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Developmental regulation of Drosophial Dad, the inhibitory SMAD for the TGFß signaling pathway

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Resumo

A via de sinalização da TGFß participa em inúmeros processos celulares tais como a proliferação e a diferenciação. No caso da *Drosophila*, esta via encontra-se dividida em dois ramos principais: o ramo da Ativina e o ramo do BMP (Proteínas Morfogenéticas Ósseas). A sua regulação inclui várias reguladores positivos e negativos, sendo um destes reguladores negativos a Smad inibitória Dad. Dad inibe esta via através da inibição da fosforilação de Mad, a Smad efetora. No entanto, a sua regulação ainda não está totalmente compreendida, nem nos mamíferos nem em *Drosophila*. Anteriormente, o nosso grupo de investigação já tinha descoberto que Gilgamesh, uma cinase associada à membrana plasmática, regulava Dad. No entanto, esta regulação ainda não está bem caracterizada. Neste trabalho, usamos duas condições de perda de função de Gish para avaliar o seu efeito na atividade e expressão de Dad. Esta perda de função foi obtida quer através da técnica RNAi como da ténica CRISPR, com o objetivo de avaliar qual delas consegue silenciar mais eficazmente Gish. Além disso, este mecanismo de regulação foi estudado em dois tecidos diferenters, olho e asa, para verificarmos se é conservado ou não. No olho, Gish impacta quer a função como a expressão de Dad, o que é fortemente verificado quer fenotipicamente como nos discos imaginais. Na asa, Gish também afeta a função e a expressão de Dad, contudo, este impacto não é tão forte como no olho. Comparando as duas técnicas, a perda de função de Gish através da técnica CRISPR leva a resultados mais fortes.

Abstract

TGFß signaling participates in numerous cellular processes such as proliferation and differentiation. The *Drosophila* TGFß pathway is divided into two branches: the Activin branch and the BMP (Bone Morphogenic Proteins) branch. The regulation of this signaling includes many positive and negative regulators, and one of the negative regulators is the I-Smad Dad (Daughters against Dpp). Dad inhibits this pathway by inhibiting the phosphorylation of Mad, the effector Smad. However, the regulation of the I-Smad activity is not yet fully understood, neither in mammals nor in *Drosophila*. Previously, our lab already concluded that Gilgamesh, a plasma membrane-associated kinase, regulates Dad. Nonetheless, this regulation is not well characterized. In this thesis, we used Gish loss-of-function conditions to evaluate its effects in Dad's function and expression. The loss-of-function of Gish was performed through the RNAi technique and the CRISPR technique, for the purpose of evaluating which one had a stronger effect in silencing Gish. In addition, this regulatory mechanism was studied in two different tissues, eye and wing, to understand if it is conserved or tissue-specific. In Drosophila eye, Gish has an impact in Dad's function and expression, which is strongly observed both phenotypically and in the imaginal discs. In *Drosophila* wing, Gish also affects Dad function and expression, however, the impact is not as strong as in the eye. Comparatively, the CRISPR technique causes a stronger Gish loss-of-function.

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Index

Figure Index

Graphic Index

Abbreviations

Ago2	Argonaute
Apt	Apterous
Babo	Baboon
BMP	Bone Morphogenetic Protein
bp	base pairs
Brk	Brinker
°C	Celsius
Cas9	CRISPR-associated protein 9
Ck1	Casein kinase 1
Co-Smad	Common-Smad
CtBP	C-terminal binding protein
Dac	Dachshund
Dad	Daughters against dpp
DAPI	4′,6-diamidino-2-phenylindole
Daw	Dawdle
DNA	Deoxyribonucleic Acid
Dpp	Decapentaplegic
dsRNA	double-strand RNA
En	Engrailed
Ey	Eyless
Eya	Eyes absent
Eyg	Eyegone
Gbb	Glass-bottom boat
GFP	Green Fluorescent Protein
Gish	Gilgamesh
gRNA	guide-RNA
Hh	Hedgehog
I-Smad	Inhibitory Smad
Mad	Mothers against dpp
Mav	Maverick
MF	Morphogenetic Furrow
MH1	Mad Homology 1
MH2	Mad Homology 2
mRNA	Messenger RNA

Myo	Myoglianin
Pax6	Paired box 6
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with Triton X100
pMad	Phosphorylated Mad
PATs	Palmitoyl acyl-transferases
Put	Punt
RI	Type I receptor
RII	Type II receptor
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	Interference RNA
R-Smad	Receptor-activated Smad
Sax	Saxophone
Scw	Screw
Ser	Serrate
siRNA	Small interference RNA
Smo	Smoothened
So	Sine oculis
TGFβ	Transforming growth factor β
Tkv	Thickveins
Toy	Twin of the eyeless
Tsh	Teashirt
UAS	Upstream activating sequence
Vg	Vestigial
Wg	Wingless
Wit	Wishful thinking

1. Drosophila as a model organism

In general, model organisms are non-human species that are deeply analyzed, studied and experimented on to better comprehend many biological processes. Then, the acquired knowledge is extrapolated to more complex organisms (Leonelli & Ankeny, 2013).

Drosophila began to be used as a model organism by Thomas Hunt Morgan and his research group in 1910, at Columbia University (Roberts, 2006).

Many attributes make *Drosophila melanogaster* an excellent model organism such as its short life cycle, its ease of maintenance (Markow, 2015) and the possibility to generate a diversity of mutant fly lines (Baenas & Wagner, 2019). Additionally, *Drosophila* is currently used to study many human diseases like neurodegenerative disorders, cancer and kidney diseases because *Drosophila* has similar organ systems to mammals (Droujinine & Perrimon, 2016) and even some physiological conservation (Allocca et al., 2018). Plus, other characteristics make *Drosophila* great for the study of human diseases: the high level of homology, and lower redundancy, that its genome presents relatively to the human; the wide range of genetic tools available to easily manipulate *Drosophila* and the fact that are many signaling pathways in humans that are conserved in *Drosophila melanogaster* (Mirzoyan et al., 2019).

Drosophila has been used in different research areas such as genetic research, neuroscience, neurodevelopment (Stephenson & Metcalfe, 2013), cancer research (Mirzoyan et al., 2019) and nutrigenomics (Baenas & Wagner, 2019).

The different stages of *Drosophila melanogaster* development are important to deepen and better understand some questions about human development.

1.1. Drosophila life cycle

Drosophila has a short life cycle, which means that from fertilization until the generation of the adult fly it takes about 10 days at 25°C (Ong et al., 2015). If the temperature is lower the life cycle has a longer duration, and if it is higher the life cycle is shorter (Deepa Parvathi V, Akshaya Amritha S, 2009). The whole life cycle consists in four different phases: embryo, larva, pupa and adult fly (Ong et al., 2015). Firstly, the egg is fertilized, then, the embryo develops inside the egg for one day. Afterward, it hatches as a larva. Next, the larva develops in the course of,

approximately, five days (Jennings, 2011), going through three different larval stages (L1, L2 and L3) (Edgar, 2006) until its pupariation (Jennings, 2011). In the pupa, a phenomenon called metamorphosis occurs where the imaginal discs develop and give rise to adult organs (Fernández-Moreno et al., 2007).

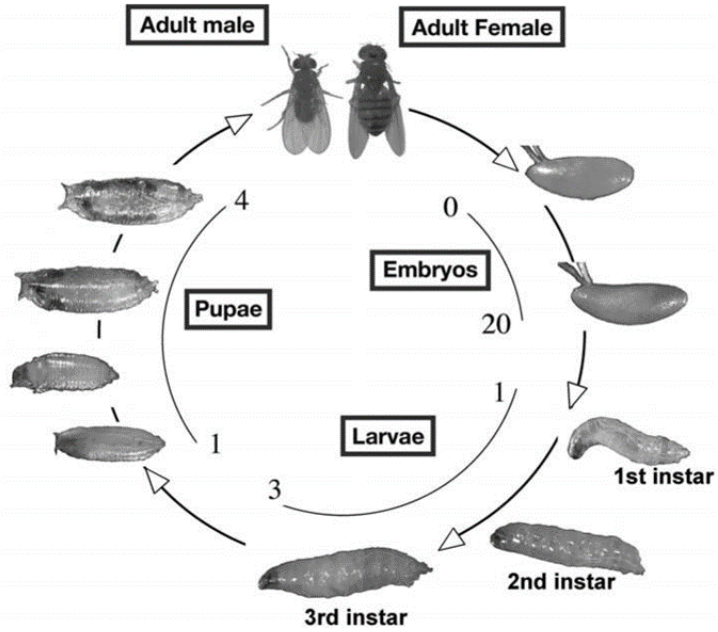


Figure 1 - Drosophila melanogaster life cycle. This cycle has a duration of about 10 days at 25°C and is comprised by 4 different main stages: embryo, larva (first instar, second instar and third instar), pupa and adult. Adapted from Fernández-Moreno et al., 2007.

2. Genetic tools

2.1. Gal4 system

The Gal4 system was created in order to allow the specific activation of a given cloned target gene (Southall et al., 2008) and is based on the yeast transcriptional activator Gal4 (the driver) and its target upstream activating sequence (UAS) which is the responder, therefore, one transgenic line expresses Gal4 and another distinct transgenic line embodies a UAS-dependent transgene (Elliott & Brand, 2008). Then, when two flies, one from each transgenic line, cross, the transcriptional activator Gal4 binds to the UAS, the target gene, in the target tissue, and the UAS-promoter is activated (Cho et al., 2014).

Initially, this system was designed in *Saccharomyces cerevisae*, with the aim of regulating transcription (Elliott & Brand, 2008). Nowadays, the Gal4 mechanism has several applications such as to prevent cellular function, express modified proteins (Southall et al., 2008), cell excision (Osterwalder et al., 2001) and the ectopic expression of any sequence of interest (Elliott & Brand, 2008).

There are many advantages in using the Gal4 system, such as the fact that this system is highly conserved between eukaryotic cells which signifies that the transcriptional activator Gal4 is able to induce transcription in numerous species. Additionally, when Gal4 is missing and the UAS is unexpressed, transgenics carrying apoptotic and toxic proteins can be originated (Elliott & Brand, 2008).

This system has several different applications, for instance, the rescuing of knockout mutations, the excessive expression of certain genes (Del Valle Rodríguez et al., 2012) and the knock-down of gene expression (Scialo et al., 2016) by the activation of RNAi cassettes. Moreover, Gal4 can also be used in gene repression when this protein's DNA binding domain links with the isolator domain of the suppressor of hairy wing (HW) (Southall et al., 2008). Therefore, Gal4 can be applied in both loss-of-function and gain-of-function studies, functioning a a chimeric protein (Elliott & Brand, 2008).

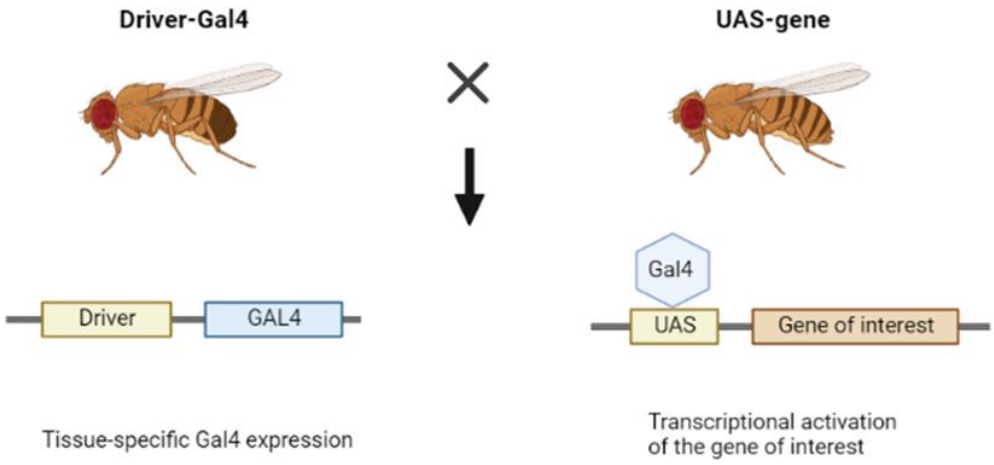


Figure 2 - UAS-Gal4 system. This system comprises two elements: one fly possesses, in its genome, the transgene that promotes Gal4 expression (the driver) and the other fly contains the UAS upstream to the transgene of interest. The progeny will express both the UAS and the Gal4 and the Gal4 protein will link to the UAS and, consequently, activate the expression of the transgene.

2.2. RNAi

The RNAi technology consists in the use of RNA interference which is created due to the degradation of mRNA promoted by complementary double-stranded small interfering RNAs (siRNA) (Xu et al., 2019) and it results in the repression of target gene expression (Zhou et al., 2003). Nowadays, this procedure allows the knockout of, approximately, 88% of *Drosophila*'s genes (Jennings, 2011).

This technique was first used in *Caenorhabditis elegans* and it was first applied in *Drosophila* as a gene silencing method in which the double-stranded RNA was inserted during early embryogenesis in the embryo (Heigwer et al., 2018). This procedure uses the Gal4-UAS system in order to generate gene fragments and clone them as inverted replicates and inactivate the target gene in specific tissues (Dietzl et al., 2007). These siRNAs are cleaved, by Dicer, into smaller fragments of 21 or 22 nucleotides. Then, these fragments associate with Ago2 (Argonaute protein) and the RISC (RNA-induced silencing complex) is formed (Song et al., 2022). The main advantage of using RISC is that it enables the silencing of any target acid nucleic sequence (Pratt & MacRae, 2009).

The junction of the Gal4-UAS system with the RNAi technique has certain advantages such as the vast heterogeneity of Gal4 lines which enables tissue or cell-type specific knock-down at any developmental phase (Kaya-Çopur & Schnorrer, 2019). Depending on the levels of Gal4 that the drivers express they are able to induce distinct silencing efficiencies in the same tissue (Yamamoto-Hino & Goto, 2013). We used this technique in some fly strains in order to study the function of Gish by analyzing what effects its silencing (loss-of-function) has in the expression and function of Dad.

