transduction, cell-cell communication and cytoskeletal organization (Williamson 1994,

Ball, Kühne et al. 2005). Thus, we next asked whether the N-terminal domain of Dad was important for Dad phosphorylation. However, the truncated version of Dad was also phosphorylated when TGFβ signaling was upregulated, which suggested that N-terminal domain of Dad was not required for Dad phosphorylation and consequently inhibition of Dad function. Therefore, we tried to determine which amino acids of Dad are phosphorylated when TGFβ signaling is upregulated. Dad has seven serines or threonines, which are followed by a proline, constituting the minimal consensus for phosphorylation by mitogen-activated protein kinases (Jacobs, Glossip et al. 1999, Sharrocks, Yang et al. 2000, Biondi and Nebreda 2003) or cyclin-dependent kinases (Kitagawa, Higashi et al. 1996, Moses, Hériché et al. 2007). Moreover, several serine/threonine sites (Ser333, Thr335, Ser488, Ser535 and Ser539) identified by Mass Spectrometry or through the conservation with Smad6/Smad7 are potential targets of phosphorylation. Thus, it would be interesting to generate flies with mutations in the different S/T sites and determine which residues are phosphorylated and required for the inhibition of Dad function. Additionally, it would be interestingly to determine which kinase is responsible for Dad phosphorylation. As I-Smads regulate fine-tune the magnitude of TGFβ family signaling, understanding the regulatory mechanisms of I-Smad function may open new avenues on TGFβ signaling coordination in cell growth, differentiation and morphogenesis in development and pathological conditions. Moreover, abnormal I-Smad expression has been proposed to play crucial roles in several diseases. Thus, it would also be relevant to determine how the expression levels of I-Smad proteins are regulated in these diseases.

4. Is *Drosophila* TGFβ signaling able to induce EMT?

Multiple non-smad signaling pathways of the TGFβ family have been described but a large part of the factors have not been characterized (Eaton and Davis, 2005; Ng, 2008). In mammals, TGFβ family can induces several non-smad signals, including mitogen-activated protein kinase (MAPK) pathways, extracellular signal regulated kinases (Erks), c-Jun amino terminal kinase (JNK) and p38 MAPK, as well as the IkB kinase (IKK), phosphatidylinositol-3 kinase (PI3K) and Akt, and Rho family GTPases (Moustakas and Heldin 2005; Zhang 2009). In *Drosophila,* the first report of non-canonical TGFβ signaling involved signals that regulate the neuromuscular junction (Easton, Cho et al. 2005). The proper development of the neuromuscular junction requires the presynaptic BMP signaling lead by Gbb, Tkv, Sax, Wit, and Mad. However, in addition to the role of canonical BMP signaling, the type II receptor Wit also stimulates the kinase LIMK1 in a Mad-independent way (Easton, Cho et al. 2005).

LIMK1 regulates development in the nervous system by phosphorylating Twinstar, which inhibits polymerization of the Actin cytoskeleton (Ohashi, Nagata et al. 2000, Ng and Luo 2004). Other signaling components shown to mediate non-canonical signaling in vertebrates are conserved in *Drosophila.* Here, we identified for the first time that TGFβ family can induce mesenchymal features in epithelial cells of *Drosophila* gland cells by influencing the activity of factors controlling cytoskeleton and cellular junctions. Cell glands strongly expressing TGFβ signaling, lost their apical-basolateral polarity and acquired mesenchymal features, characterized by Actin reorganization, stress fiber formation and cell detachment. Furthermore, TGFβ signaling seemed to regulate these cellular alterations through the activation of Erk signaling. Such mechanism has been proposed for EMT in mammals, suggesting strong evolutionary conservation (Xu, Lamouille et al. 2009). Curiously, a downregulation of *bnl* (a *Drosophila* homologue of the mammalian FGFs) strongly reverted TGFβ-induced phenotype, inhibiting Mmp1 expression, Actin stress fiber formation and promoting apical constriction. However, *bnl* was not able to regulate Erk signaling, which suggests that *bnl* functions downstream or in parallel of Erk signaling, as a regulator of TGFβ-induced phenotype. The canonical TGFβ/Smad signaling directly regulates the expression of different EMT-inducing transcription factors, including Snail/Slug, Twist and ZEB1/2. These transcriptional factors inhibit the expression of epithelial protein markers and stimulate mesenchymal characteristics (Xu, Lamouille et al. 2009, Moustakas and Heldin 2012). In the future, it would be interesting to investigate if the regulation of EMT-inducing transcription factors by canonical TGFβ/Smad signaling is conserved in *Drosophila*. Therefore, it would be important to activate simultaneously the R-Smads of both BMP and Activin branches and determine which target genes are upregulated. The deep characterization of EMT-induced by canonical and non-canonical TGFβ/Smad signaling in *Drosophila* may allow a better mechanistically characterization of the different factors and pathways that cooperate with TGFβ signaling. Thus, the fast and easy *Drosophila* model system might be used to screen therapeutic drugs able to revert the phenotype induced by TGFβ signaling and its interactors (Vidal, Wells et al. 2005, Gasque, Conway et al. 2013, Willoughby, Schlosser et al. 2013, Markstein, Dettorre et al. 2014). The combination of genetic analysis and therapeutic drug treatments will be a very powerful tool for the advances in personalized medicine.

