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Studies on the Hippo Pathway:
new insights about a multifaceted signalling

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Abstract

The Hippo pathway is a well-known master regulator of cell growth and proliferation. More and more studies have shed light on the centrality of Hippo functions, as this signalling is able to respond to different stimuli and translate them into distinct transcriptional outputs. Therefore, it is clearly implicated in a number of important processes, which alteration has consequences on the correct specification of the single cell, as well as the whole tissue. Even if the core of the signalling has been extensively characterized, it remains unclear which are the "co-workers" that permit the Hippo pathway to answer to so many different stimuli and act as a coordinator of the growth/differentiation balance.

Taking advantage of the *Drosophila* model, which has witnessed most of the discoveries on this signalling pathway, this thesis aims to add some new knowledge about the Hippo pathway molecular mechanisms in different contexts, from development to disease.

In the first part I have studied the dynamics of the Hippo core kinase protein Warts in the development of the pupal eye. I have found out a critical time point in which the expression and the localization of Warts change suddenly, suggesting the intervention of upstream regulators modulating its activity in an extremely narrow time window.

The second goal was investigating the role of the Hippo pathway in the neurodegenerative Gaucher disease. Indeed, I have produced some preliminary results which demonstrate a growth deficit associated with a massive reduction of some Yki targets, supporting a *Hyper-Hippo* condition underlying this neuropathic syndrome.

Finally, I have evaluated the transcription factor Orthodenticle as a co-factor of Yorkie in driving tissue overgrowth, and my findings support a model of interaction of these two molecules based on Yki conformational changes.

Altogether, my results lay the foundation for new important studies on the molecular mechanisms ruling Hippo pathway activity.

1. Introduction

Normal growth and growth impairment: where are the *Hercules pillars* located?

A correct balance between cell growth, proliferation and apoptosis controls the final size of developing organs, defining cell size and number. Each organism is the result of an elaborated exchange in growth and differentiation signals between cells and tissues. The stimuli arise from outside the body, as well as from the neighbouring cells, and the correct balance between them generate a perfect *being*. Indeed, it is important understanding that development is a flexible process, influenced by multiple factors, during which many molecules respond to the different signals in order to guarantee the right proportions within a tissue. Despite that, sometimes something goes wrong, and the unbalance of some crucial players in this process may set the conditions for a pathological evolution. The limit existing between a normal and an impaired condition is still not perfectly defined, and many molecules responsible for answering to the stimuli remain unknown. Among the numerous pathways involved in the modulation of growth, the Hippo signalling is keeping high the level of attention.

To better understand the purpose of this thesis, I will introduce the Hippo pathway in different contexts, as it has been inferred from studies carried out in *Drosophila*.

1.1. The Hippo Pathway

The Hippo pathway is a master regulator of growth. It was first discovered in *Drosophila melanogaster* in a genetic screening that identified the main players of this signalling, given that their mutations caused an over-growth phenotype (Harvey et al., 2003; Pantalacci et al., 2003; Tapon et al., 2002; Wu et al., 2003; Xu et al., 1995) (Fig. 1). Nowadays, most of the components have been successfully identified in mammals, proving the pathway is highly

conserved. It was initially defined as the "Salvador-Warts-Hippo (SWH) pathway" as these were the first proteins identified that constitute the core kinase cassette (Harvey and Tapon, 2007). Hippo (Hpo) and Warts (Wts) are the serine/threonine kinases, whereas Salvador (Sav), with Mob as tumour suppressor (Mats) are the scaffold proteins. Once it is activated by autophosphorylation, because it is a member of the sterile-20 family kinase, Hpo can directly activate by phosphorylation Wts, which is responsible for the inactivation of the final effector of this kinase cascade, Yorkie (Yki). To contrast the constant activation of Hpo due to autophosphorylation, other mechanisms are involved, mostly unknown. Among them, recent studies have pointed the attention on STRIPAK (Striatin-Interacting Phosphatase And Kinase), that thanks to a feedback loop is able to inhibit Wts through its phosphatase activity (Bae et al., 2017; Zheng et al., 2017). On the contrary, there is also evidence of a Hpo-independent activation of Wts that occurs by the two kinases Happyhour (Hppy) and Misshapen (Msn), members of the MAP4K subfamily (Li et al. 2014; Meng et al. 2015; Zheng et al., 2015). The Rho-type guanine nucleotide exchange factor Pix (PAK-interacting exchange factor) and GPCR kinase-interacting protein (Git) have also been suggested to influence Hpo kinase activity by facilitating Hpo dimerization and autophosphorylation (Dent et al. 2015). One key regulatory mechanism of the Hippo pathway core kinase cassette is related to the localisation of Hpo and Wts. It has been demonstrated that when the kinases are localised at the apical membrane of the cell, this results in an enhancement of their activity on Yki (Deng et al. 2013; Sun et al., 2015a).

Yki is the last component of the core of the pathway and is a transcriptional coactivator (Huang et al., 2005). Its inhibition occurs through phosphorylation at three separate serine residues: S111, S168 and S250. Among these, serine S168 appears to be the most important one because it is bound by the 14-3-3 phospho-peptide binding proteins, that lead to the retention of Yki in the cytoplasm and to its degradation, thereby suppressing its transcriptional activity (Oh and Irvine, 2008; Ren et al., 2009). This important mechanism is extremely conserved, as also Yes-Associated Protein 1 (YAP) and the Transcriptional coactivator with PDZ-binding motif (TAZ), the Yki homologues in humans, show equivalent sites of inhibition at S127 and S89 respectively (Chen et al., 2015). Yki carries out its function

in the nuclei where it drives the expression of numerous genes involved in cell cycle regulation, such as *cyclin E* and the inhibitor of apoptosis *dIAP1*, the growth promoter *myc* and the growth and cell survival-promoting miRNA *bantam*, which drive cell proliferation and cell survival (Harvey et al., 2003; Huang et al., 2005; Jia et al., 2003; Neto-Silva et al., 2010; Pantalacci et al., 2003; Tapon et al., 2002; Thompson and Cohen, 2006; Wu et al., 2008; Zhang et al.,2008b; Ziosi et al., 2010). Because of its fundamental function, Yki is tightly regulated, and its localisation plays a dramatic role in this process. It has been demonstrated that Yki moves from the cytoplasm to the nucleus in a dynamic way guaranteed by a different ratio in nuclear import/export, that is peculiar for each phase of the cell cycle (Manning et al., 2018). Beside this, another class of target genes transcribed by Yki includes some upstream negative regulators of Yki such as *expanded* (*ex*) (Hamaratoglu et al., 2006), *kibra* (Genevet et al., 2010), *crumbs* (*crb*) (Hamaratoglu et al., 2009), *four-jointed* (*fj*) (Cho et al., 2006) and *wts* (Jukam et al., 2013), which constitute a negative feedback loop that maintains signalling homeostasis.