2.3. CRISPR

The CRISPR/Cas9 system is a recent technique used in gene therapy (Uddin et al., 2020). The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) technology is a genome editing system and it has several applications such as gene deletions, gene insertions and gene replacements (Zhang & Showalter, 2020). Essentially, the CRISPR/Cas9 system can be used to find and slice specific target DNA regions (Ayanoğlu et al., 2020). This technique is composed of a guide RNA (gRNA), which is an extremely gene-specific sequence, and

the Cas9 endonuclease (Zhang & Showalter, 2020), which is able to cause double-stranded DNA breaks, thus, enabling genome editing (Redman et al., 2016). The gRNA has complementarity and associates with a specific target DNA sequence adjacent to the PAM (protospacer adjacent motif) sequence. The latter is necessary to direct Cas9 to its target sequence. The gRNA and Cas9 bind thereby forming a complex and gRNA and, then, Cas9 does a double-stranded cleavage 3 bp before the PAM sequence (Zhang & Showalter, 2020).

In *Drosophila melanogaster*, the CRISPR technique can be used in a tissue-specific way and, by using the UAS-Gal4 system, Drosophila is able to express Cas9 in any target tissue (Meltzer et al., 2019).

One of the advantages of using CRISPR to do the knock-down of a certain gene is that this technique actually promotes the complete knockout of that gene as opposed to the RNAi technique which only allows partial knockdown (Meltzer et al., 2019).

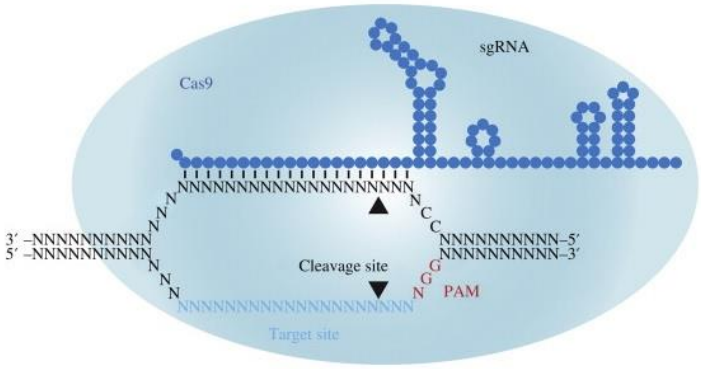


Figure 3 - CRISPR/Cas9 technique. Cas9 is directed to its target sequence by the PAM motif and it does a double-stranded cleavage in its target.

3. Imaginal discs

Imaginal discs are formed by epithelial tissue (de Celis & García-Bellido, 2013) and there are nineteen of them (Aldaz & Escudero, 2010). Throughout the development of the embryo, assembles of ectodermal cells, proliferate, grow and give rise to the imaginal discs (de Celis & García-Bellido, 2013) through invagination of the ectodermal membrane (Aldaz & Escudero, 2010). These discs are composed by two layers, the

squamous layer which refers to the peripodial membrane, and the columnar layer which corresponds to the disc proper (Pallavi & Shashidhara, 2005).

There are many advantages that make the imaginal discs great for biomedical studies, such as their acessibility and their similarity with the epithelial cells that cover the majority of human organs (Beira & Paro, 2016).

In this work, it was used and studied two types of imaginal discs: the eye imaginal discs and the wing imaginal discs.

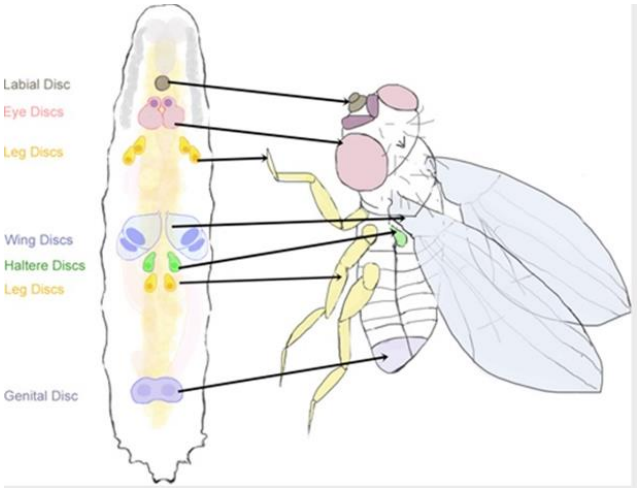


Figure 4 - Representation of the main imaginal discs. They are sac-like structures that give rise to the adult structures. Adapted from Jaszczak & Halme, 2016.

3.1. Eye development

The evolution of science allowed the development of several genetic tools to study, in more depth, the *Drosophila*'s eye imaginal discs, it being one of the many particularities that make its use, for cell growth and differentiation studies, very beneficial. Also, their accessibility and the fact that the eye is composed, mostly, by epithelial tissue, which corresponds to the same cell type that is part of a lot of human organs, therefore, this makes the imaginal discs a strong weapon to be used in further biomedical research (Beira & Paro, 2016).

The *Drosophila* compound eye is composed of, approximately, 750 subunits called ommatidia (Tsachaki & Sprecher, 2012). Each ommatidium contains eight photoreceptor neurons (Karpilow et al., 1996), four non-neuronal cone cells and two pigment cells (Cagan, 2009).

The eye imaginal discs, which are composed by a monolayer of epithelial cells (Spratford & Kumar, 2014) that invaginate from the dorsal pouch and originate a pair of eye-antennal discs, during embryogenesis (Domínguez & Casares, 2005).

During the three larval stages, different genes are expressed in order to do the specification of the imaginal discs. This expression starts by the signaling originated by the Hox genes, which, in turn, activate hedgehog (hh), wingless (wg) and decapentaplegic (dpp) (Beira & Paro, 2016). In the first instar, two Pax6 genes homologs are expressed: eyeless (ey) and twin of the eyeless (toy) (Domínguez & Casares, 2005), which are vital for the development of the eye of Drosophila (Jacobsson et al., 2009), stimulating cell growth and division (Czerny, Halder et al. 1999). Both of these genes are vital for the development of the eye, since, on one hand, the absence of ey leads to adults with loss-of-phenotype in the eye and, on the other hand, toy promotes ey expression (Domínguez & Casares, 2005).

Then, during the second larval stage, cells continue to proliferate and it begins to be possible to discern two different regions: the disc proper and the peripodial membrane, composed by columnar and squamous cells, respectively (Aldaz et al., 2010). Afterward, at the end of the second instar, another group of genes (early retinal genes, which are eya, so and dac) start to being expressed (Domínguez & Casares, 2005). During the third instar, the morphogenetic furrow, which moves from the posterior to the anterior area of the imaginal disc, is an indicator of differentiation since anterior to the morphogenetic furrow cells are undifferentiated (Bonini, 1997) and, as the furrow progresses, it creates changes in the shape of the cells and transient cell cycle arrest (Baker et al., 2014). Therefore, posterior to the morphogenetic furrow, cells sustain a series of differentiation processes, promoted by hh, in order to become adult ommatidial cells (Bonini, 1997).

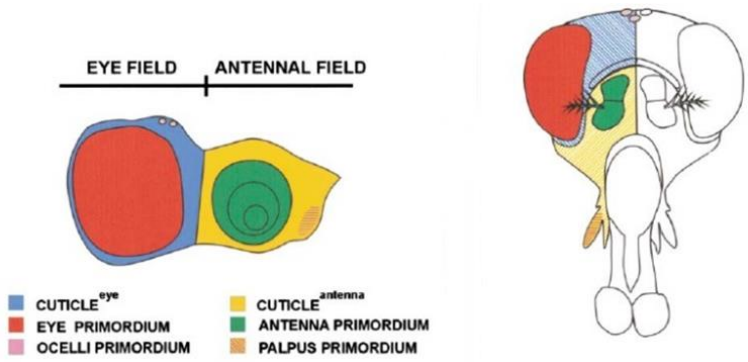


Figure 5 - Correspondence between the components of the eye-antennal imaginal disc and the adult head of *Drosophila*. They eye-antennal imaginal disc generates, in the adult fly, the eye, the antenna, the palpus and the cuticle of the head. Adapted from Kenyon et al., 2003.

3.1.1. Retinal determination genes

The retinal determination network of *Drosophila* corresponds to a group of genes that are involved in many cellular processes such as the coordination of cell proliferation rates, regulation of the initiation and progression of the morphogenetic furrow and specification of cell fate (Kumar, 2010). The retinal determination genes are: *optix*, *eyegone* (*eyg*), *eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*) (Hanson, 2001) and their activation is regulated by both *ey* and *toy* (Halder et al., 1998) which are selector genes (Silver & Rebay, 2005) also known as the fruit fly Pax6 genes (Zhu et al., 2017). *ey* and *toy* are very similar, although, they present a strong difference in their C-terminal, which causes *ey* to be a more powerful transcriptional factor than toy (Weasner et al., 2009) and also gives rise to *ey* and *toy* having different target genes (Punzo et al., 2004). *toy* is able to directly control the expression of *ey* which initiates the eye development process (Punzo et al., 2004), however, *toy* can also promote the formation of ectopic eyes even in the absence of *ey*, which makes us conclude that these two genes play redundant roles in the formation of the eye and they both can partially substitute each other (Jacobsson et al., 2009).

This network is expressed anterior to the morphogenetic furrow and prior to the beginning of the differentiation of the retina (Halder et al., 1998). All of these genes were identified as vital for the correct formation of the retina because they can promote the formation of ectopic eyes in other tissues besides the eye (Firth & Baker, 2009), for example, *optix* is able to induce the formation of ectopic eyes in the antenna (Seimiya & Gehring, 2000) and *ey* is also able to do the same in other tissues (Baker et al., 2018). Plus, the depletion of *toy* results in the loss of the adult compound eye (Blanco et al., 2010). All of these results are indicators that these genes are required for the precise formation of the retina.

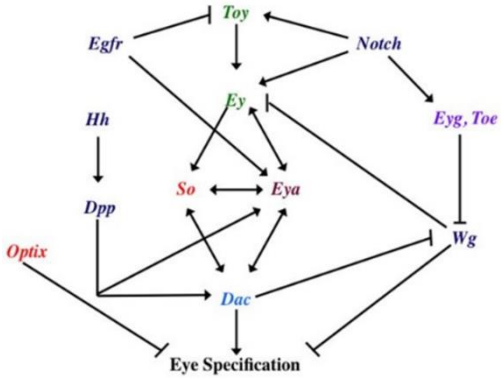


Figure 6 - Retinal determination network. These genes interact with each other through positive and negative regulation. Adapted from Weasner et al., 2004.

3.1.2. Early retinal genes

The main genes involved in retinal differentiation are: *so*, *eya* and *dac* (Silver & Rebay, 2005) and they begin to be expressed during the late second larval stage, in the posterior domain of the eye (Domínguez & Casares, 2005). The expression of these genes is induced by *ey* (Shen & Mardon, 1997). The expression of dac begins at the anterior region of the eye imaginal disc and, then, progresses to the posterior area (Mardon et al., 1994).

All of these genes are able to regulate *ey*, for instance, *eya* suppresses *ey* in the cells posterior to the morphogenetic furrow, *dac* has the ability to repress *ey* inside the morphogenetic furrow in early stages of development and *so* blocks *ey* expression posterior to the furrow and promotes its expression anterior to the morphogenetic furrow, depending on *so* binding site (Atkins et al., 2013).

eya and *dac* are able to activate *ey* and induce ectopic eye formation in other tissues that are not the eye (Yang et al., 2009), however, *so* alone is not capable of inducing ectopic eye formation (Silver & Rebay, 2005). When *dac* and/or *so* are expressed together with *eya*, the ectopic eye formation is stronger than with *eya* expression alone (Davis & Rebay, 2017). Plus, *eya* is enough to promote ectopic eye formation (Jemc & Rebay, 2007). The expression of *eya* and *so* is promoted by *ey*, however, the first ones are also able to control each other's expression (Pignoni et al., 1998). A study using dpp loss-of-function mutants, showed that *eya* is able to rescue the adult eye phenotype and its activity is independent of *dpp*, which indicates that *dpp* functions upstream of *eya* (Curtiss & Mlodzik, 2000). In addition, the dissipation of both *eya* and *so* results in the loss of the adult eye (Bonini, 1997). Moreover, *dac* also plays a role in the movement of the morphogenetic furrow, since it was shown that the depletion of this gene ahead of the furrow results in cell death (Bonini, 1997).

dpp and *hedgehog* also have a key role in retinal differentiation, thus, they can be considered as important as *eya*, *so* and *dac* for the correct differentiation of the retina in *Drosophila*. *hedgehog* signaling is activated by the expression of *dac* and, in turn, it activates *dpp* (Brás-Pereira et al., 2016). Although Hh and Dpp pathways do not control *ey* expression, they are vital for the expression of the early retinal genes. Mutations on either of these signaling pathways affects the progression of the morphogenetic furrow, however, mutations on both of them, stop the progression of the furrow and inhibit eye differentiation, which means that they have partially redundant functions in eye development (Curtiss & Mlodzik, 2000). As noted before, both *hedgehog* and *dpp* are equal stakeholders in the progression of the morphogenetic furrow. Hedgehog is in charge of activating *dpp*'s expression and initiating differentiation by stimulating the

progenitor cells posterior to the furrow to start a series of differentiation processes. *Dpp*, then, begins to be expressed by cells in the posterior boundary and lateral margins of the eye imaginal disc, before the movement of the morphogenetic furrow, however, its expression is maintained as the furrow progresses through the eye field (Bonini, 1997).

3.1.3. Eye differentiation and its regulation

Eye differentiation is a process where cells differentiate from posterior to anterior region (Treisman, 2013). The first genes involved in the retinal differentiation to be expressed are *wingless* (*wg)* and *dpp*, which are expressed since the first instar and because, at that point, the discs are considerably small, cells receive both signals. *Dpp* is expressed along the anterior-posterior boundary and *wg* is expressed at the dorsal-ventral boundary. However, they have opposite actions: *dpp* allows the activation of the early retinal genes while wg inhibits this activation. As both of these genes are expressed so prematurely, the onset differentiation is prevented (Beira & Paro, 2016) because *wg* can repress *dpp* (Estella & Mann, 2008). Then, during the second instar, *wg* is expressed in order to prevent the expression of *eya* by *dpp*. However, when the eye disc grows, in the third instar, and the posterior cells became out of the *wg* range of action, *wg* is confined to the anterior lateral margins (Legent & Treisman, 2008). *Wg* and *dpp* also regulate the expression of *Hth* (*Homothorax*) which, along with *ey* and *tsh* (*Teashirt*) maintains the proliferation of cells and prevents premature eye differentiation. Plus, when these three genes are activated at the anterior region of the eye disc, *eya*, *so* and *dac* are repressed (Domínguez & Casares, 2005). As the differentiation carries on, *Hth* and *Tsh* are restricted to the most anterior region of the eye disc. Therefore, a pre-proneural domain is formed anterior to the morphogenetic furrow. This domain is determined by the loss of *Hth* and the presence of *eya*, *so* and *dac* (Legent & Treisman, 2008) in order to delimit the region between the undifferentiated cells and the pre-proneural cells (Beira & Paro, 2016).