5. Final considerations

Through my PhD research I have identified novel inputs into the TGFβ family signaling for organ size regulation, differentiation, morphogenesis and epithelial mesenchymal transition. I have identified that TGFβ signaling regulates the ribosome biogenesis and the downregulation of this signaling inhibits cell growth and differentiation. In contrast, the upregulation of TGFβ family signaling is associated with several defects in cell polarity and Actin reorganization, leading to a mesenchymal phenotype. The regulation of TGFβ signaling is done by different negative regulators, including Ago, Brk, CtBP and Dad. One of these negative regulators is the Inhibitory Smad of the TGFβ signaling, Dad. Transcription of *dad* is positively regulated by TGFβ signaling. Nevertheless, Dad can negatively regulate TGFβ pathway preventing Mad activation. The N-terminal domain of Dad is required for Dad function and its transient localization in the membrane. However, a strong upregulation of TGFβ signaling induces Dad phosphorylation, stabilizes it in the membrane and inhibits its function as negative regulator of TGFβ signaling. This work will help to better understand how different signaling pathways interact with TGFβ signaling to regulate its functions during development and how an inappropriate regulation of this pathway could lead to disease conditions like cancer.

6. References

Ball, L. J., R. Kühne, J. Schneider-Mergener and H. Oschkinat (2005). "Recognition of Proline-Rich Motifs by Protein–Protein-Interaction Domains." Angewandte Chemie International Edition **44**(19): 2852-2869.

Biondi, R. M. and A. R. Nebreda (2003). "Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions." Biochemical Journal **372**(Pt 1): 1-13.

Bray, S. (1999). "DPP on the brinker." Trends Genet **15**(4): 140.

Campbell, G. and A. Tomlinson (1999). "Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*." Cell **96**(4): 553-562.

Chao, J.-L., Y.-C. Tsai, S.-J. Chiu and Y. H. Sun (2004). "Localized Notch signal acts through *eyg* and *upd* to promote global growth in *Drosophila* eye." Development **131**(16): 3839-3847.

Domínguez, M. and J. F. d. Celis (1998). "A dorsal/ventral boundary established by Notch controls growth and polarity in the *Drosophila* eye." Nature **396**: 276.

Easton, R. M., H. Cho, K. Roovers, D. W. Shineman, M. Mizrahi, M. S. Forman, V. M. Y. Lee, M. Szabolcs, R. de Jong, T. Oltersdorf, T. Ludwig, A. Efstratiadis and M. J. Birnbaum (2005). "Role for Akt3/Protein Kinase Bγ in Attainment of Normal Brain Size." Molecular and Cellular Biology **25**(5): 1869-1878.

Ekas, L. A., G.-H. Baeg, M. S. Flaherty, A. Ayala-Camargo and E. A. Bach (2006). "JAK/STAT signaling promotes regional specification by negatively regulating *wingless* expression in *Drosophila*." Development **133**(23): 4721-4729.

Gasque, G., S. Conway, J. Huang, Y. Rao and L. B. Vosshall (2013). "Small molecule drug screening in *Drosophila* identifies the 5HT2A receptor as a feeding modulation target." Scientific Reports **3**: srep02120.

Ge, J., D. A. Rudnick, J. He, D. L. Crimmins, J. H. Ladenson, M. Bessler and P. J. Mason (2010). "Dyskerin Ablation in Mouse Liver Inhibits rRNA Processing and Cell Division." Molecular and Cellular Biology **30**(2): 413-422.

Gesualdi, S. and T. Haerry (2007). "Distinct signaling of *Drosophila* Activin/TGF-beta family members." Fly (Austin) **1**(4): 212-221.

Giordano, E., I. Peluso, S. Senger and M. Furia (1999). "minifly, A *Drosophila* Gene Required for Ribosome Biogenesis." The Journal of Cell Biology **144**(6): 1123-1133.

Hanyu, A., Y. Ishidou, T. Ebisawa, T. Shimanuki, T. Imamura and K. Miyazono (2001). "The N domain of Smad7 is essential for specific inhibition of transforming growth factor-β signaling." The Journal of Cell Biology **155**(6): 1017-1028.

Hasson, P., B. Müller, K. Basler and Z. e. Paroush (2001). "Brinker requires two corepressors for maximal and versatile repression in Dpp signalling." The EMBO Journal **20**(20): 5725-5736.