Yki is able to bind DNA only when complexed with transcription factors; the most well characterised is the TEAD family member Scalloped (Sd) (Goulev et al., 2008; Wu et al., 2008). Notably, even if the overexpression of both Yki and Sd leads to an overgrown tissue, that can be rescued by the depletion of Sd, this interaction is not exclusive. It is known that Yki has different binding partners, such as Homothorax (Hth), with which leads the expression of *bantam* and *myc* in two different larval discs (Peng et al., 2009; Slattery et al., 2013; Ziosi et al., 2010). Sd has also some functions independent of Yki: as an example, it binds Vestigial (Vg) in the developing wing and drives the transcription of some wing specification genes (Halder et al., 1998). In this way it results in an opposite outcome for the cells, respect to what would be dictated by the Sd-Yki combination (Halder and Carroll, 2001). Moreover, it was observed that the loss-of-function (LOF) of Sd restores a normal growth condition also in a Yki LOF background. As a consequence, the most corroborated model is a *default repression* operated by Sd, in alliance with other corepressor factors such as the Tondu-domain-containing growth inhibitor (Tgi), in which the target genes of the Hippo pathway are actively repressed in the absence of Yki (Koontz et al., 2013). This model can explain why

growth is severely compromised by the LOF of Yki but not by that of Sd, as the loss of Yki represses, while loss of Sd derepresses, growth-promoting genes (Zecca and Struhl, 2010).

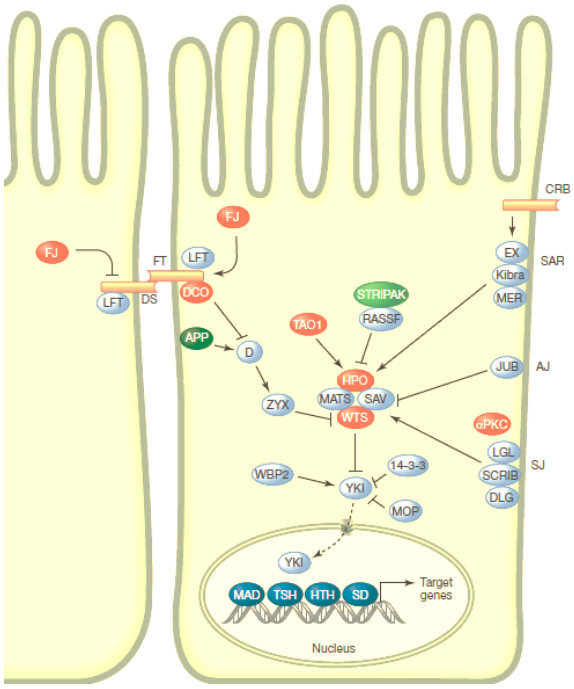

Fig. 1 The Drosophila Hippo pathway (adapted from Harvey and Hariharan, 2012; permission requested to Cold Spring Harbour Laboratory Press).

Since the discovery of the kinase core components, more and more upstream regulators have been found to be implicated in the modulation of the Hippo pathway. These include transmembrane proteins, structural components of the cell such as the actin cytoskeleton, junctional proteins and cell polarity components. From that, it is deducible the ability of this pathway to integrate multiple cell-cell communication signals coming from outside, as well as from the inner compartments, and to translate these inputs into a transcriptional response. The Hippo pathway regulators were deeply studied first of all in the epithelial development, where apical-basal and planar cell polarity are key processes in the balance between cell proliferation and differentiation, and where the inter-cell communication results fundamental to guarantee the normal organisation and growth within a tissue.

Fat (Ft) is a large atypical cadherin able to modulate growth and planar cell polarity in Hippo-dependent and independent ways (Bennett and Harvey, 2006; Bryant et al., 1988; Reddy and Irvine, 2008; Sopko and McNeill, 2009; Silva et al., 2006; Tyler and Baker, 2007). The counterpart of Ft is Dachsous (Ds), another cadherin with which Ft forms a heterodimer at the adherens junction of the cell. Both are able to send signals to the Hippo pathway, even if in a different way. Ft signalling requires the myosin Dachs (Cho and Irvine, 2004). Dachs, and also Zyxin, another protein that concentrates at focal adhesions and along the actin cytoskeleton, work downstream of Ft and bind Wts, thus influencing its protein levels (Cho et al., 2006; Rauskolb eta t., 2011). Furthermore, Ft is able to inhibit Yki in a cell-autonomous way affecting the levels of membrane-localised Ex (Bennett and Harvey, 2006; Cho et al., 2006; Tyler and Baker, 2007). Ft activity is regulated by its interaction with Ds, which is modulated by phosphorylation of the extracellular domains of Ft and Ds by the kinase Fj, which localises at the Golgi and is itself a Yki target (Brittle et al., 2010; Casal et al., 2006; Cho and Irvine, 2004; Ma et al., 2003; Matakatsu and Blair, 2004; Matakatsu and Blair, 2006; Silva et al., 2006; Simon et al., 2010; Strutt and Strutt, 2002; Willecke et al., 2006). In this way, Fj promotes the activity of Ft and reduces that of Ds, and this may explain why a different gradient of Fj and Ds is visible along the imaginal wing disc cells, that is responsible, with the other cell polarity modulators, for regulating proliferation along the tissue.