The formation of the morphogenetic furrow (MF) is promoted by high levels of *dpp* at the posterior region of the eye disc, which represses *wg* (Silver & Rebay, 2005). The morphogenetic furrow progresses from the posterior region of the eye disc to the anterior margin (Frankfort & Mardon, 2002). Posteriorly to the MF, a row of newly-formed R8 cells is present (Greenwood & Struhl, 1999). *Dpp* expression occurs only from a group of cells moving along with the MF. Firstly, during the first instar, *dpp* is expressed at the ventral margin, then, at the beginning of the second

instar, *dpp* is expressed along the posterior and lateral margins. However, at this stage, *dpp* activates *hh* which is involved in the MF progression (Sarkar et al., 2018). Hh is expressed in the MF and behind it (Treisman & Heberlein, 1998) and is able to cross the anterior-posterior boundary in order to promote the expression of *dpp* (Beira & Paro, 2016). *Dpp* also is involved in the repression of *hth* (Lopes & Casares, 2010).

When the MF progresses, R8 cells (atonal-expressing cells) recruit the other photoreceptors (R2, R3, R4 and R5) by the activation of the EGF receptor pathway (Domínguez & Casares, 2005).

The separation between *dpp* and *wg* signals is implemented by Notch, at the beginning of the second instar, when Notch promotes cell proliferation when *wg* levels are high. Notch is activated at the dorsal-ventral compartment of the eye disc (Domínguez & Casares, 2005).

Another important gene to refer is *engrailed* (*en*) which is expressed in all cells in the posterior region which allows the establishment of the anterior-posterior boundary. Plus, *en* directs the expression of *Hh* (Beira & Paro, 2016).

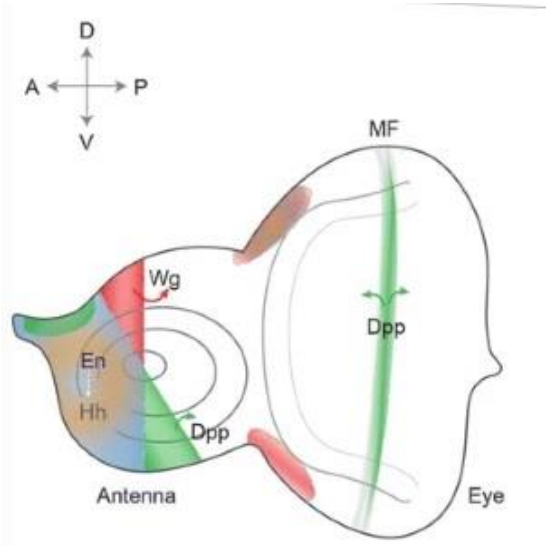


Figure 7 - - Domains of expression of the morphogenes involved in eye differentiation. *En* guides the expression of *Hh* which, in turn, induces *Dpp* expression. *Dpp* and *wg* mutually repress each other. *Dpp* is expressed along the A-P boundary and *wg* is expressed at the dorsal-ventral border. Adapted from Beira & Paro, 2016.

3.1.4. Gilgamesh (Gish)

Gilgamesh (gish) is the ortholog of human casein kinase 1 γ (CK1-γ) in *Drosophila* (Gault et al., 2012). CK1 is highly conserved and is involved in many cellular processes such as DNA-repair, cell proliferation and differentiation, apoptosis and vesicular trafficking (Knippschild et al., 2014). Gish is a protein associated with the plasmatic membrane (Nerusheva et al., 2008) and it is involved in glial cell migration, sperm partitioning, the regulation of polarized membrane trafficking (Gault et al., 2012) and cell proliferation (Li et al., 2020). Additionally, this protein plays a key role in various signalling pathways such as the Wingless signalling pathway (Han et al., 2014). The migration of glial cells in *Drosophila* is advanced when mutations occur in the locus of Gilgamesh, therefore, some level of disorganization of the glial cells happens anteriorly on the eye disc as the R cell axons lengthen (Hummel et al., 2002).

Being a cinase, Gish interacts with other proteins such as the protein Expanded, promoting its phosphorylation and depletion (Fulford et al., 2019).

3.2. Wing development

Just like any other appendage, the *Drosophila* wing is obtained from the imaginal discs, which are sac-like structures derived from epithelial tissue (Ruiz-Losada et al., 2018) and precursors of many of *Drosophila*'s adult organs (Aldaz et al., 2010). More specifically, the wing imaginal discs appears during embryogenesis from an agglomeration of, approximately, thirty cells in the second thoracic segment. Then, this group of cells invaginate and proliferate in a high rate in order to originate a mature wing disc, by the end of the third instar (Tripathi & Irvine, 2022). Just like the eye imaginal disc, in the wing imaginal disc, by the end of the third larval stage, two different surfaces can be distinguished: the peripodial membrane, which is thinner, and the disc epithelium, which is thicker. The latter is highly important for the wing development since it has a key role in the development of the wing hinge and blade (Blair, 2007). During pupal stages, the cells of the wing imaginal discs experiences differentiation and the adult wing and notum emerges (Tripathi & Irvine, 2022). Also, throughout the pupal stages, occurs the evertion of the disc epithelium and the apposed dorsal and ventral epithelia of the wing blade are formed (Blair, 2007).

The majority of the wing disc is composed by epithelial cells, however, it also has neurons, glia, tracheal and muscle precursors cells (Tripathi & Irvine, 2022).

The thoracic appendage primordia (wing, haltere and legs) are limited to their corresponding thoracic segments due to the inhibition of the abdominal Hox proteins (Ruiz-Losada et al., 2018).

Like in every imaginal disc, there are two separate areas: the anterior and the posterior compartments, which are originated from two distinct cell lineages. This responsibility is assigned to the *engrailed* (*en*) gene which is a selector gene, in other words, segregates anterior and posterior compartment cells because its activity in the posterior compartment makes the cells in that area secrete some adhesion molecules that make them unmixable with the cells in the anterior compartment. In the posterior section, *en* activates *hedgehog* (*hh*). This last gene produces a protein that has no action in the posterior compartment, however, it can progress across the dorsal-ventral barrier to the anterior area. Here, this protein induces the expression of *wg* and *dpp* (Morata, 2001) which are two morphogens that are responsible for the development and patterning of the wing (Parker & Struhl, 2020). Moreover, in order to define the dorsal-ventral border, it is also necessary the *apterous* (*ap*) gene which, in turn, activates the gene *fringe* (*fng*) and the ligands Serrate and Delta that trigger the Notch signaling. Finally, Notch activates vestigial (vg) and wg at the dorsal-ventral barrier (Morata, 2001).

This signaling pathways and genes all have their specific roles in the development of the wing. For example, the Dpp signaling pathway is largely responsible for controlling the growth of the wing disc. dpp is expressed by a band of cells next to the anterior-posterior border, thus, creating a gradient in both anterior and posterior directions (Aegerter-Wilmsen et al., 2007). Notch, in turn, is vital for maintaining the lineage segregation in the dorsal-ventral border (Morata, 2001).

Drosophila melanogaster, like all insects, has veins in its wings, which are cuticular structures that differentiate in specific patterns in the wings of insects (De Celis, 2003). The veins in the wing are formed by different signaling pathways like Notch (Johannes & Preiss, 2002), BMP, EGF, Hedgehog and Wnt (Blair, 2007). The veins are visible on both surfaces of the wing and they are grouped into the following categories: five longitudinal veins (L1 to L5), two smaller abbreviated veins (L0 and L6), three crossveins (the anterior, posterior and the humeral crossveins), and a vein that is located along the anterior margin. The wings also have intervein cells, although the they disappear just after the flies come out of the pupal cases. On one hand, the development of the longitudinal veins is controled by both EGFR signaling and BMP signaling. Hedgehog signaling also participates in the formation of proveins: firstly, interveins are specified by high levels of Hh, then, they emit a signal to the adjacent

that will promote the genesis of proveins. On the other hand, BMP signaling also intervenes in the development of the crossveins and the Wingless signaling has a key role in the formation of the marginal veins (Blair, 2007).

The wing development involves multiple genes and pathways that are, even now, being more intensively studied in order to be fully understood, like the case of *wingless*.

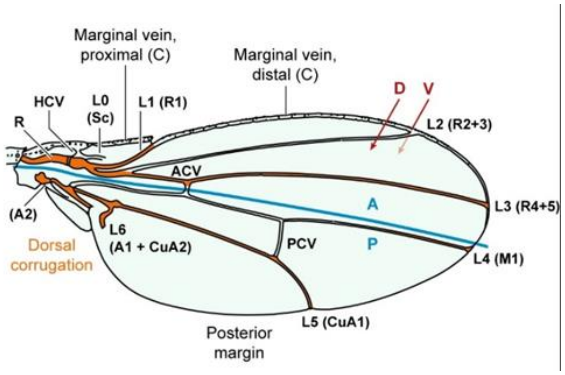


Figure 8 - Representation of the wing veins. L0-L6 correspond to the longitudinal veins, ACV to the anterior crossvein, PCV to the posterior crossvein and HCV to the humeral crossvein. Adapted from Blair, 2007.

3.2.1. Wingless pathway

The Wingless (Wg) signaling pathway, the mammalian Wnt oncoproteins ortholog (Bejsovec, 2018), is involved in different aspects of *Drosophila* development such as patterning, tissue growth and cell proliferation. This pathway, during embryogenesis, alongside *Hedgehog*, plays a key role in patterning and segmentation of the embryo (Swarup & Verheyen, 2012). Also, Wg signaling is involved in the development of multiple adult structures such as legs, antennae, genitalia, wing and eye imaginal discs (Jenny & Basler, 2014).

wg is a morphogen, therefore, it is produced by cells and, then, distributed along its target tissues. Hereafter, a concentration gradient is formed and this gradient instigates the expression of its target genes in a concentration-dependent manner (Hatori et al., 2021). *wg* and *dpp*, both morphogens, regulate each other. For instance, if the expression of *dpp* is high in the wing, it will lead to a lowering of *wg* expression in the scutellum region (Morimura et al., 1996). Moreover, Dpp signaling is vital for the expression of *wg* target genes (in the wing pouch area) as well as the expression of wg

itself, directly or indirectly, via Mad. Mad, the main transducer of Dpp signaling, is also involved in the Wingless signaling, by repressing it (Li et al., 2020). Consequently, one of *dpp*'s functions is to diminish or to even obstruct the expression of *wingless* (Morimura et al., 1996).

In the wing imaginal disc, *wg* is expressed in two different regions called outer ring and inner ring in the hinge, in the dorsal-ventral boundary of the wing blade and in the dorsal area of the notum (Swarup & Verheyen, 2012). *wg* progresses from the dorsal-ventral border forward in order to pattern the cells in both dorsal and ventral sections in a concentration-dependent manner, because it is a morphogen, as already said above. However, the range of wg action depends of the target gene in question. For example, if it is *Distal-less*, *wingless* has a short-range action, but, if it is *optomotor blind*, *wg* acts long-range (Howes & Bray, 2000).

During the second instar, *wg* is expressed at the ventral area of the wing imaginal disc in order to specify the wing field, while vein (a EGFR ligand) is expressed at the dorsal area to specify the notum. *Vein* restricts the *wg*'s expression to the ventral region and, on the other hand, *wg* confines *vein*'s expression to the dorsal region (Swarup & Verheyen, 2012).

In the dorsal region, *vein* induces the expression of *apterous* (ap) which, in turn, activates Notch along the dorsal-ventral boundary. Afterwards, Notch induces the expression of *vestigial* (*vg*) which has two main functions at the wing imaginal disc: specificies the wing blade and, together with Notch, promotes the expression of *wg* at the dorsal-ventral border. Then, being a morphogen, *wg* activates its target genes in a concentration-dependent manner in order to pattern the wing margin. At the third larval stage, the wing blade starts to grow and *vg*'s expression is promoted in cells that do not belong in the Notch signaling domain by its quadrant enhancer (vgQE), which activity depends on *vg*, *wg* and *dpp* (Swarup & Verheyen, 2012).

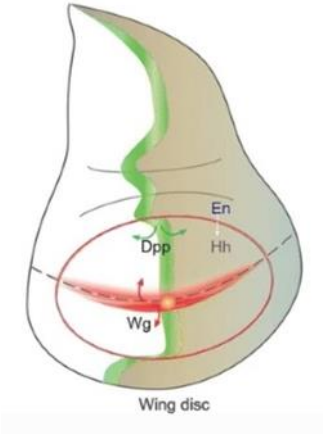


Figure 9 - Wing imaginal discs and its morphogenes expression domains. *Dpp* is expressed alongside the A-P border and *wg* is expressed along the D-V boundary. Adapted from Beira & Paro, 2016.

4. TGFß signalling pathway

The transforming growth factor-ß (TGF-ß) consists in a superfamily that plays a key role in numerous cellular processes including cell differentiation, cell proliferation and apoptosis (Hamaratoglu et al., 2014). The TGFß pathway is present in both vertebrates and invertebrates (Raftery & Sutherland, 1999), such as *Drosophila*, and is conserved in animals (Upadhyay et al., 2017). This pathway can be divided in two main branches: the Activin branch and the BMP branch (or Dpp branch) (Ozakman & Eleftherianos, 2019) and is activated by specific ligands, which are different in both branches. In Drosophila, on one hand, the BMP branch has bone morphogenetic proteins as ligands (Upadhyay et al., 2017) that are: Decapentaplegic (Dpp), Glass-bottom boat (Gbb), and Screw (Scw). On the other hand, the Activin branch has the following ligands: Activin-ß (Actß), Dawdle (Daw), Myoglianin (Myo) and Maverick (Mav) (Ozakman & Eleftherianos, 2019).

This pathway is initiated when a ligand binds to a type II receptor, which are the same in both branches- Punt (Put) or Wishful Thinking (Wit)- causing its phosphorylation, in both BMP and Activin branches (Upadhyay et al., 2017). Once phosphorylated, that type II receptor binds to type I receptor, which is, then, activated. (Yu et al., 2002). In the BMP branch, these type I receptors are Tkv and Sax and in the Activin branch there is only one- Baboon (Babo) (Upadhyay et al., 2017). Afterward, the type I receptor promotes the phosphorylation of its specific receptor-activated Smad (R-Smad) (Peterson & O'Connor, 2014) which binds with the common Smad Medea (co-Smad) giving rise to a complex. Lastly, this complex is translocated into the nucleus, where the expression of distinct target genes is modulated (Upadhyay et al., 2017).