Hazelett, D. J., M. Bourouis, U. Walldorf and J. E. Treisman (1998). "decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc." Development **125**(18): 3741-3751.

Hevia, C. F. and J. F. de Celis (2013). "Activation and function of TGFβ signalling during *Drosophila* wing development and its interactions with the BMP pathway." Developmental Biology **377**(1): 138-153.

Inoue, H., T. Imamura, Y. Ishidou, M. Takase, Y. Udagawa, Y. Oka, K. Tsuneizumi, T. Tabata, K. Miyazono and M. Kawabata (1998). "Interplay of Signal Mediators of Decapentaplegic (Dpp): Molecular Characterization of Mothers against dpp, Medea, and Daughters against dpp." Molecular Biology of the Cell **9**(8): 2145-2156.

Itoh, S., M. Landström, A. Hermansson, F. Itoh, C.-H. Heldin, N.-E. Heldin and P. ten Dijke (1998). "Transforming Growth Factor β1 Induces Nuclear Export of Inhibitory Smad7." Journal of Biological Chemistry **273**(44): 29195-29201.

Jacobs, D., D. Glossip, H. Xing, A. J. Muslin and K. Kornfeld (1999). "Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase." Genes & Development **13**(2): 163-175.

Jaźwińska, A., N. Kirov, E. Wieschaus, S. Roth and C. Rushlow (1999). "The *Drosophila* Gene *brinker* Reveals a Novel Mechanism of Dpp Target Gene Regulation." Cell **96**(4): 563-573.

Kamiya, Y., K. Miyazono and K. Miyazawa (2008). "Specificity of the inhibitory effects of Dad on TGF-β family type I receptors, Thickveins, Saxophone, and Baboon in *Drosophila*." FEBS Letters **582**(17): 2496-2500.

Kenyon, K. L., S. S. Ranade, J. Curtiss, M. Mlodzik and F. Pignoni (2003). "Coordinating Proliferation and Tissue Specification to Promote Regional Identity in the *Drosophila* Head." Developmental Cell **5**(3): 403-414.

Kitagawa, M., H. Higashi, H. K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, S. Nishimura and Y. Taya (1996). "The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2." The EMBO Journal **15**(24): 7060-7069.

Li, L., S. Anderson, J. Secombe and R. N. Eisenman (2013). "The *Drosophila* ubiquitin-specific protease Puffyeye regulates dMyc-mediated growth." Development (Cambridge, England) **140**(23): 4776-4787.

Li, W., W. Li, L. Zou, S. Ji, C. Li, K. Liu, G. Zhang, Q. Sun, F. Xiao and D. Chen (2017). "Membrane targeting of inhibitory Smads through palmitoylation controls TGF-β/BMP signaling." Proceedings of the National Academy of Sciences.

Lin, X., Y.-Y. Liang, B. Sun, M. Liang, Y. Shi, F. C. Brunicardi, Y. Shi and X.-H. Feng (2003). "Smad6 Recruits Transcription Corepressor CtBP To Repress Bone Morphogenetic Protein-Induced Transcription." Molecular and Cellular Biology **23**(24): 9081-9093.

Ma, H. and T. Pederson (2008). "Nucleophosmin Is a Binding Partner of Nucleostemin in Human Osteosarcoma Cells." Molecular Biology of the Cell **19**(7): 2870-2875.

Marinho, J., F. Casares and P. S. Pereira (2011). "The *Drosophila Nol12* homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth." Development **138**(2): 349-357.

Markstein, M., S. Dettorre, J. Cho, R. A. Neumüller, S. Craig-Müller and N. Perrimon (2014). "Systematic screen of chemotherapeutics in *Drosophila* stem cell tumors." Proceedings of the National Academy of Sciences **111**(12): 4530-4535.

Massague, J. (2012). "TGFβ signalling in context." Nat Rev Mol Cell Biol **13**(10): 616-630.

Moberg, K. H., D. W. Bell, D. C. R. Wahrer, D. A. Haber and I. K. Hariharan (2001). "Archipelago regulates Cyclin E levels in *Drosophila* and is mutated in human cancer cell lines." Nature **413**(6853): 311-316.

Moses, A. M., J.-K. Hériché and R. Durbin (2007). "Clustering of phosphorylation site recognition motifs can be exploited to predict the targets of cyclin-dependent kinase." Genome Biology **8**(2): R23-R23.

Moustakas, A. and C.-H. Heldin (2012). "Induction of epithelial–mesenchymal transition by transforming growth factor β." Seminars in Cancer Biology **22**(5): 446-454.

Nakayama, T., L. K. Berg and J. L. Christian (2001). "Dissection of inhibitory Smad proteins: both N- and C-terminal domains are necessary for full activities of Xenopus Smad6 and Smad7." Mechanisms of development **100**(2): 251-262.