The above mentioned Kibra and Ex, together with the FERM domain protein Merlin (Mer), form a complex localised at the apical junctions that regulates the Hippo signalling. First of all, Ex is able to bind directly the WW domains of Yki through its PPxY motifs. In this way, the final effector is retained next to the apical domain and inhibited in a faster way, skipping the kinase core of the pathway (Badouel et al., 2009; Oh et al., 2009). Wts has been shown to be recruited at the apical membrane by both Mer and Ex to facilitate the interaction with Hpo and Sav, where these latter are activated directly (Genevet et al., 2010; Sun et al., 2015a; Yin et al., 2013). Downstream of Mer and Ex there is also the sterile-20 kinase Tao, which can directly activate Hpo (Poon et al., 2011).

Taken together, these observations show a redundancy in the regulation of the Hippo signalling, that appears to be fundamental to guarantee a compensation effect in case of a mutation occurring in one of the members of the pathway. It has to be noticed that the ability

of Mer to bind Wts and relocate it next to the apical membrane is directly related to the interaction between Mer and the actin cytoskeleton (Yin et al., 2013).

The actin cytoskeleton regulates and is regulated by the Hippo pathway. The signalling acts in this context as a bridge connecting the mechanical inputs to the proliferative outcome. Even if most of the mechanisms that lay behind are still to uncover, it has been demonstrated in the wing imaginal disc that mutations disrupting the apical component Ex, as well as the kinase core components Hpo, Wts and Mats, cause an increase in the filamentous actin, maybe through the involvement of Spectrins, connection proteins linking the actin cytoskeleton and the cell membrane. On the other hand, a deficit in the actin capping proteins leads to a higher activity of Yki (Deng et al. 2015; Fernandez et al., 2011; Sansores-Garcia et al., 2011; Wong et al., 2015).

Crumbs (Crb) is a large single-pass transmembrane protein with a short intracellular domain with a FERM motif able to directly bind the FERM domain of Ex, thus recruiting it at the apical membrane and promoting its activity (Chen et al., 2010; Robinson et al., 2010). Its mutation causes an overgrowth phenotype, characterised by the decrease of many Yki targets, especially *ex* (Ling et al., 2010; Richardson and Pichaud, 2010). Crb is important for the apical-basal polarity determination within a tissue. It is required for the proper localisation of Crb on the neighbouring cells and acts in a cell-cell contact-dependent manner that leads to the activation of the atypical Protein Kinase C (aPKC) and Par-6 (Izaddoost et al., 2002; Morais-de-Sà et al., 2010; Walter and Pichaud, 2010; Tanentzapf et al., 2000).

The Jub protein, member of the Ajuba protein family, is an adaptor that localises at the adherens junction. Jub physically interacts with Wts and Sav, inhibiting Yki phosphorylation and, in this way, promoting cell proliferation (Das Thakur et al., 2010). *jub* mutants show a growth deficit associated with an increase in apoptosis, typical traits of Yki mutants. It can be inferred that Jub is in part responsible for the contact-dependent inhibition of growth; the formation of the adherens junction recruits Jub and impedes the action of Jub on Wts, thereby allowing the Hippo pathway to repress Yki/YAP and cell proliferation. Another important modulator of the pathway is Lethal giant larvae (Lgl). It localises at the basolateral membrane of epithelial cells, and with Discs large (Dlg) and Scribble (Scrib) is a fundamental player in cell polarity determination. The genes encoding these polarity proteins are defined

as “neoplastic tumour suppressor genes” as their mutant forms cause loss of basolateral markers and the expansion of apical markers, leading to defects in apical-basal cell polarity, and result in massively overgrown imaginal discs and brains (Froldi et al., 2008; Humbert et a., 2008; Grzeschik et al., 2010). The link with the Hippo pathway is given by the evidence that *lgl* mutant clones in some regions of the imaginal wing and eye discs are characterised by an increased proliferation and a decrease of apoptotic response associated with the relocation of Yki in the nuclei and over-expression of the Hippo target genes (Grzeschik et al., 2010; Parsons et al., 2014; Zhao et al., 2008).

In conclusion, it is evident how the Hippo pathway fulfils a central role in regulating cell proliferation. The intricate connection between its members and many different molecules that act at multiple levels within the cell allows this signalling pathway to be exquisitely sensitive to perturbation of normal tissue and cellular integrity and, at the same time, it is able to answer in a very accurate way and to finalise the signals into different cellular outcomes. This ensures that any deviation from the normal tissue and cellular architecture in development can be effectively restored by compensatory cell proliferation mediated by the Hippo pathway effector Yki.

1.1.1 The Hippo pathway as cell fate decision maker: pupal photoreceptors

Two dimensions decide cell specification: *time* during development, and *space* within the tissue. Depending on the context, the Hippo signalling can suppress growth and induce cell differentiation in organ development, mediate stress-induced apoptosis or promote tissue-resident stem cell proliferation, and tissue repair. The different outcome is conditioned by at least two factors: first, Yki, as well as its mammalian orthologue YAP, has multiple binding partners, and second, the transcriptional output of the Hippo signalling is cell-type dependent.

In this session I will focus on the role of Hippo in cell fate specification during pupal and adult eye development.

In the fly compound eye, each of the about 800 ommatidia is a single optical unit that contains 8 photoreceptor cells (PRs). The extremely expanded membrane of these cells, the rhabdomeres (R), expresses a distinct type of *Rhodopsin* (Rh), that is the photosensitive protein responsible for the visual properties of the eye. The type of *Rhodopsin* expressed depends on the type of photoreceptor. Two of them are coupled: R7 and R8, which are the inner PRs. Each R7 and R8 expresses only one type of Rh between Rh3, Rh4, Rh5, Rh6 in a tightly regulated manner (Hardie, 1985; Cook and Desplan, 2001; Rister et al., 2013). Of note, the two PRs share a common optic path, with R7 positioned above R8 (Fig2).