The last two serines of the SSXS motif of the R-Smads, which is located at their C-terminal, are phosphorylated (Chen et al., 2006) by the type I receptors (Van de Laar & Loeys, 2017).

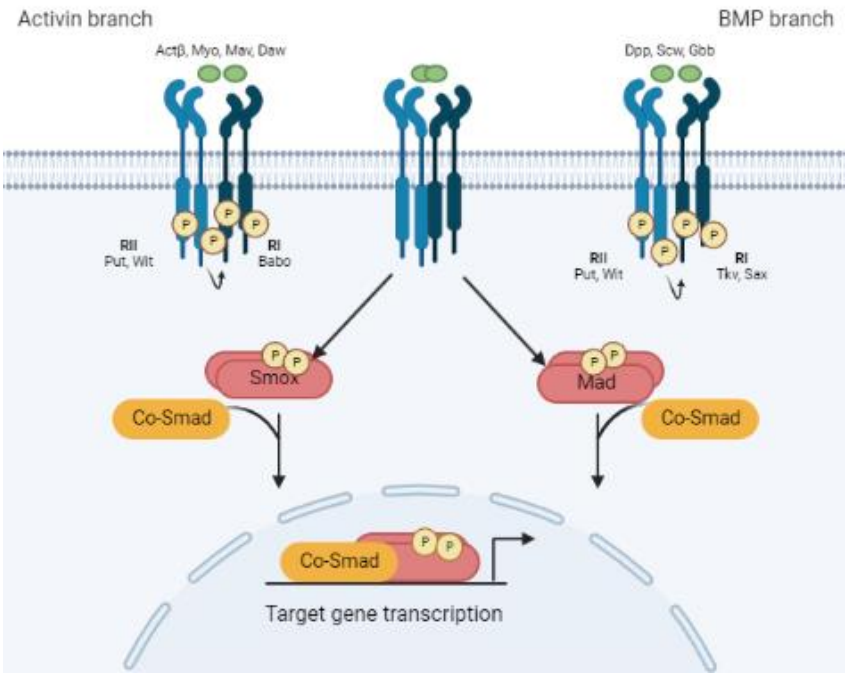


Figure 10 - TGFß signaling pathway. This pathway is divided into two branches: the Activin branch and the BMP branch. The ligands of the Activin branch are Myoglianin (Myo), Dawdle (Daw) and Activin-ß (Actß) while the ligands of the BMP branch are Decapentaplegic (Dpp), Glass-bottom boat (Gbb) and Screw (Scw).

4.1. Activin branch

Although the Activin pathway is not yet as well understood as the BMP branch, it is still very important during development, in *Drosophila*, in processes such as cell growth, neuronal function (Parker et al., 2004), cell proliferation and apoptosis (Chen et al., 2002) and cellular metabolism (Ghosh & O'Connor, 2014). For instance, a study that used mutants in which the Activin pathway was eliminated, showed that that deletion gave rise to smaller larvae (Kim & O'Connor, 2021). Another set of experiments from our lab demonstrated that salivary glands that contain low levels of Put, display tissue undergrowth (Martins et al., 2017). Besides that, it is also known that an Activin pathway ligand, Activin-ß, is expressed by peripheral nervous system neurons and it leads to hemocyte proliferation (Makhijani et al., 2017).

In *Drosophila melanogaster*, this branch has a specific R-Smad called Smox (Santos et al., 2016) which is the ortholog of Smad2 and Smad3 (Brummel et al., 1999).

In Drosophila, the growth and patterning of the wing disc require the involvement of the vertebrate homologous of R-Smad2 and Smad3, Smad2, since the depletion of Smad2 caused deformation of wing discs (Peterson & O'Connor, 2013). Additionally, a further study revealed that only when the expression of Smad2 in the wing discs is very low is Baboon able to phosphorylate Mad (the transducer of the BMP branch), or else,

when the expression of Smad2 in wing discs is elevated, Baboon can not phosphorylate Mad, which leads to BMP loss-of-function phenotypes. Plus, the depletion of Smad2 can diminish the growth of the wing blade. Thus, it can be inferred that Mad's activation can be controlled by Smad2 expression levels (Hevia & de Celis, 2013)- this refers to the cross-talk between the two branches of the TGFß signalling (Upadhyay et al., 2017).

4.2. BMP branch

Dpp is the most important morphogen in the BMP family and it is the *Drosophila* ortholog of vertebrate BMP-2/4 (Setiawan et al., 2018). Dpp is crucial for the development of *Drosophila* since it has numerous crucial functions such as specifying the position of wing veins, stimulating tissue growth in the wing, regulating the expression of target genes (Bosch et al., 2017), patterning of the embryo and the imaginal disc (Upadhyay et al., 2017). Also, it plays a key role in tissue homeostasis (Hamaratoglu et al., 2014) and it promotes, during embryogenesis, the dorsal-ventral patterning (Raftery & Sutherland, 1999).

The Co-Smad orthologous to the Medea, in vertebrates, corresponds to the Smad4 (Goldstein et al., 2011) and Mad is the *Drosophila* ortholog of Smad1/5 in vertebrates (Urrutia et al., 2016).

As *dpp* is a morphogen, that means that it disperses from a specific place (Matsuda et al., 2021) and, therefore, can regulate the expression of target genes in a concentration-dependent manner (Bosch et al., 2017). This *dpp* gradient is formed because of its fast diffusion through the extracellular space (Strigini & Cohen, 2000). Although *dpp* and *wg* are both morphogens, their dispersal occurs in very distinct ways. *Dpp* dispersal takes place along the anterior-posterior axis, in a band of cells situated anteriorly of the A-P axis, creating a gradient in both sections that is responsible for wing patterning and growth (Matsuda et al., 2021). The genes whose expression *dpp* regulates are: *optomotor blind* (*omb*), *spalt* (*sal*), *daughters against dpp* (*dad*) and *brinker* (*brk*), although, *brk* also has the capacity to repress *dpp*'s vertebrate ortholog, BMP-4 (Minami et al., 1999), because *brinker* can negatively modulate Dpp-dependent genes by binding itself to them (Schwank et al., 2008). However, a study showed that *brk* can, also, regulate dad when the BMP signaling is inactive (Weiss et al., 2010).

Various studies proved that *Dpp* is directed conected to wing disc size since the wing discs that presented high Dpp signaling were the same discs were tissue overgrowth was observed (Restrepo et al., 2014). Another experiment showed that

TkvQ253D , an activated Dpp receptor, quickened cell proliferation (Martín-Castellanos & Edgar, 2002). On top of that, *dpp* levels are linked to the width and length of the wing (Barrio & Milán, 2017).

Now, focusing only on the eye, other experiments demonstrated that BMP signaling in the eye disc epithelium is more powerful than in the peripodial epithelium (Firth et al., 2010). Plus, an ectopic *dpp* expression is capable to, by itself, promote the progression of the morphogenetic furrow in the eye imaginal discs and originate a duplicated eye disc (Pignoni & Zipursky, 1997). Furthermore, *dpp* together with *hedgehog* control glial migration in the eye discs (Velarde et al., 2021). Therefore, we can conclude that the BMP signaling is essential for numerous vital cell processes, including in the eye discs, in *Drosophila*.

5. I-Smads

5.1. Structure and inhibition of TGFß signalling

TGF-ß signaling pathway has several negative regulators called inhibitory Smads (I-Smads). In mammals, these I-Smads are Smad6 and Smad7 (Hariharan & Pillai, 2008). I-Smads have a MH1 (N-terminal Mad Homology 1) domain, which has the ability to bind to DNA, and a MH2 (C-terminal Mad Homology 2) domain, which does not connect to DNA but instead with proteins (Attisano & Lee-Hoeflich, 2001), and these two domains are separated by a less conserved linker region (Nakayama et al., 2001). Only their MH2 domain is conserved between I-Smads (Miyazawa & Miyazono, 2017) and their MH1 domain is very divergent (Mochizuki et al., 2004) since Smad6 and Smad7 have a 36,7% partial conservation in this domain (Hanyu et al., 2001). In R-Smads, the MH1 and MH2 domains interrelate in order to mutually repress each other, however, this inhibition is blocked when the receptor is phosphorylated (Hanyu et al., 2001).

I-Smads suppress Dpp signaling by binding to the intracellular domain of activated type I receptors of this pathway, thus, blocking the activation of R-Smads by these receptors, inhibiting the formation of the complex constituted by the R-Smad and the Co-Smad and, consequently, the translocation of this complex to the nucleus (Nakayama et al., 2001). The MH1 domain has very different functions like nuclear translocation and the inhibition of the MH2 domains (Miyazono & Shimanuki, 2008). Also, this same region is important for the inhibition of BMP signaling in Smad6 (Lin, Liang et al. 2003) and, in Smad7, plays a key role in the inhibition of TGFß signaling (Itoh, Landström et al. 1998). However, the MH2 domains are, also, very important for

the roles that the I-Smads play since, in both Smad6 and Smad7, it is indispensable for the inhibition of BMP and TGFß signaling (Hanyu, Ishidou et al. 2001).

These I-Smads regulate the TGF-ß pathway through negative loops (Yan et al., 2009). Smad6 preferably only inhibits the BMP branch (Miyazawa & Miyazono, 2017), by competing with the R-Smad for type I receptors (Li et al., 2017) while Smad7 inhibits both BMP and Activin branches (Miyazawa & Miyazono, 2017). The inhibition by Smad7 is a more complex process because this Smad prevents the complex, constituted by the R-Smad and the type I receptor, to bind with their target DNA, by binding to that same DNA, therefore, it does not induce the expression of its target genes (Li et al., 2017).

The association between type I receptors and R-Smads can be blocked by different mechanisms. Firstly, BAMBI, which is a transmembrane protein with an extracellular domain close to the type I receptors but without the intracellular kinase domain, is able to repress the connection between type I receptors and the R-Smads, thereby, preventing its phosphorylation. (Miyazawa & Miyazono, 2017). Additionally, when I-Smads cooperate with Smurf (a Smad-interacting ubiquitin ligase), the receptors suffer ubiquitination and proteasomal degradation (Katakawa et al., 2016). Plus, Smurf1 promote the ubiquitination and degradation of Smad1/5 and type I receptors by the mediation of Smad6. (Zhang et al., 2007).

Smad6 intervenes in other processes of inhibition of TGF-ß through I-Smads. For instance, Smad6 is also able to associate with phosphorylated Smad1 and block the formation of the complex between R-Smad and Co-Smad. Ultimately, Smad6 also acts in the nucleus, where it enlists co-repressors like CtBP and histone deacetylase and transcriptional repressors in order to prevent the transcription of target genes (Zhang et al., 2007). These co-repressors associate with Smad6 in its PxLDL domain (Lin et al., 2003).

In *Drosophila*, Dad is the only Inhibitory Smad, being the orthologous of Smad6 and Smad7 (Kamiya et al., 2008) and regulates cell growth and differentiation (Djike & Hill, 2004). Dad is a 568 aminoacid Smad protein that has a MH1 domain, a linker domain and a MH2 domain (Figure X). Dad's N-terminal is rich in proline residues, therefore, it may be a very interesting region to study since it might be important for many intermolecular interactions such as cell-cell communication, signal transduction and cytoskeletal organization (Williamson, 1994, Ball et al., 2005). These proline-rich regions are involved in the formation of complexes between different proteins as a consequence of phosphorylation (Williamson, 1994).

Dad's expression is regulated by the TGF-ß signaling pathway (Dijke & Hill, 2004). However, Dad is also able to regulate negatively this pathway and, more specifically, the BMP branch (McCabe et al., 2004) through two different mechanisms: by associating itself with membrane proteins in a palmitoylation-dependent manner (W. Li et al., 2017) and by inhibiting the phosphorylation of Mad (H. Li et al., 2016). Dpp signaling is negatively regulated by Dad (Tsuneizumi et al., 1997) in two distinct ways: Dad can either inhibit Mad activation by blocking its phosphorylation by Tkv (Inoue et al., 1998) or Dad can supress the translocation of Mad to the nucleus (Gilbert, 2012).

Another important protein involved in TGF-ß inhibition is Gliotactin, whose overexpression activates Tkv and Mad and, consequently, leads to cell death and cell migration. Tkv's activation occurs due to reclusion of Dad. Plus, a decrease in Dad's activity promotes activation of Mad (Sharifkhodaei & Auld, 2021).

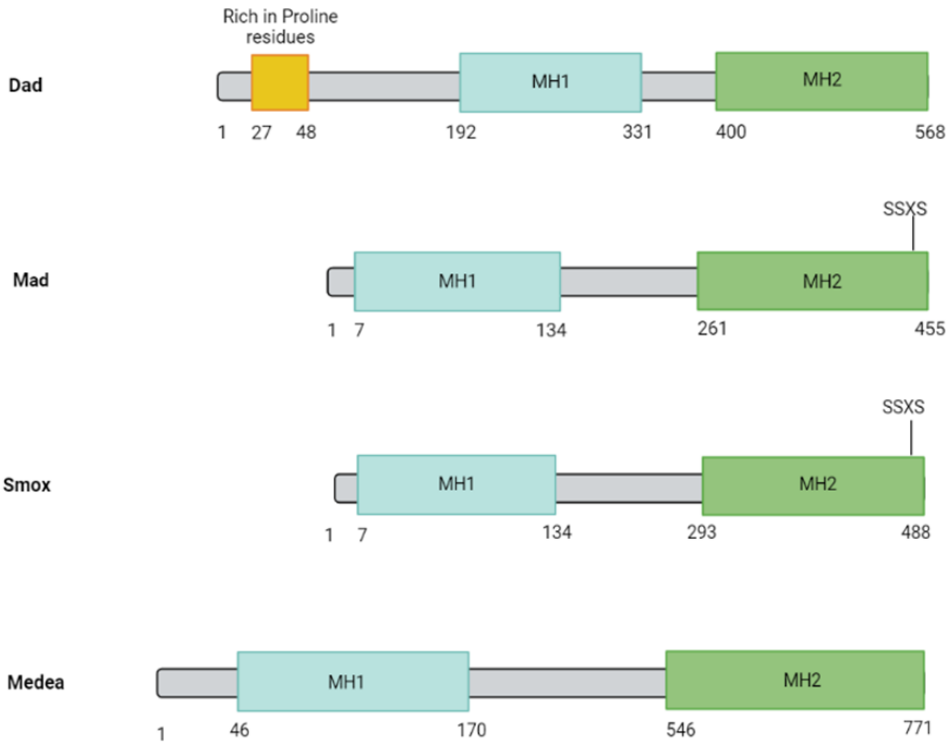


Figure 11 - Structural organization of the main I-Smads. Structure of Dad, Mad, Smox and Medea. Type I receptors phosphorylate Mad and Smox in the SSXS motif in the MH2 domain.