Ng, J. and L. Luo (2004). "Rho GTPases Regulate Axon Growth through Convergent and Divergent Signaling Pathways." Neuron **44**(5): 779-793.

Oeffinger, M., D. Zenklusen, A. Ferguson, K. E. Wei, A. El Hage, D. Tollervey, B. T. Chait, R. H. Singer and M. P. Rout (2009). "Rrp17p Is a Eukaryotic Exonuclease Required for 5′ End Processing of Pre-60S Ribosomal RNA." Molecular Cell **36**(5): 768-781.

Ogunjimi, A. A., D. J. Briant, N. Pece-Barbara, C. Le Roy, G. M. Di Guglielmo, P. Kavsak, R. K. Rasmussen, B. T. Seet, F. Sicheri and J. L. Wrana (2005). "Regulation of Smurf2 Ubiquitin Ligase Activity by Anchoring the E2 to the HECT Domain." Molecular Cell **19**(3): 297-308.

Ohashi, K., K. Nagata, M. Maekawa, T. Ishizaki, S. Narumiya and K. Mizuno (2000). "Rho-associated Kinase ROCK Activates LIM-kinase 1 by Phosphorylation at Threonine 508 within the Activation Loop." Journal of Biological Chemistry **275**(5): 3577-3582.

Ready, D. F., T. E. Hanson and S. Benzer (1976). "Development of the *Drosophila* retina, a neurocrystalline lattice." Developmental Biology **53**(2): 217-240.

Roberts, A. B. (1998). "Molecular and Cell Biology of TGF-β." Mineral and Electrolyte Metabolism **24**(2-3): 111-119.

Roberts, A. B. and M. B. Sporn (1993). "Physiological Actions and Clinical Applications of Transforming Growth Factor-β (TGF-β)." Growth Factors **8**(1): 1-9.

Romanova, L., A. Grand, L. Zhang, S. Rayner, N. Katoku-Kikyo, S. Kellner and N. Kikyo (2009). "Critical Role of Nucleostemin in Pre-rRNA Processing." Journal of Biological Chemistry **284**(8): 4968-4977.

Rosby, R., Z. Cui, E. Rogers, M. A. deLivron, V. L. Robinson and P. J. DiMario (2009). "Knockdown of the *Drosophila* GTPase Nucleostemin 1 Impairs Large Ribosomal

Subunit Biogenesis, Cell Growth, and Midgut Precursor Cell Maintenance." Molecular Biology of the Cell **20**(20): 4424-4434.

Saller, E. and M. Bienz (2001). "Direct competition between Brinker and *Drosophila* Mad in Dpp target gene transcription." EMBO Reports **2**(4): 298-305.

Sharrocks, A. D., S.-H. Yang and A. Galanis (2000). "Docking domains and substrate-specificity determination for MAP kinases." Trends in Biochemical Sciences **25**(9): 448-453.

Tortoriello, G., J. F. de Celis and M. Furia (2010). "Linking pseudouridine synthases to growth, development and cell competition." FEBS Journal **277**(15): 3249-3263.

Tsai, Y.-C. and Y. H. Sun (2004). "Long-range effect of upd, a ligand for Jak/STAT pathway, on cell cycle in *Drosophila* eye development." genesis **39**(2): 141-153.

Tsuneizumi, K., T. Nakayama, Y. Kamoshida, T. B. Kornberg, J. L. Christian and T. Tabata (1997). "*Daughters against dpp* modulates dpp organizing activity in *Drosophila* wing development." Nature **389**(6651): 627-631.

Vidal, M., S. Wells, A. Ryan and R. Cagan (2005). "ZD6474 Suppresses Oncogenic RET Isoforms in a *Drosophila* Model for Type 2 Multiple Endocrine Neoplasia Syndromes and Papillary Thyroid Carcinoma." Cancer Research **65**(9): 3538-3541.

Williamson, M. P. (1994). "The structure and function of proline-rich regions in proteins." Biochemical Journal **297**(2): 249-260.

Willoughby, L. F., T. Schlosser, S. A. Manning, J. P. Parisot, I. P. Street, H. E. Richardson, P. O. Humbert and A. M. Brumby (2013). "An in vivo large-scale chemical screening platform using *Drosophila* for anti-cancer drug discovery." Disease Models & Mechanisms **6**(2): 521-529.

Xu, J., S. Lamouille and R. Derynck (2009). "TGF-β-induced epithelial to mesenchymal transition." Cell research **19**(2): 156-172.

Xu, J., A. H. Wang, J. Oses-Prieto, K. Makhijani, Y. Katsuno, M. Pei, L. Yan, Y. G. Zheng, A. Burlingame, K. Brückner and R. Derynck (2013). "Arginine Methylation Initiates BMP-Induced Smad Signaling." Molecular Cell **51**(1): 5-19.