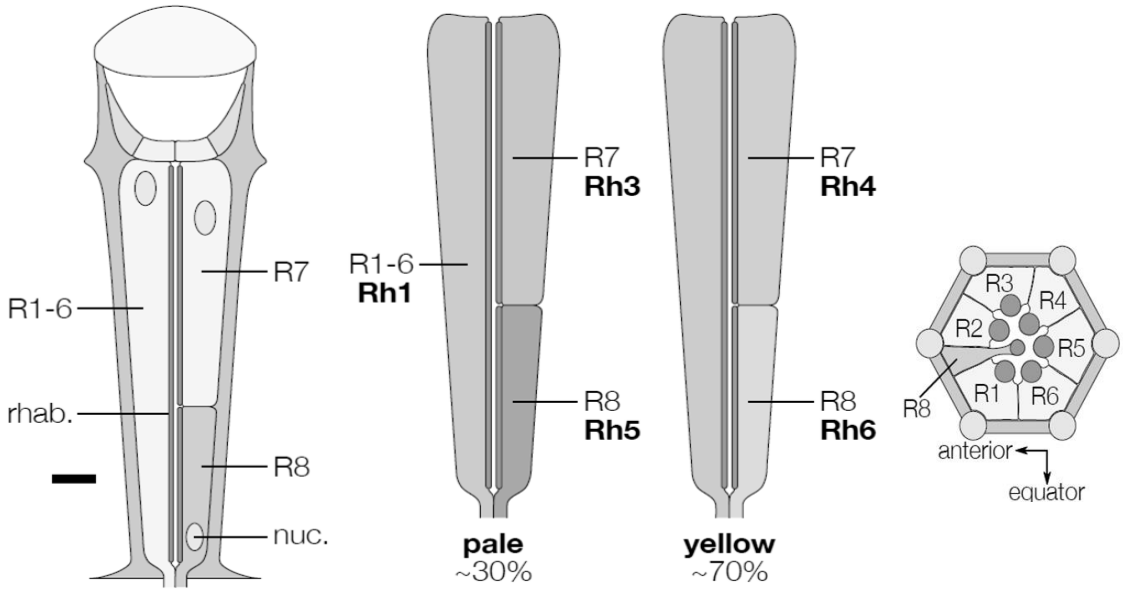

Fig.2 *Anatomy of the Drosophila photoreceptors: it is highlighted the position and the type of rhodopsin expressed by each PRs in the optic unit. On the left a vertical section, on the right a cross section* (*adapted from Wolff and Ready, 1993*; *permission requested to Cold Spring Harbour Laboratory Press*).

The activation in the expression of the different Rh appears to be randomly but constantly maintained with a proportion of 70% PRs defined as *yellow* and of a 30% PRs defined as *pale*. At the same time, as in other sensory systems, the expression is mutually exclusive (Fortini and Rubin, 1990; Mazzoni et al., 2004; Montell et al., 1987). In the pale subtype, R7 expresses the UV-sensitive Rh3 and R8 the blue-sensitive Rh5. In the yellow subtype, R7 expresses a distinct UV-sensitive Rh4 while R8 expresses the green-sensitive Rh6. The first

choice is made by R7 cells, that then, through a still unclear mechanism, induce the expression of Rh5 or Rh6 in the R8 cells (Mikeladze-Dvali et al., 2005).

It has been shown that Wts and Yki play a critical role in promoting the expression of Rh5 and Rh6 in the R8 cells. They work in a bi-stable negative feed-back loop that involves other molecules, such as the two transcription factors Orthodenticle (Otd) and Traffic jam (Tj), both promoters of Rh5 expression. On one side, Wts represses the activity of Melted (Melt), an insulin signalling modulator and known activator of Rh5 expression, and turns on the expression of Rh6. The activity of Wts results from upstream activators such as Mer, Kibra and Lgl. Conversely, on the other side, Melt represses Wts, activating in this way Yki that leads to Rh5 expression through Otd and Tj. In a redundant way, Yki promotes the expression of Melt and represses its inhibitor Wts, in order to guarantee a unique and robust cell fate decision (Jukam and Desplan, 2011; Jukam et al., 2013; Mikeladze-Dvali et al., 2005). More recently, Sd was found to act as a direct repressor of Melt and Rh5 genes in yellow PRs; at the same time, in pale PRs, Sd is required to promote the translation of Rh5 protein through a 3'UTR-dependent and microRNA-mediated process. In these ways, Sd can drive context-dependent cell fate decisions through opposing transcriptional and post-transcriptional mechanisms (Xie et al., 2019). This represents just one example of how the Hippo pathway is enrolled to promote different cell outcomes, and how this is related to the presence of unique factors that cooperate with the Hippo components.

1.1.2 Deregulation of the Hippo pathway

The impairment of the Hippo pathway is a recurrent characteristic of many diseases, above all, many types of cancers. The first evidence was obtained in mice, where both the hyper-activation of YAP or the inhibition of the upstream regulators of the pathway led to the development of many cancerous masses in different tissues, with the peculiar involvement of the liver (Camargo et al., 2007; Dong et al., 2007; Song et al., 2010). Looking at the literature, there aren't many mutations identified among the Hippo components, but it appears clear that the alteration of this pathway, and the consequent over-proliferation and the escape from apoptosis, are typical traits of many tumour conditions where YAP and TAZ

are found hyper-expressed (Wang et al., 2010; Xu et al., 2009). The hyper activation of YAP and TAZ is able to transform, both in *in vitro* and *in vivo* models, the wild-type context into a pro-tumoral one, increasing genomic instability and growth potential, impeding cell-contact inhibition and promoting invasiveness (Chan et al., 2008; Fernandez-L et al., 2012; Zhao et al., 2012). The higher levels of YAP and the concomitant nuclear localisation, synonym of its effective activity ongoing, are correlated with poor prognosis in many tumours, such as non-small cell lung cancer, breast cancer, gastric and colorectal cancer, hepatocellular and renal carcinoma, many types of sarcoma and even in leukemia (Basu-Roy et al., 2016; Bos et al., 2009; Chen et al. 2015; Lam-Himlin et al., 2006; Liang et al., 2014; Lo Sardo et al., 2018; Masayuki et al., 2017; Shutte et al., 2014; Wang et al., 2015). For some of these tumours, it has been demonstrated that YAP inhibition gives rise to a less invasive and differentiated phenotype (Chai et al., 2017; Fitamant et al., 2015; Li et al., 2016). In general, cancers show an impairment of the kinase core, associated with a hyper activation of YAP and so, with a hyper expression of its targets.