6. Aims

The main goal of this work was to evaluate and characterize several aspects of Dad's function, the Drosophila melanogaster's I-Smad, by analyzing its global expression, subcellular localization and pattern of expression. Then, knowing that Gilgamesh (Gish) is one of Dad's genetic partners from previous experiments, we wanted to understand to what extent Gish impacted Dad's function. Therefore, two different techniques (RNAi and CRISPR/Cas9) were used in order to induce Gish loss-of-function both in the presence or absence of Dad overexpression and the results were analyzed in two different tissues, eye and wing, in order to evaluate the specificity of this regulatory mechanism.

7. Materials and methods

7.1. Fly lines and genotypes

All crossings were initiated in the same day and all of them were maintained under the same conditions: at 25ºC.

The stocks used were: optixGal4; MKRS/TM6B, UAS-lacz, optixGal4; UAS GFP-DadL6/ SM6^TM6B, UAS-GishRNAi HMS01609, nubGal4-UAS-CD8-GFP, nubGal4-UAS-DadL6-GFP, nubGal4-UAS-Cas9, GishTKOGS04372, GishTKO342446.

When the observation of adult retinal phenotypes was necessary, in all different conditions, a stereo microscope (Stemi 2000, Zeiss) connected to a digital camera (Nikon Digital Sight DS-2Mv) was used.

7.2. Larval dissection and Immunohistochemistry

Both eye imaginal discs and wing imaginal discs were obtained through the dissection of the larvae. Then, the imaginal discs are prepared for immunohistochemistry by using the standard protocol from the "Cell Growth and Differentiation" group.

Firstly, the larvae dissection was performed using PBS (Phosphate-Buffered Saline) on ice. Afterwards, for the fixation of the imaginal discs, it was used PFA 20% (paraformaldehyde) diluted in PBS. The fixation period is different, depending on the nature of the imaginal discs: 20 minutes for the eye imaginal discs and 30 minutes for the wing imaginal discs. Then, in order to remove the fixative, three washes of 5 minutes each were executed, using PBS-T 0.1% (PBS with 0.1% Triton X100). After fixation, the imaginal discs were incubated with the primary antibodies in PBS-T for two hours at room temperature or, in certain cases, like when using pMad, overnight at 4ºC. The primary antibodies used were: anti P-Smad1/5 (pMad) 41D10 at 1:100 developed in rabbit (Cell Signaling 9516), anti Ecad (Ecad) at 1:100 developed in rat and anti 22c10 at 1:100 developed in mouse (Developmental Studies Hybridoma Bank, DSHB).

After this incubation, the imaginal discs were subjected, again, to three washing processes in PBS-T 0.1% during 5 minutes each, at room temperature. Hereafter, the imaginal discs were incubated with the secondary antibodies for 1 hour and 30 minutes at room temperature. In order to identify each of the primary antibodies it was used

the Alexa-Fluor conjugated secondary antibodies from Molecular Probes and DAPI was used to stain the DNA.

At last, the imaginal discs were stored in Vectashield (Antifade Mounting Medium with DAPI, BIOZOL) at 4℃.

7.3. Confocal microscopy and image processing

Afterwards, the images of the imaginal discs were obtained using the Leica SP5II laser confocal microscopy using the 40x objective. The posterior analysis of the imaginal discs was performed using the LAS AF software by Leica Microsystems CMS GmbH and all images were processed with Adobe Photoshop 2020.

7.4. Adult wings preparation, image obtainment and wing area quantification

When the observation of adult wing phenotypes was needed, the wings were obtained by using ethanol (96%) and placing the chosen flies (with the intended phenotype) in that solution, in a well. Afterwards, the wings were cut from the flies, still sunken in the ethanol solution, in a glass slide and the adult flies were discarded. Then, after the ethanol solution has dried, a certain amount of glycerol was added to the slide, over the wings.

In order to obtain the adult wings images presented in the results, the Olympus Cx31 upright brightfield microscope was used. The analysis of the wings was executed using the Mosaic 2.3 software and all the acquired images were processed with Adobe Photoshop 2020.

Posteriorly, the wing area quantification was done by using the Fiji software, to calculate the areas and the GraphPad Prism software to organize the data and obtain the graphics.

8. Results

8.1. Gish affects Dad's function and expression in the eye

Firstly, the aim was to understand to what extent the loss-of-function of *gish*, induced by RNAi, would affect the function of an overexpressed Dad-GFP fusion protein. Previous results from our group have shown that overexpression of Dad inhibits differentiation in imaginal eye discs, as well as in adult eyes (Eusebio et al., 2018), which resembles the phenotype caused by loss-of-function of the dpp signaling. Thus, if Gish can impact Dad at the level of its expression pattern, or expression levels, or by modulating Dad's subcellular localization we expected that GishRNAi would modify the eye phenotypes caused by Dad-GFP overexpression.
We specifically targeted the overexpression of Dad to the developing eye. Therefore, primarily, the retinas of adult flies were evaluated. As control we used the optix-Gal4 driver which is expressed anteriorly to the morphogenetic furrow from late L2 on. We crossed this driver with UAS-Lacz and the resultant retinas (Figure 12. A) did not show any significant changes compared to the wildtype flies.
The phenotype of adult flies was analyzed in two conditions: optix> GFP dadOE; Lacz (DadOE) and optix> GFP dadOE, GishRNAi (DadOE+GishRNAi).
DadOE means that UAS-Dad-GFP was used as a way to achieve Dad overexpression. Dad was fused with the green fluorescent protein (GFP) which allowed us to determine the localization of Dad. As expected, we observed a strong inhibition of retina formation, which we categorised in three different classes: canonic DadOE (Figure 12. B), micro ventral (Figure 12. D) and small reduction of the retina (Figure 12. H). The first category is denominated canonic because it is the most predominant one. In addition, the second class is called micro ventral for the reason that some additional differentiation in the ventral area of the retina can be seen. Lastly, the small reduction of the retina refers to, as the name indicates, a subtle decrease of the area of the retina compared to the wildtype. In DadOE+GishRNAi the RNAi technique was used in order to do the silencing of Gish together with Dad overexpression. In this condition, the same three classes as in DadOE were obtained - canonic DadOE (Figure 12. C), micro ventral (Figure 12. E) and small reduction of the retina (Figure 12. I) and the obtained phenotypes are very similar to the ones acquired in DadOE in the same classes. Moreover, two new classes emerged: weak ventral (Figure 12. F) and significant ventral (Figure 12. G). Micro ventral, weak ventral and significant ventral differ in the area of differentiation in the ventral region: the significant ventral

corresponds to the biggest area in this zone and the micro ventral refers to the smallest. In this condition, the additional area of differentiation in the ventral region is bigger than in DadOE because, in the latter, only the micro ventral class exists while, in DadOE+GishRNAi, the weak ventral and significant ventral are also present, therefore, the area of differentiation in the ventral zone is, often, more prominent in DadOE+GishRNAi.

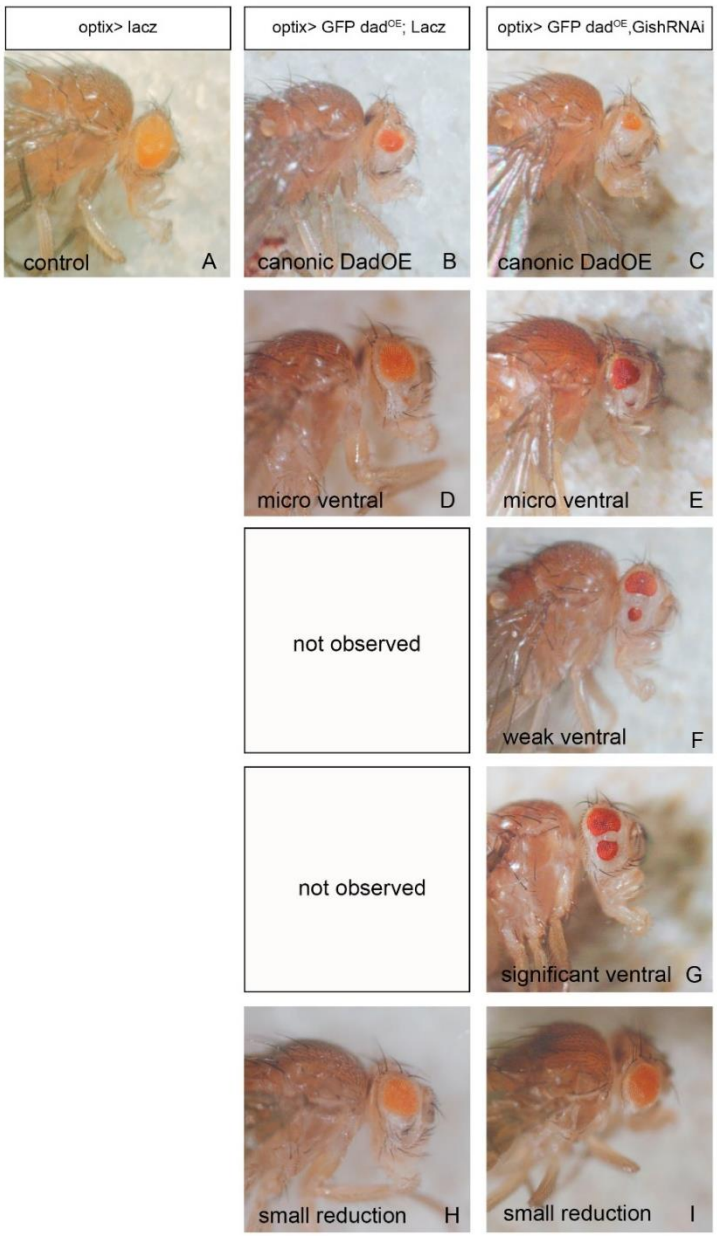
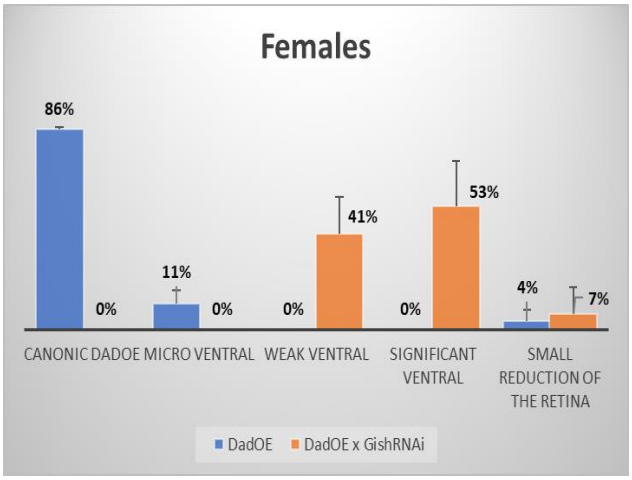


Figure 12 - Impact of the overexpression of Dad and the loss-of-function of Gish through RNAi in the adult retinas. (A) Control (optix> Lacz). (B,C) In both DadOE and DadOE+GishRNAi the canonic DadOE, (D,E) micro ventral and (H,I) small reduction of the retina classes are observed. In DadOE+GishRNAi the (F) weak ventral and (G) significant ventral classes are also present.

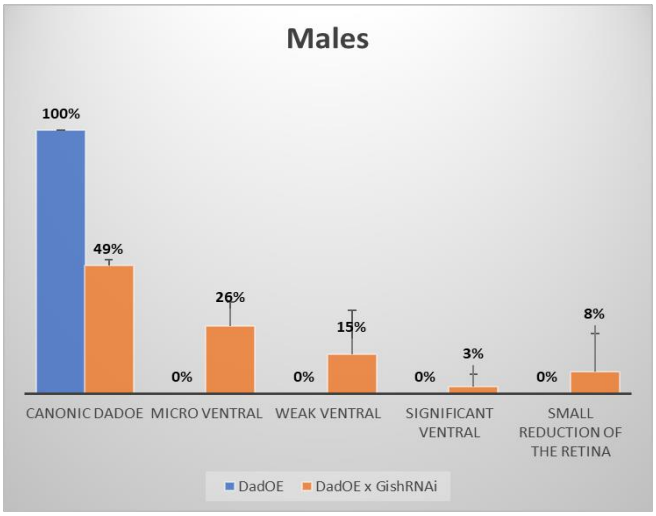
Afterward, in order to quantify the phenotypic classes of the adult retinas of the previously described genotypes, we counted how many adults had each phenotype both in DadOE and in DadOE+GishRNAi. To provide a better visualization and consequent understanding of the results, the data was organized firstly by gender and, then, by the classes described above.

Overall, considering both genders, it is obvious that in DadOE, as said before, the only obtained phenotypes are: canonic DadOE, micro ventral and small reduction of the retina (when compared to the wildtype). However, in DadOE+GishRNAi, all five classes were acquired. On one hand, referring to DadOE, only females can exhibit the three categories previously remarked- the overwhelming majority present the canonic DadOE phenotype (86%), 11% are in the micro ventral category and 4% manifest little reduction of the retina- while males only display the canonic DadOE phenotype.



Graphic 1. Quantification of the different eye phenotypic classes found in female adult flies in DadOE and in DadOE+GishRNAi. In DadOE, the majority of adults (86%) belong to the canonic DadOE class, 11% to the micro ventral class and only 4% in the small reduction of the retina one. In DadOE+GishRNAi only the weak ventral (41%), significant ventral (53%) and small reduction of the retina (7%) classes are present.

On the other hand, in DadOE+GishRNAi, only the males present all five classes and the females only display three of them (weak ventral, significant ventral and a small reduction of the retina). Being exact, in the male group (Graphic 2), the predominance is, again, in the canonic DadOE class with 49%, 26% exhibit micro ventral retina, 15% display weak ventral retina, 3% present significant ventral retina and 8% little reduction of the retina. Nevertheless, the majority of females (Graphic 1) belongs to the significant ventral category (53%), followed by the weak ventral class (41%) and, then, the small reduction of the retina (7%).



Graphic 2. Quantification of the different eye phenotypic classes found in male adult flies in DadOE and in DadOE+GishRNAi. In DadOE only the canonic DadOE phenotype is observed. In DadOE+GishRNAi all classes are present: the majority belongs to the canonic DadOE class (49%), micro ventral with 26%, weak ventral with 15%, significant ventral with 3% and small reduction of the retina with 8%.