More recently, it has been shown the involvement of the Hippo pathway in neurodegenerative diseases. It is known that the pathway plays a role in the proper development of brain tissues and controls neuronal and glial growth (Ahmed et al., 2015; Cappello et al., 2013; Ciani et al., 2003; Huang et al., 2016; Lavado et al., 2014; Lin et al., 2012; Sakuma et al., 2016). What was unknown is its role in maintaining neuronal health. Even if evidence is still few, it has been demonstrated that YAP functions as a neuroprotector in many pathological conditions. YAP is fundamental for astrocytic proliferation and its knock out leads to death of neocortical neurons (Huang et al., 2016). Together with SOCS3 (suppressor of cytokine signalling 3), it prevents reactive astrogliosis and inflammation (Huang et al., 2016). In Poly-Q mediated neurodegeneration, YAP is able to reduce the pathogenicity of inclusion bodies both by activating its targets *cyclin E* and *bantam* and also negatively regulating immune deficiency and Toll pathway (Dubey and Tapadia, 2017). In Huntington disease (HD) YAP is found deeply decreased in the nuclei of brain neurons, and the only form detectable was the phosphorylated, inactive one. At the same time, MATS and LATS, the human orthologues of Hpo and Wts respectively, are found active in the cortical neurons of the same HD patients, thus demonstrating that the activation of the upstream

components of the Hippo pathway, together with a strong downregulation of YAP, can significantly contribute to transcriptional dysregulation and neuronal death in HD patients (Mueller et al., 2018; Yamanishi et al., 2017). In mouse models of Amyotrophic Lateral Sclerosis (ALS), mutations in Sod1 are associated with increased MST1 activity in motor neurons. The positive feedback between oxidative stress and MST1 may fuel this status and enhance apoptosis of affected neurons (Lee et al., 2013; Rawat et al., 2013).
Altogether, these studies shed light on another function of the Hippo pathway as a possible mediator of neuronal impairment, thus confirming a central role for this signalling in many different tissues, both in normal growth and in pathological landscapes.

Until few years ago, the common thought described the Hippo pathway characterised by two functional states: active, when YAP/Yki is phosphorylated by Warts and can't enter nucleus; inactivated, when YAP/Yki is unphosphorylated and, therefore, is able to enter nucleus and interact with tissue-specific DNA-binding transcription factors to turn on target genes' expression. Recent evidence has instead shed light on a different model no more based on a simple "on/off" status. These studies concluded that the majority of YAP/TAZ/Yki molecules constantly shuttle between the nucleus and cytoplasm in a rapid and dynamic way (Ege et al., 2018; Elosegui-Artola et al., 2017; Kofler et al., 2018; Manning et al., 2018). The Hippo pathway works by controlling the rate at which this happens in different contexts in a cell-specific manner, but also depending on the physiological and pathological conditions. This system appears much more sensible to the background variations, and offers a refined response, which can lead to a broader spectrum of nuclear to cytoplasmic YAP/TAZ/Yki ratios and, therefore, different levels of target genes' transcription activation. The strong variability that follows from cell to cell doesn't change the role of the Hippo components, where the upstream ones are generally defined as "tumour suppressors", while the downstream effector YAP/Yki is well-known as a "growth promoter".

1.2 *Drosophila* as a model

The fact that 6 Nobel prizes were won by scientists who have used *Drosophila* as a model is only a confirmation of the importance that this tiny organism fulfils in the scientific field.
Since its first application for studying genetics adopted by Thomas Hunt Morgan, *Drosophila* has been used to address many questions about normal and pathological conditions. The major advantage of using the fruit fly is given by the multiple powerful genetic tools developed along the years, from gene knock-out to gene over-expression, that permit different analyses within a tissue, or a cluster of cells, or in the whole organism, in a defined time window. The high amenability of this organism is accompanied by an easier way to do it and to analyse the results, a statistic relevance, lower genetic redundancy and *low-cost* trials, that have no comparison with the other model systems used. This explains also why *Drosophila* is often exploited for genetic screens that have the purpose to find out the function of countless genes and to uncover the mechanism that lay behind a certain developmental process (St. Johnston 2002). At the same time, the knowledge acquired from these studies has a profound impact on the understanding of human normal tissues development and pathological conditions causes, because of the large genome and function homologies between flies and other species, including humans. More than 75% of genes involved in human diseases have a homologue in *Drosophila* (Reiter et al., 2001). Numerous studies use the fly as a vehicle to understand the mechanisms by which an oncogene or a tumour suppressor gene modulate the outcome of a tissue; dysregulating these genes in *Drosophila* allows studying multiple aspects of cancer transformation, including the invasive potential and the metastatic phase.

Drosophila is currently used as a good model for Parkinson Disease, Alzheimer Disease, Tauopathies and Polyglutamine (polyQ) diseases (Lu and Vogel, 2009). These human pathologies with an age-related onset are caused by progressive neuronal loss caused by inflammation of the neuronal compartment. The short life cycle of *Drosophila* represents an incredible advantage, making it possible analysing neurodegenerative mechanisms from early initiation events to the terminal stages. Then, fly's eyes and photoreceptor neurons

have proven to be a good tool for examining neurodegeneration arising from gene mutation; indeed, degeneration of photoreceptor neurons indicate neuronal cell loss, and can be easily quantified by the decrease in the number of ommatidia (Jackson et al., 1998). The combination of molecular analysis and behavioural assays, along with a strong statistics, make *Drosophila* a suitable and reliable model.

The fruit fly has a life cycle characterised by four phases: the embryo, the larva, the pupa, and the adult. Different tissues and different organs, as well as the entire organism, can be manipulated and used to study genes and biological processes. In this thesis I will describe the eye as one of the most suitable organs, commonly used as a model, and protagonist of many different experiments in my project.

1.2.1 *Drosophila* eye

The *Drosophila* eye has always been a theatre for dramatic discoveries: from the white-eyed fly and the consequent X chromosome mapping and X-linked recessive inheritance at the beginning of the century, to the most recent Hippo pathway and its role in controlling growth (Morgan, 1910; Stevens, 1905; Tapon et al. 2002; Wilson, 1905). The larval diploid epithelial tissues are defined as imaginal discs. The imaginal discs originate in the embryo and undergo an incredibly rapid process of proliferation and specialisation, giving rise to the highly complex precursors of the adult organs in a time window of a few days. The eye-antennal disc is a monolayer epithelium that gives rise to the entire visual system, the olfactory organs and the head epidermis (Fig. 3) (Baker, 1978; Haynie and Bryant, 1986; Gehring, 1966; Ouweneel, 1970; Vogt, 1946; Weismann, 1864). The overall shape that changes from small water droplet to an oval one around the second instar larva, is the result of oriented cell divisions, morphogen gradients, individual cell changes and mechanical forces acting within the tissue (Hariharan, 2015; Romanova-Michaelides et al., 2015; Shingleton, 2010).

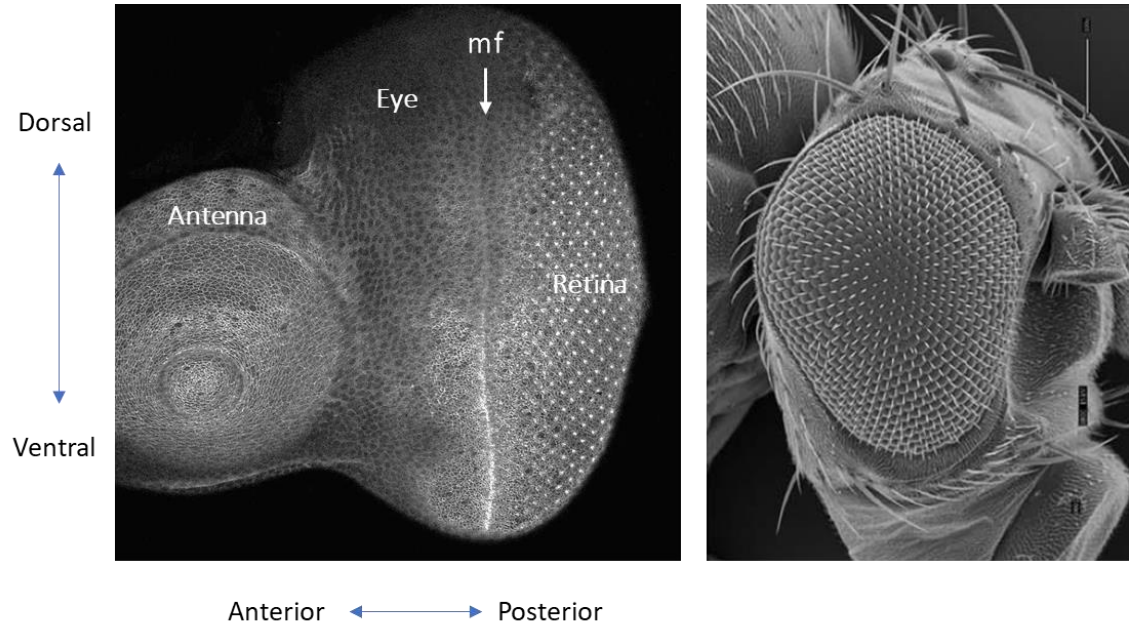

Fig. 3 *Eye disc (ed) and adult eye (ae). The confocal image shows a staining for the basolateral cell membranes. Photo taken at the Transmission microscope. mf: morphogenetic furrow*

It is divided into dorsal and ventral compartments and cell fate is established by the retinal determination network (RDN) (Kumar, 2010). During the third larval instar, a groove called the morphogenetic furrow (mf) sweeps progressively across the eye imaginal disc from posterior to anterior, creating a *wave* of differentiation, where the cells behind the mf are undifferentiated, whereas the cells after the mf are going to become the single ommatidia (Tomlinson et al., 1987; Wolff et al., 1993). In this way, the cell division patterning, since that moment symmetric, becomes asymmetric from the third stage. The progression of the furrow is guaranteed by multiple signalling pathways: Hedgehog (Hh), Decapentaplegic (Dpp) and Epidermal Growth Factor (EGF), all play important roles (Roignant et al., 2009). The first mitotic wave sets the number of ommatidia. R8 are the founding photoreceptor cells, followed by R2/R5 and R3/R4. The second mitotic wave is required to generate the remaining cells necessary to complete each unit of the eye: R1/R6/R7 and the cone cells. The R8 cell plays a crucial role in the recruitment of pairs of all other photoreceptors through a sequential use of the EGF receptor pathway (Freeman, 1996). The growth continues till the early stages of the pupal phase, when all the remaining cells not specified to be photoreceptors or cone cells, adopt one of the three pigment cell fates (Cagan and Ready,

1989). All the excess cells are eliminated by apoptosis occurring during the mid-pupal stage, such that only a single secondary cell exists along the long faces of each ommatidium and that only a single tertiary pigment cells occupies the vertices (Larson et al., 2010; Miller and Cagan, 1998; Wolff and Ready, 1991b). Notably, the morphogens seem to play a role not only in promoting proliferation but also in preventing organs from growing beyond a predetermined maximum size (Hariharan et al., 2015; Shingleton et al., 2010). These synchronised growth signals will generate the compound adult eye, composed of about 600-800 ommatidia. It is important to know that these signals come also from "above". The peripodial epithelium is a squamous monolayer coat, connected with the eye-antennal disc along the edges by a narrow band of cuboidal margin cells, which are part of the peripodial epithelium too (Chen, 1929; Lim and Choi, 2004; McClure and Schubiger, 2005). Hh and Dpp are secreted by the peripodial cells and sent to the disc proper cells through actin filaments and microtubules; disruption of this layer of cells indeed leads to disruption of the entire morphology of the eye (Cho et al., 2000; Gibson and Schubiger, 2000). Each ommatidium derives from cell clusters of around 20 cells, called rosettes, that form in the mf. After the separation of few of them, the remaining will form the pre-cluster, which is the ommatidium precursor (Wolff and Ready, 1991). The core of the adult ommatidium, as described above, is composed of eight photoreceptors (R1-R8), four cone cells and two primary pigment cells. Each ommatidium then shares some other pigment cells and three mechano-sensory bristles with the surrounding neighbours (Waddington and Perry, 1960). The R1-R6 rhabdomeres compose the outer region, whereas R7 and R8 are positioned centrally. The entire morphology is well defined as an asymmetric trapezoid (Dietrich, 1909). The photoreceptor axons are sent into the brain optic lobe and the signal output reaches the lamina, for the R1-R6, or the medulla, in the case of R7-R8 (Clandinin and Zipursky, 2002; Fischbach, 1983; Mollereau et al., 2001). The expression or the lack of expression of the transcription factor Spinless in each R7 cell dictates the random activation of rhodopsin. About 35% of ommatidia will express UV-Rh3 in the R7 and blue-sensitive Rh5 in the R8 cells; the others 65% will express UV-Rh4 in the R7 and green-sensitive Rh6 in the R8 cells (Johnston et al., 2011; Johnston and Desplan, 2014; Rister et al., 2013; Wernet et al., 2006).