These results lead to the previous conclusion that, in males, the phenotype observed in the DadOE category is more severe than in females, because all males present the canonic DadOE phenotype (Graphic 2). This can be because the Gal4 system achieves higher induction of expression levels in males. Furthermore, to strengthen this inference, in DadOE+GishRNAi, there is a stronger rescue of the retina in the ventral area in females.

These results allow the conclusion that Gish affects Dad's function mainly because there is a stronger rescue of the retina in the ventral area when Gish is silenced through RNAi which means that, in a Gish's loss-of-function condition, Dad becomes less effective in its functions such as the inhibition of cell growth and cell differentiation.

Hereupon, after analyzing the adult phenotypes, we evaluated several aspects of earlier eye development such as the extension of differentiation, the subcellular localization of Dad, the global expression of Dad (which is given by the intensity of GFP) and the pattern of Dad's expression. All of these points were analyzed in the context of the L3 eye imaginal discs.

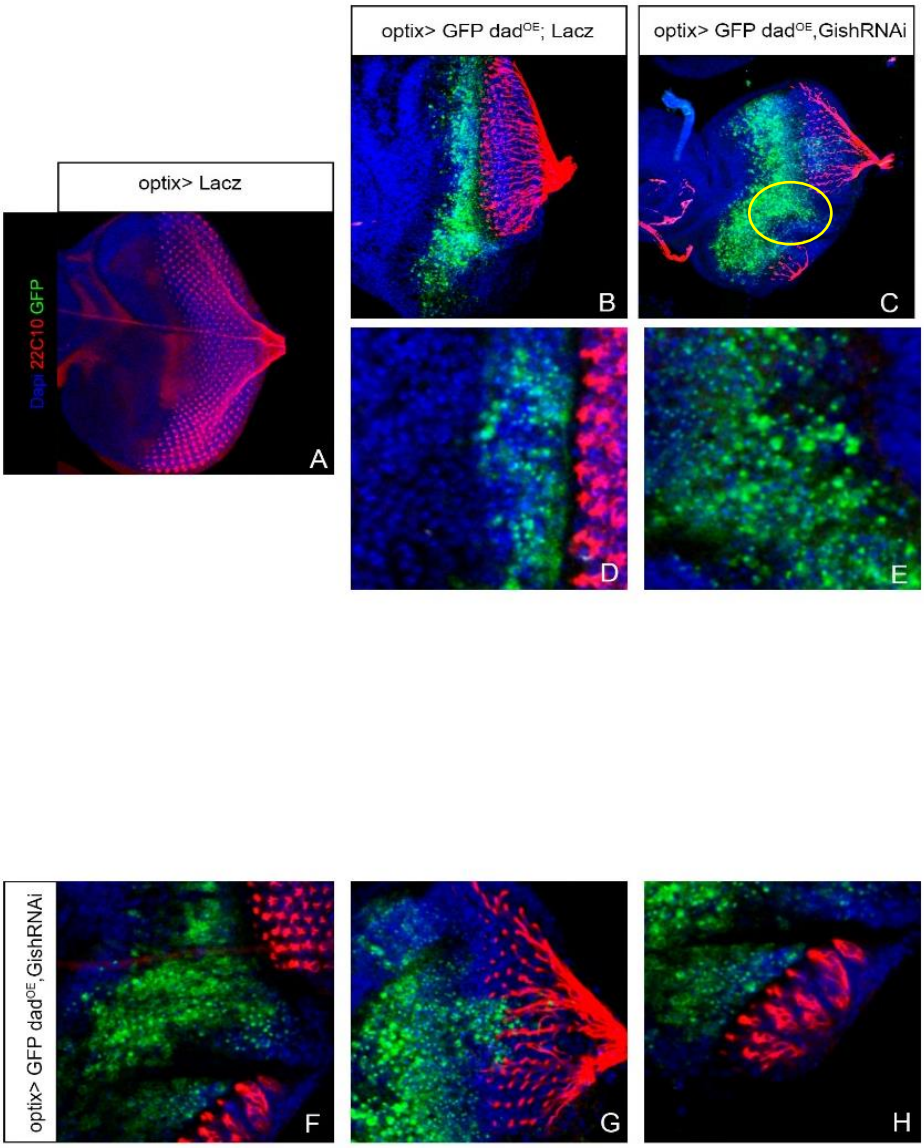


Figure 13 - Extension of differentiation and subcellular localization, global expression and pattern of expression of Dad in eye imaginal discs. (A) Control (optix> Lacz). (C) In DadOE+GishRNAi the expression levels of Dad are higher than in DadOE alone (B). (D,E) In both DadOE and DadOE+GishRNAi Dad is present in the nucleus, in the cytosol an associated with the plasmatic membrane. (F) In DadOE+GishRNAi, Dad is found between the dorsal and the ventral regions and (G) in the posterior region of the morphogenetic furrow. (H) In DadOE+GishRNAi, some additional differentiation is observed in the ventral area.

In DadOE, Dad is localized mainly in the nucleus and in the cytosol, with a small pool associated with the plasma membrane (Figure 13. D). Then, it was analyzed if, in the presence of DadOE+GishRNAi, any differences were found in terms of subcellular localization of Dad. The result was that Dad was still mainly present in the nucleus and in the cytoplasm (Figure 13. E), as well as in the plasma membrane region (Figure 13. F). Thus, no significant differences were visualized in the subcellular localization of Dad in both conditions.

Overexpression of Dad inhibited differentiation in imaginal eye discs (Figure 13. A, B), which was detected by expression of 22C10 a photoreceptor-specific marker. This inhibition is stronger in the disc's margins causing the appearance of a curved morphogenetic furrow. Interestingly, when it comes to the differences, one of the dissimilarities observed in the imaginal discs corresponds to the additional differentiation observed in their ventral area, since there is an increase of differentiation in this region in DadOE+GishRNAi (Figure 13. H), which is in concordance with the phenotypes observed in adult flies.

Moreover, the intensity of expression levels of Dad, which is significantly greater in DadOE+GishRNAi (Figure 13. C), is another difference observed when comparing the eye discs of DadOE and of DadOE+GishRNAi Lastly, in terms of its expression domain, Dad-GFP signals are more broadly detected in the eye disc of the DadOE+GishRNAi condition (Figure 13. C) since Dad is also present between the ventral and the dorsal regions in this imaginal disc (Figure 13. F) (which is marked by the ellipse in Figure 13. C), which is not detected in DadOE imaginal discs (Figure 13. B). In addition, Dad is also found in the posterior region of the morphogenetic furrow (Figure 13. G), which is not common.

These results highlighted the importance to study if this regulatory mechanism is preserved or if it is tissue-specific, thus, they led to the analysis of DadOE+GishRNAi wing phenotypes and wing imaginal discs.

8.2. Gish affects Dad's activity and expression in the wing

As pointed out before, one of the main experimental goals was to verify if Dad's regulation by Gish is tissue-specific or otherwise broadly conserved across distinct tissues and organs. With that in mind, this experience comprises basically the same principles as the experience described above to understand if Gish impacts Dad and if so, in what magnitude, however, it occurs in the wing and in the wing imaginal discs. The RNAi technique was used again in order to promote Gish loss-of-function. Therefore, the first course of action was to analyze the phenotype of adult wings to understand if the knockdown of Gish impacts the adult phenotypes of Dad overexpression. For that, the driver used was nubbin-Gal4 (nub-Gal4) which is expressed in the larval wing pouch and in the wing blade. Then, this driver was crossed with Lacz to setup the control of this experience. The control wings are very similar to the wild-type wings. Subsequently, the wings of two more genotypes were also analyzed: nub> GFP dadOE; Lacz, in which Dad was overexpressed (DadOE) and nub>

GFP dadOE, GishRNAI in which Dad was overexpressed plus Gish was silenced through RNAi (DadOE+GishRNAi).

The results show clear differences between the size and shape of the control wing (A) and both wings in which Dad was being overexpressed (Figure 14. B and C). Both DadOE wing (Figure 14. B) and DadOE+GishRNAi wing (Figure 14. C) are smaller when compared to the control wing (Figure 14. A) and they also lack the normal patterning of vein formation, which is regulated by Dad and Dpp signaling (Matsuda & Shimmi, 2012). Besides, in the DadOE+GishRNAi condition, the wing is slightly bigger than the wing in the DadOE condition, however, it maintains the same shape.

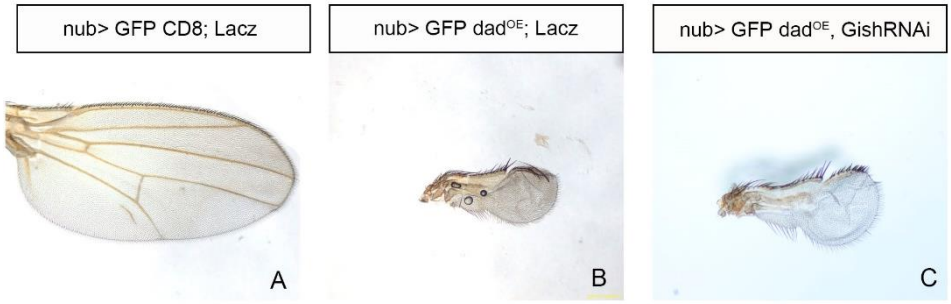
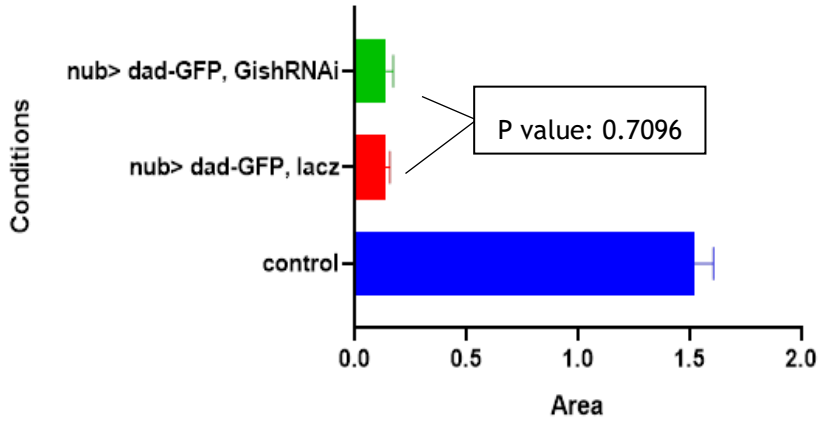


Figure 14 - The loss-of-function of Gish was not able to rescue the control wing phenotype. (A) Control (nub> GFP CD8; Lacz). (B) The DadOE wing is significantly smaller than the control wing. (C) The DadOE+GishRNAi wing is very similar to the DadOE wing.

The decrease in size of the DadOE wing proves that Dad is active and functions by limiting the tissue growth in the wing. Additionally, the small difference in the size of the DadOE+GishRNAi wing relatively to the DadOE wing, demonstrates that when there is a loss-of-function of Gish, Dad's function, i.e., its ability to restrict cell proliferation, is affected.

Then, in order to get a clearer view of the results and confirm the conclusion taken before, the areas of twenty different wings of each condition were calculated and the obtained results are shown in the graphic below (Graphic 3). As observed, these results are in accordance with the phenotypes observed: the control wings (Graphic 3. Blue) are much bigger than the wings of DadOE (Graphic 3. Red) and DadOE+GishRNAi (Graphic 3. Green) and the DadOE (Graphic 3. Red) wings are very similar, in terms of area, with the DadOE+GishRNAi wings (Graphic 3. Green). In fact, the mean area of the control wings is 1.523 mm^2 while the mean area of the DadOE is 0.137 mm^2 and the DadOE+GishRNAi's mean area is 0.140 mm^2. Therefore, the DadOE+GishRNAi wings are only 2.2% bigger than the DadOE wings, which is not a significant difference.



Graphic 3. The wings' area decreases when Dad is overexpressed and the loss-of-function of Gish does not significantly rescue this phenotype. The control (blue) wings are significantly bigger than the DadOE (red) and the DadOE+GishRNAi (green) wings. The DadOE+GishRNAi wings are slightly bigger than the DadOE wings.

However, as these results are not much strong and the differences are not that severe, this regulatory mechanism was also evaluated in the context of the wing imaginal discs, in L3. Here, some of the same aspects (the global expression of Dad, Dad's subcellular localization, and the pattern of expression of Dad) as the previous experiment were evaluated in order to analyze if Dad's regulation by Gish is tissue-specific or not. The localization of Dad is possible to determine due to the Dad-GFP fusion (GFP is a green fluorescent protein). Plus, we also analyzed the pattern of anti-pMad labelling, which is an antibody that specifically recognises the phosphorylated Mad and, consequently, allows the recognition of the BMP signaling activity.

The first observation that stands out is the fact that, just like as in the adult wings, the DadOE (Figure 15. B and B') and the DadOE+GishRNAi wing imaginal discs (Figure 15. C and C') are smaller than the control (Figure 15. A and A'). Secondly, it is clear that the wing pouch region (marked by the yellow dashes in Figure 15) of the control imaginal disc (Figure 15. A and A'- yellow dashes) is bigger than wing pouch region of the DadOE (Figure 15. B and B'-yellow dashes) and the DadOE+GishRNAi (Figure 15. C and C'- yellow dashes) wing imaginal discs, which agrees to what was seen in the wing

phenotypes: the control wing is bigger than the other two conditions (since the wing pouch gives rise to most of the adult wing structure).

The global expression of Dad (which is given by the intensity of GFP) is higher in the DadOE+GishRNAi imaginal disc (Figure 15. C) comparatively to the control (Figure 15. A) and the DadOE (Figure 15. B) wing imaginal discs. Therefore, we can infer that the knockdown of Gish, through the RNAi technique, increases the stability/expression of Dad. However, despite the intensity of its expression being higher in DadOE+GishRNAi, its pattern of expression is the same in both DadOE (Figure 15. B) and in DadOE+GishRNAi (Figure 15. C), or else, Dad's dispersion throughout the wing imaginal disc is the same in both conditions: it is confined to the wing pouch area. Moreover, when it comes to the subcellular localization of Dad, once again, no differences were viewable: either in DadOE (Figure 15. D) or in DadOE+GishRNAi (Figure 15. E) Dad is located both in the nucleus, in the cytosol and associated with the plasmatic membrane.

In addition, we can perceive that, while the standard pattern of expression of pMad (two bands in the wing pouch area) is detectable in the control imaginal disc (Figure 15. A'), it is not visible in the wing pouch region of DadOE (Figure 15. B') and DadOE+GishRNAi (Figure 15. C'). This indicates that the BMP signaling is not active in the presence of Dad.