1.3 The Gaucher disease

The Gaucher disease is the most common lysosomal storage disorder, affecting approximately 1 out of 50,000 births worldwide (Martins et al., 2009). It is a rare autosomal recessive disorder caused by the mutation of the *GBA1* gene located on the chromosome one. This gene encodes the acidic β-glucocerebrosidase (GCase), which is a lysosomal enzyme responsible for the cleavage of glucosylceramide and glucosylsphingosine into glucose and ceramide and sphingosine. Mutations of the GCase lead to the accumulation of the substrates inside the cells and, often, to systemic manifestations that involve spleen, liver, bone marrow, lungs and nervous system (Cox et al., 2015). Even if it is a single gene mutation, the pathological phenotypes resulting from that are multiple.

The clinicians distinguish three categories (Grabowski et al., 2008). The Type 1 (GD1) is the most common and doesn't involve the neuronal compartment, so it is so far considered a systemic, non-neuropathic form. It can be asymptomatic, with the onset at any age. Although GD1 is considered the non-neuropathic form, neurological symptoms including peripheral neuropathy and Parkinsonism have been reported in systematic studies. (Capablo et al., 2008). Recent studies have identified some *GBA1* mutations as one of the major risks factor responsible for the increased incidence of Parkinson disease (Eblan et al., 2005; Goker-Alpan et al., 2004; Lwin et al., 2004; Sidransky, 2006). The Type 2 (GD2) is the acute neuropathic form, characterised by a severe involvement of the nervous system that often leads to death within few years after birth (Stone et al., 2000). The Type 3 (GD3) is instead described as the chronic neuropathic form, which patients show neurological defects already during the infant age that persist throughout the entire life (Sidransky and Lopez, 2012). However, these three categories are not tightly separated, indeed there are many cases described in literature regarding intermediate variants especially between GD2 and GD3: because of that, different types of GD may alternatively be considered as part of a phenotypic continuum (Cherin 2006; Sidransky 2004). It remains unclear how the alteration of a single gene can create such a variability in the pathological condition, and the molecular mechanisms that lay behind are under study.

1.3.1 Molecular basis

The *GBA1* gene is located on chromosome 1q21-22, separated by 16 kb from a GBA pseudogene with 96% identity (Bru Cormand et al., 1997; Horowitz et al., 1989). More than 300 mutations have been described so far (Smith et al., 2017). The different variants can be more represented in particular ethnic groups as well as in particular pathological phenotypes. For example, N370S is mostly causative of GD1 and is particularly frequent among Ashkenazi Jewish, and rare among Chinese and Japanese people (Hruska et al., 2008). The Asian ethnic groups show more frequently the L444P mutation, associated with GD2 and GD3 (Pastores et al., 2000).

Normally GCase is synthesised on the rough endoplasmic reticulum (ER) by the ribosomes attached to the membrane. Then it is translocated inside the reticulum where it undergoes some important modifications and, after folding, it is addressed to the lysosomes through a specific transport. The development of a catalytically active enzyme is dependent on GCase glycosylation, especially with the occupancy of one site located at Asn19 (Berg-Fussman et al., 1993; Grace et al., 1990; Pol-Fachin et al., 2016). The N-glycosylation is necessary for the conformational stability of specific sites. The active site consists of a catalytic dyad, composed of two important residues, Glu 340 and Glu 235. The site is not always exposed, but the change in pH modifies the conformation of the protein, leading to the accessibility of the catalytic dock. The optimal pH for GCase activity is around 4.7-5.9, which is consistent with its lysosomal function (Lieberman et al., 2007; Liou et al., 2006; Tan et al., 2014). Around neutral pH (7.4), typical of the ER, the enzymatic activity is inhibited. The rearrangement of the enzyme is guaranteed by the flexibility of the loop that composes the protein, responsible also for the bound of the GCase to the membrane (Yap et al., 2015). Especially the loop 3 moves from an extended loop conformation to a helical shape when in acidic conditions (Lieberman et al., 2009). The mechanism, elucidated also through crystallography analysis, shows that, at neutral pH, residue Tyr 313 binds via hydrogen interactions Glu 325 and acts as a gate to keep the active site closed. When GCase reaches the lysosomal compartment and is ready to bind the substrates, Tyr 313 changes conformation to bind Glu 340 in the active site. This change causes the disruption of the hydrogen binding pattern of the Asp 315

residue, allowing loop 3 to take the helical conformation, thus inducing the opening of the catalytic site (Kacher et al., 2008; Lieberman, 2011; Lieberman et al., 2009). At the entrance of the active site there are several hydrophobic residues responsible for the vesicular transport of the enzyme and for its anchoring at the lysosomal membrane. Indeed, unlike the other mammalian lysosomal proteins, the transport of GCase from the ER to lysosomes does not follow an M6P-dependent pathway but needs the Lysosomal Membrane Protein 2 (LIMP2), and occurs into vesicles (Gonzalez et al., 2014). GCase binds a coiled-coil domain in the lumen region of LIMP2 at the neutral pH of the ER, and the protein complex persists until its entrance into the lysosome, where the low pH mediates its dissociation. (Reczek D, et al.,2007). The hydrophobic residues may also facilitate the interaction between GCase and the activator protein, Saposin C (Wilkening et al., 1998). Several studies found the Saposin C involved in the activation of the catalytic properties of GCase (Ho and O'Brien, 1971; Tamargo et al., 2012; Vaccaro et al., 2010). Mutations in the residues 443-445, such as L444P, lead to a more severe pathological phenotype (Atrian et al., 2008). Since now, the only mutant structure analysed is the N370S, that doesn't show dramatic alterations in the catalytic site, which explains also the mild phenotype arising from that. It has an increased thermostability, showing that the optimum pH shifts from 4.5 to 6.4, and as a result the loop 3 maintains the extended conformation across the pH range (Offman et al., 2010; Steet et al., 2006; Wei et al., 2011).