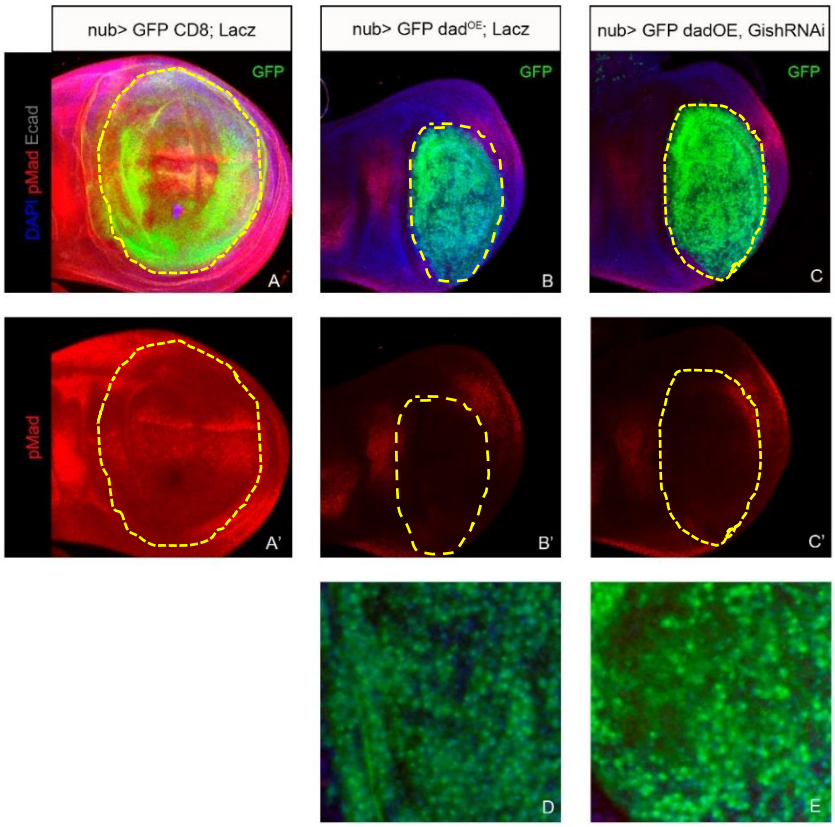


Figure 15 - Subcellular localization, global expression and pattern of expression of Dad and pattern of anti-pMad labelling. (A and A') Control (nub> GFP CD8; Lacz). (C) In DadOE+GishRNAi the global expression of Dad is the highest. (A, B and C) Dad is located in the wing pouch region in all conditions. (D and E) In both DadOE and DadOE+GishRNAi, Dad is located in the nucleus, in the cytosol and associated with the plasmatic membrane. In the control (A') the standard pattern of anti-pMad labelling is observed, however, in DadOE (B') and in DadOE+GishRNAi (C') this labelling is absent.

Since the outcomes of the loss-of-function of Gish were stronger in the eye tissue, this may be an indicator that the regulation of Dad by Gish is stronger in that. Plus, all of these results demonstrated that Gish affects Dad's function and expression which allows the conclusion that this regulatory mechanism is conserved because occurs both in the eye and in the wing tissue.

8.3. Gish loss-of-function through CRISPR is stronger than through RNAi

The experiments described above suggest that Gish function could be required for Dad to efficiently inhibit Dpp signaling. Thus, we hypothesised that inhibiting *Gish* function could, on its own, induced phenotypes similar to *Dpp* overexpression. Moreover, the tissue-specific CRISPR technique was used in order to evaluate if Gish knockout by mutation is stronger through this mechanism than with the use of the RNAi technique. Using the RNAi lines we observed that *gish* silencing induced visible adult wing phenotypes, but we failed to observe significant eye alterations (not shown). Furthermore, the experiments were done in the wings and not in the eye because the Gish loss-of-function phenotypes in the wing have already been described and they are significant (Gault et al., 2012). Nevertheless, it would be interesting to test, in the future, the Gish knockout induced the driver line, optixGal4; UAS-Cas9, under construction. The driver used in this set of experiments in the wing was the nub-Gal4, which is expressed in the wing pouch and in the wing blade since the second larval stage (L2). The driver was crossed with Lacz as a control (nub> Cas9, Lacz), and micrographs of the adult wings were acquired (Figure 16. A). These wings did not show any dissimilarities compared to the wildtype ones. Then, more wings were analyzed in two distinct lines of GishTKO: $Gish^{TKOGS04372}$ and $Gish^{TKO342446}$ which express distinct gRNAs targeting different regions of the *gish* gene locus. Both of these lines were crossed with nubGal4-UAS-Cas9 which allows the knockout of Gish by the CRISPR technique.

There is a difference in the size of the wings represented in each condition, since in both lines of GishTKO (Figure 16. B and C), the wings appeared to be slightly smaller

than the control wing (Figure 16. A). However, this difference does not stands out, only visually.

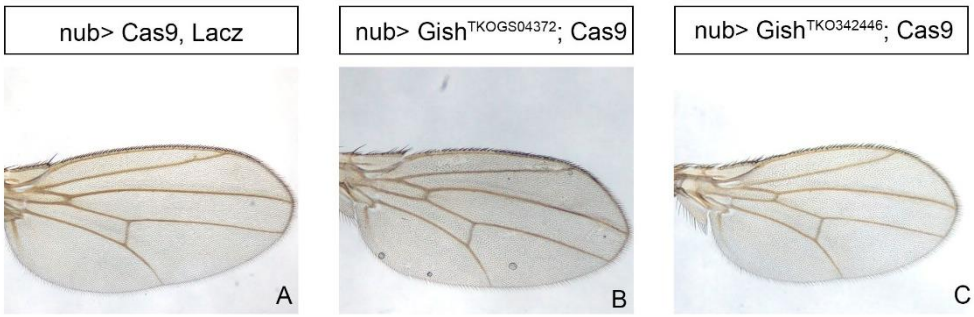
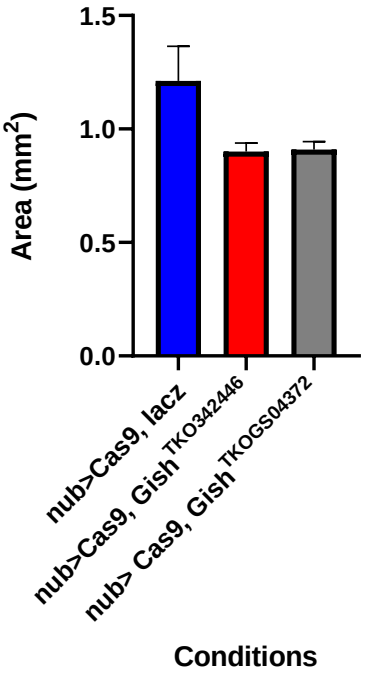


Figure 16 - The knockout of Gish results in smaller wings. (A) Control (nub> Cas9, Lacz). Both (B) Gish^{TKOGS04372} and (C) Gish^{TKO0342446} present smaller wings.

On top of that, in order to get a clearer vision of the size difference between the three conditions, twenty-five different wings of each category were analyzed, their area was calculated and the data is shown in Graphic 4 As pointed before, wings from the GishTKO conditions (Graphic 4. red and gray), are smaller than the control (Graphic 4. blue), which can be seen in the graphic or in the information given by the calculated statistics. The mean area of the control was 1.212 mm^2, in the Gish^{TKO342446} line was 0.9005 mm^2 and in the Gish^{TKOGS04372} line was 0.9097 mm^2. Plus, the maximum area obtained in the nub>Cas9 Lacz condition was 1.604 mm^2, in the Gish^{TKO342446} was 0.9670 mm^2 and in the Gish^{TKOGS04373} was 0.9840 mm^2. Therefore, the wings in the Gish^{TKOGS04372} line are, on average, 34,73% smaller than the control ones and the wings in the Gish^{TKO342446} are 33,57% smaller than the control wings. Moreover, the difference between the two GishTKO lines is only 1,16%. Therefore, all of this data confirm that the control wings are bigger than the wings in the GishTKO lines and, in the latter, there is no significant difference in the size of the wings, however, in order to be more



Graphic 4. The knockout of Gish leads to a small decrease in the wing's area/size. The control wings (blue) are bigger than both lines of Gish knockout: Gish^{TKOGS0342446} (red) and Gish^{TKOGS04372} (grey).

specific, the Gish^{TKO342446} line presents a stronger phenotype than the Gish^{TKOGS04372} line.

Additionaly, the wing bristles were analyzed. In *Drosophila*, the bristles derive from the division of a single hypodermal cell (SONDHI, 1963). The role that Gish plays in trichome formation has already been characterised (Gault et. Al, 2012). The authors demonstrated that Gish is able to regulate cellular morphogenesis and trichome formation by controlling the local where Rab11 does its vesicle recycling function. The mechanism is not yet fully understood, but what is known is that Gish is located in the prehair base and it is able to direct the trafficking of Rab11 endosomes and vesicles to that area. When this vesicle recycling near the prehair base is polarized, the nucleation does not diffuses laterally and duplicated trichomes are not formed. However, when *Gish* mutations occur, this trafficking is disturbed which results in diffuse membrane trafficking of nucleation activity and repeated trichome formation (Gault et al., 2012).

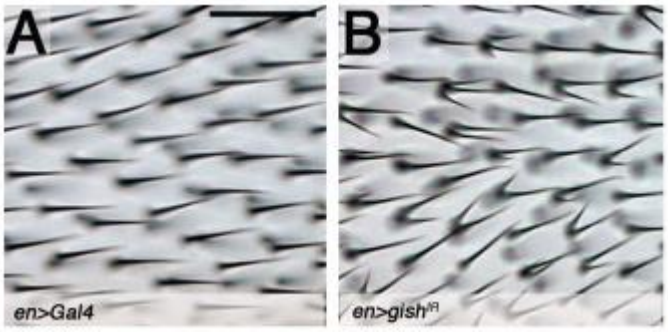


Figure 17 - Gish mutations lead to trichome duplication. (A) Control (en>Gal4). (B) Gish knockdown leads to trichome duplication. Adapted from Gault et al., 2012.

In this figure from the paper mentioned above, the image A (Figure 17. A) is referred to the control in which single distally oriented trichomes are visible and the image B (Figure 17. B) shows the loss-of-function of Gish through the RNAi technique where multiple distally oriented trichomes are visible.

After evaluation of the experiment conducted in our lab, it is obvious that the control (Figure 18. A and A') has less bristles than both lines of GishTKO (Figure 18. B and C), in which seems to be a bristle/trichome duplication (Figure 18. B' and C') which is consonance with previously obtained results (discussed in the referred paper). However, in the paper, the silencing of Gish is acquired through the RNAi technique while, in our experience, Gish was inactivated through the CRISPR technique.

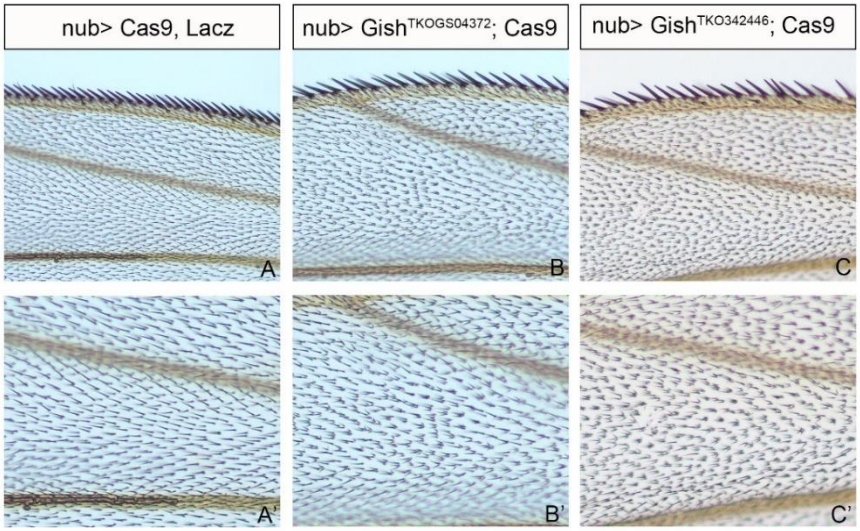


Figure 18 - The loss-of-function of Gish leads to bristle duplication. (A, A') Control (nub> Cas9, Lacz). (B, B', C and C') Both lines of Gish's knockout results in a very similar bristle duplication.

Nevertheless, the results concerning the bristles were very much alike to those obtained through the RNAi technique, which leads to the conclusion that, in this specific aspect, both techniques are highly similar in what concerns Gish's loss-of-function.

Subsequently, the L3 wings discs were evaluated to see if these differences in the phenotype of the wings were also visible in the context of the wing imaginal discs and if so, if those differences were stronger in the imaginal discs. We observed that there is a small difference in the size of the wing discs of both GishTKO lines (Figure 19. B and C) comparatively with the control (Figure 19. A), Plus, the wing pouch area (represented by the white dashes in every imaginal disc) of the control wing is also bigger than the wing pouch area of the imaginal discs of both GishTKO lines which is consistent with the adult wings phenotype.
In this experiment, anti-pMad (phosphorylated Mad) was used as an antibody which identifies the BMP signalling activity in *Drosophila* tissues because this antibody stains the phosphorylated and, therefore, active Mad. In all three genotypes, two bands of anti-pMad are visible in the wing pouch region (Figure 19. A', B' and C'), which is the normal pattern of expression of pMad, which indicates that, in all three conditions, the BMP signaling was activated. However, in $Gish^{TKOGS04372}$, these bands are somewhat less intense (Figure 19. B'), thus, suggesting that the BMP pathway could be less active when there is a knock-out of Gish, which means that Gish affects the function of the BMP signaling. However, we would not observe a consistent decreased pMad signal when *gish* was knocked-out by $Gish^{TKO342446}$. Although further independent experiments

and larger sampling is required, these results suggest that the silencing of Gish (through CRISPR) leads to a lower Mad phosphorylation.

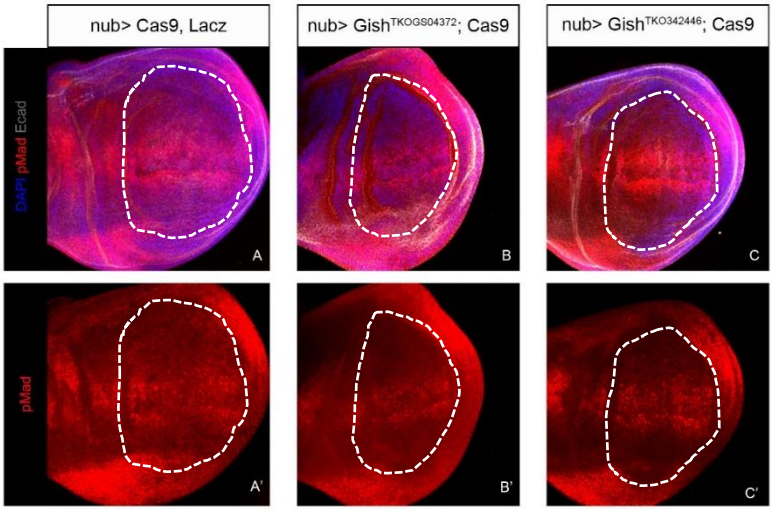


Figure 19 - The knockout of Gish leads to smaller wing imaginal discs and affects Mad phosphorylation. (A, A') Control (nub> Cas9, Lacz). (B) The Gish^{TKOGS04372} presents a smaller wing imaginal disc and (B') the intensity of the pMad signal is slightly weaker. (C) In the Gish^{TKO342446} line the imaginal discs are even smaller and the pMad signal (C') is similar to the control.

9. Discussion and Conclusion

The *Drosophila Melanogaster*'s genome encodes a single I-Smad: Dad, which regulates TGFß signaling, more specifically, the BMP branch (Upadhyay et al., 2017) through the inhibition of the Dpp signaling (Kamiya et al., 2008). The expression of Dad is promoted by *Dpp* and Dad antagonizes Dpp signaling, which means that Dad's expression induces negative feedback of the Dpp signaling. This inhibition mechanism conducted by Dad occurs in two different ways: by inhibiting Mad phosphorylation by Tkv or by preventing the hetero-oligomerization and consequent nuclear translocation of Mad (Inoue et al., 1998). Phenotypically, the inhibition of Dpp signaling by Dad results in reduced adult retinas (Eusebio et al., 2018). In fact, this was observed in adult animals during *Dad* overexpression (Figure 12. B).

The vertebrate orthologs of Dad, Smad6 and Smad7, have a similar function as negative regulators of the TGFß signaling (Uchida et al., 2000). Smad6 is located both in the nucleus and in the cytoplasm and Smad7 is, mostly, located in the nucleus (Hanyu et al., 2001). Our results show that Dad is also located both in the nucleus and in the cytosol, in eye imaginal disc cells (Figure 13. D).

Dad's function is modulated by palmitoylation. Palmitoylation refers to a post-translational modification where a palmitic acid is added to substrate proteins through a thioester bond. This modification regulates the function of these proteins at membranes and is regulated by palmitoyl acyl-transferases (PATs). More specifically, the palmitoyltransferase dHIP14 palmitoylates Dad, modifying its membrane function (Li et al., 2017). However, it is also important to study other mechanisms that regulate Dad's function. With that in mind, our lab has already done a screen with genes involved in processes of growth, differentiation and proliferation and discovered that Gilgamesh (Gish) was a genetic partner of Dad that interfered with its function.

Gish is a kinase involved in Hedgehog and Wg signaling (Li et al., 2016) orthologous to the human CK1 (Gault et al., 2012). Gish phosphorylates certain substrates like Dishevelled, APC and Smo in order to regulate the functioning of these pathways (Nye et al., 2020, Li et al., 2016). Hedgehog signaling is activated by the phosphorylation of the Smo C-terminal by Gish and depends on its membrane association, therefore, Gish acts as a positive regulator of this signaling (Li et al., 2016). Since Gish is already proven to be involved in the regulation of the Hedgehog and Wingless pathways, it has a strong potential to be a part of other signaling pathways and participate in the phosphorylation of other proteins such as Dad and, therefore, regulate them.

Therefore, the aim of this work was to understand and evaluate the effect that Gish has on Dad's function.

Hereupon, the first step was to confirm that Dad's overexpression affected the phenotype of adult retinas. After achieving this goal (Figure 12. B), the next aim was to analyze if the loss-of-function of Gish, induced by the RNAi technique, also had repercussions on the adult retinas phenotype.

Therefore, in the first place, the evaluation of the phenotype of adult flies of three different conditions was performed: Optix> Lacz (control), DadOE and DadOE+GishRNAi. In DadOE, considering both females and males, the canonic DadOE phenotype was obtained, as expected, and two more classes: small reduction of the retina (relative to the wildtype flies) and a phenotype called micro ventral. However, in the DadOE+GishRNAi flies, two more classes were observed, besides the three already discussed: weak ventral (Figure 12. F) and significant ventral (Figure 12. G), which indicates a stronger rescue of the retina in the ventral area, compared to DadOE adult flies. Thus, this more intense rescue that happened when Gish was silenced through the RNAi technique allows the conclusion that Gish does control Dad's activity because when there is a loss-of-function of Gish, Dad's activity is weaker. Now, comparing females and males in DadOE, it is noticeable that the phenotype is more drastic in males because they only present the canonic DadOE phenotype (Graphic 1), while females, besides this phenotype, also present the small reduction of the retina (compared to the wildtype) and the micro ventral phenotypes (Graphic 2). These last results indicate that despite the retina not being similar to the control, it also has not decreased as much in size as in the males, and/or there is still some retinal differentiation present in the ventral area. Furthermore, in DadOE+GishRNAi, in females, only the classes where a rescue of the retina in the ventral area occurred were acquired, while in males all five classes exist (canonic DadOE, small reduction of the retina, micro ventral, weak ventral and significant ventral). Plus, still regarding males, the most predominant class is the canonic DadOE one. On that account, these results induce the inference that, in females, there is a more frequent and stronger rescue of the retina, in the ventral area, than in males where, despite also presenting these classes, the rescue is less frequent and weaker. Therefore, this leads to the conclusion that males have more severe phenotypes both in DadOE and in DadOE+GishRNAi.

Then, it was evaluated if this regulatory mechanism also existed in the context of the eye imaginal discs. For such, the same genotypes were tested and different aspects

of Dad were evaluated: its global quantity, the pattern of expression and subcellular localization.

After examining the results obtained, some differences were seen such as the global quantity of Dad, which is notably superior in DadOE+GishRNAi (Figure 13. C), when compared to DadOE (Figure 13. B). Therefore, these data agrees with the previous results and adds the conclusion that Gish also has an impact on Dad's pattern of expression in addition to affecting its activity. This result supports the relevance of performing Western Blot analysis of Dad expression levels in the context of control vs GishOE conditions, in future experiments. Another dissimilarity observed was in terms of Dad's localization along the eye imaginal disc because in DadOE+GishRNAi, Dad expression is maintained in patches posteriorly to the morphogenetic furrow between the dorsal and ventral areas (Figure 13. C, F and G), which is not a common outcome in DadOE. However, there is no difference concerning Dad's subcellular localization since Dad is present in the nucleus and in the cytoplasm in both conditions (Figure 13. D and E). Thus, it can be suggested that, in terms of Dad's expression, this regulatory mechanism acts only concerning Dad's protein stability throughout the imaginal disc and not in Dad's subcellular localization.
Another conclusion that can be drawn is that Gish affects Dad's function because in DadOE+GishRNAi the differentiation in the ventral area is more frequent (Figure 13. H), and in the context of the adult retinas, there is a stronger rescue of the retina in the ventral region (Figure 12. F and G). Indeed, some Dad is not functional because in the presence of more Dad, which occurs in DadOE+GishRNAi, the extent of differentiation should be inferior, however, that is not the case: the differentiation in the ventral area is higher in terms of area and in terms of frequency (Figure 12. F and G, Graphic 1 and 2).

With all of these results in mind, it is possible to infer that Gish is, indeed, involved in the regulation of Dad's function since it affects its pattern of expression and its inhibition promotes an additional differentiation in the ventral area. As Gish is a cinase, its potential mechanism to repress Dad's function and stability could involve Dad's phosphorylation. However, it is not explicited if this interaction between Gish and Dad might be direct or indirect. One hypothesis to discover if this interplay is direct, includes the immunoprecipitation technique, which will only result if this interaction has a stable duration. It would also be interesting to evaluate if Gish overexpression phosphorylates Dad by western blotting analysis.

Then, we moved on to do a similar experiment in the wings and wing imaginal discs. Our aim was to comprehend if the regulatory mechanism that Gish has over Dad is tissue-specific or conserved. Thus, it was evaluated if the knockdown of Gish through the RNAi technique had any impact in wing phenotypes. For that, the following conditions were used: nub> GFP CD8; Lacz (as the control), nub> GFP dad^{OE}; Lacz (DadOE) and nub> GFP dad^{OE}, GishRNAi (DadOE+GishRNAi).

As expected, the wing in the presence of Dad overexpression (DadOE) is smaller (Figure 14. B). Plus, the wing of the DadOE+GishRNAi condition is very similar in terms of size and shape (Figure 14. C) to the DadOE wing, however, it is slightly bigger. On one hand, this size difference between the control and the DadOE wings proves, again, that Dad indeed affects tissue growth and cell proliferation. On the other hand, the size difference between the DadOE wing and the DadOE+GishRNAi wing demonstrates that Gish, indeed, somehow, affects Dad's function.

Then, in order to corroborate these results, the areas of twenty different wings of each condition were calculated and it was confirmed that, in fact, the wings of DadOE+GishRNAi are slightly smaller than the DadOE wings.

Afterward, this regulatory mechanism was also studied in the context of the wing imaginal discs, in L3. The same points of the first experience were evaluated: the global expression of Dad, Dad's subcellular localization and the pattern of expression of Dad. As pMad was used as an antibody, its pattern of expression was also analyzed.

The results showed that the DadOE (Figure 15. B) and DadOE+GishRNAi (Figure 15. C) wing imaginal discs are smaller than the control (Figure 15. A), which proves that Dad overexpression interferes with cell proliferation and tissue growth. Plus, the fact that the DadOE+GishRNAi (Figure 15. C) imaginal disc is slightly bigger than the DadOE (Figure 15. B) imaginal disc is an indicator that Gish loss-of-function affects Dad function, leading to a small increase in the tissue growth.

Additionally, the wing pouch area of DadOE (Figure 15. B) and DadOE+GishRNAi (Figure 15. C) is considerably smaller than the control (Figure 15. A) which is in accordance with the phenotypes observed in the adult wings because, since the wing pouch originates most of the adult wing; when this region is smaller, the resulting wing will be smaller either. This decrease of the wing pouch area is because the Dpp signaling, which is responsible for the growth of the wing disc, more specifically, the wing pouch, is inhibited in these two conditions as a consequence of Dad overexpression. This conclusion is also supported by the pMad pattern of expression, which is absent in the wing pouch area of the DadOE (Figure 15. B') and

DadOE+GishRNAi (Figure 15. C) wing imaginal discs. This indicates that the BMP signaling is not activated in these conditions on account of Dad overexpression.

Dad's expression is higher in the DadOE+GishRNAi imaginal disc (Figure 15. C) comparatively to the DadOE imaginal disc (Figure 15. B). However, its pattern of expression is the same in both conditions (Figure 15. B and C), like Dad subcellular localization (Figure 15. D and E). This allows us to infer that Gish knockdown leads to higher Dad expression, which indicates that Gish affects Dad expression, however, Gish does not have an effect in Dad pattern of expression or its subcellular localization.

To sum up, Gish impacts Dad global expression and has a small effect in its function in th wing tissue. Therefore, comparing these results with those obtained in the eye, we can conclude that Gish knockdown, through the RNAi technique, has a stronger influence in Dad's overall activity and expression in the eye. However, this regulatory mechanism is not tissue-specific, instead, it is conserved, because Gish affects Dad functions and expression both in the eye and in the wing tissues.

Lastly, we wanted to verify if the CRISPR technique was stronger in silencing Gish than the RNAi technique.

First and foremost, the adult wings phenotypes were analyzed in order to compare them and see differences between the genotypes. The results showed that, visually, in terms of size/area, the wings of the GishTKO lines were only slightly smaller than the control wings, therefore, there was not an outstanding difference (visually) in the phenotypes to allow a strong conclusion. Therefore, the area of twenty-five wings of each condition was calculated to get more evident results. In fact, the results lead to the conclusion that the differences are not insignificant and confirmed that the knockout of Gish promotes a decrease in the area of the wings. Therefore, it can be inferred that, when there is a loss-of-function of Gish through the CRISPR technique, the wings are smaller compared with the control, which can be an indicator that the silencing of Gish affects cell proliferation and tissue growth. As said before, since the Gish loss-of-function on its own, obtained through the RNAi technique, did not generate strong phenotypes, it can be speculated that CRISPR is more successful in silencing Gish than the RNAi technique.

Afterwards, the bristles in the wings were also evaluated. Here, the differences are more considerable between the control and the GishTKO lines since there is, clearly, a bristle duplication in both GishTKO lines. This bristle duplication occurs in both techniques, CRISPR and RNAi, in the same magnitude. Therefore, this aspect is not able to dictate which one of these techniques is stronger when it comes to silencing Gish.

The next step was the analysis of the wing imaginal discs in L3, which were stained with pMad (and Ecad and DAPI) in order to see if the knockout of Gish had any visual impact on the imaginal discs and if it had any effect on the BMP pathway of the TGFß signaling. Firstly, in terms of size, the wing imaginal discs belonging to the GishTKO lines are smaller than the control one, mainly the imaginal disc of the $Gish^{TKO342446}$ condition. In addition, the wing pouch area of the imaginal discs of both GishTKO conditions is smaller than the wing pouch area of the control imaginal disc, which is in accordance with the obtained phenotypes: the loss-of-function of Gish causes a decrease in the area of the wing pouch region of the wing imaginal discs of both GishTKO lines. In addition, as pMad stains the phosphorylated Mad, it signals the activation of the BMP pathway. After analyzing the obtained images, it is easily understood that the BMP pathway is activated in all three conditions since there are two bands of pMad in the wing pouch area in all three imaginal discs, which is the standard pattern of pMad's expression. Nonetheless, these bands are a bit less intense in the $Gish^{TKOGS04372}$ line suggesting that the knockout of Gish led to a less active BMP pathway, which signifies that Gish affects the function of *Dpp*.

The overall conclusions that were taken from this last experiment were that the knockout of Gish through the CRISPR technique might promote stronger phenotypes than the RNAi technique. In future experiments, it will be interesting to evaluate the contribution of Gish to Dad overexpression phenotypes by Gish CRISPR-mediated knockout.

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