The final protein is composed of three non-contiguous domains (Fig. 4): domain I (residues 1-27, 383-414), consisting of a three stranded anti-parallel β-sheets, flanked by a loop and a perpendicular strand; domain II (residues 30–75, 431–497), an Ig-like fold formed by two β-sheets; and domain III (residues 76–381, 416–430), a central (β/α)8 TIM barrel common to most members of the glycoside hydrolases, holder of the catalytic site (Pol-Fachin et al., 2016). Mutations affecting the two non-catalytic domains are not known, whereas the domain III is protagonist of many alterations that can influence the distance between Glu235 and Glu340, as well as the N-glycosylation, both leading to the alteration of the catalytic dyad.

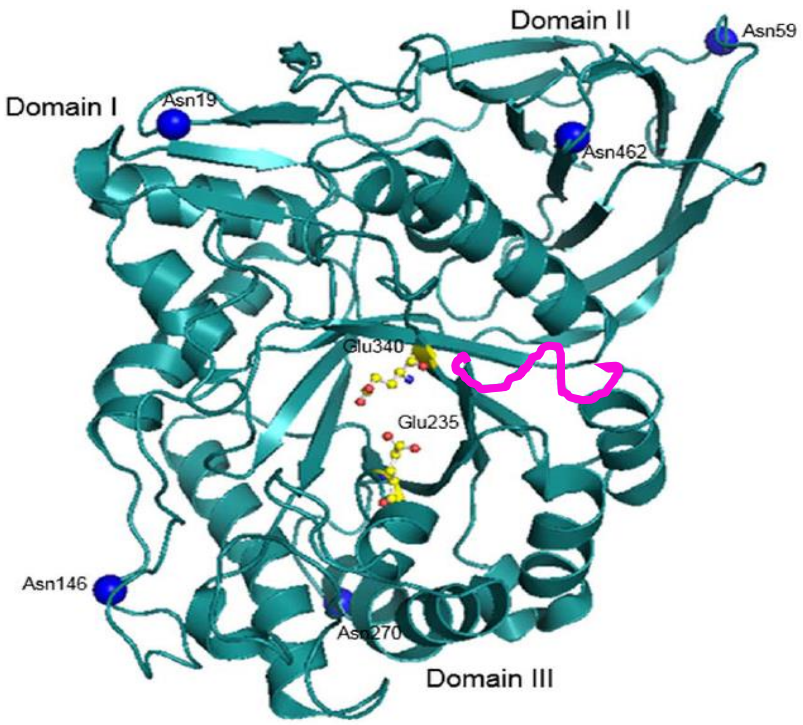

Fig. 4 X-ray structure of acidic-β-glucosidase showing the main components of the enzyme: N-glycosylation sites are presented as blue spheres. The active site catalytic dyad is shown as a ball-and-stick model. The loop 3 is evidenced in pink (Adapted from Smith et al., 2017 and with permission provided by Elsevier and Copyright Clearance Centre _ license number 4698851381818).

The *Gaucher cells*, typical trait of Gaucher patients, are macrophages, in other words cells which lysosomal functionality is essential. These cells show enlargement of the lysosomes, due to the failed catalysis of the GCase substrates, and a partial or total retention of the unfolded enzyme into the ER compartment. A damage in the lysosomal function produces as a consequence the impairment of the autophagy mechanisms (Aflaki et al., 2016a; Barak et al., 1999; Pandey et al., 2017). The result is the activation of the stress response within the cell, which finally leads to the activation of the inflammatory pathway and often to cell death (Aflaki et al., 2016b; Sun et al., 206; Vitner et al., 2012).

1.3.2 Gaucher studies in *Drosophila*

Drosophila melanogaster shows two orthologues for the human *GBA1*: CG31148 and CG31414, defined as *dGBA1a* and *dGBA1b* (Suzuki et al., 2015). The *dGBA1a* form is expressed primarily in the midgut, whereas *dGBA1b* is broadly expressed throughout development in a wide range of tissues, including the larval and adult brain and the fat bodies (Davis et al., 2016). They are ~2 and ~4 kb in size respectively, and occupy the same *locus* on chromosome 3, separated by the CG31413 gene, encoding an oxidase expressed only in the male accessory gland (FlyBase.org).

The true homologue is *dGBA1b*, sharing 35% of identity and 88% of similarity with the human gene (Maor et al., 2013). Its function is highly conserved: it encodes the *Drosophila* GCase enzyme and it is responsible for the metabolism of the glucosylceramide and glucosylsphingosine. In the last few years, it has been used as a model to study the Gaucher disease, as most of the pathological traits are perfectly reproducible in the fly. The *Drosophila* mutant for dGBA1b shows indeed an enlargement of the size and the number of the lysosomes, combined with the activation of the ER stress response (Kinghorn et al., 2016; Martinez et al., 2016; Suzuki et al., 2013). Moreover, some researchers evidenced the activation of the Unfolded Protein Response (UPR) and the consequent neuroinflammation typical of the neuronopathic Gaucher (Cabasso et al., 2019; Hindle et al., 2017; Maor et al., 2013). More and more studies aimed at better understanding the relation Gaucher-Parkinson, because of that it was analysed the α-Synuclein content in the adult brain of *GBA* loss of function individuals. This accumulation was then associated to the reduced climbing ability and lifespan of the mutant flies (Davis et al., 2016; Kawasaky et al., 2017; Maor et al., 2019).

Together, these observations illustrate how *Drosophila* can be used as a good model for studying the Gaucher syndrome and to fulfil the lack of information regarding the mechanisms that lead to the more severe neuronopathic form.

2. Aim of the study

The Hippo pathway plays a central and dynamic role in the cell. It is implicated in many important processes, which alteration has consequences on the correct development of the single cell as well as the whole tissue. Even if the major components of the signalling are known, it remains unclear which are the "co-workers" that permit the Hippo pathway to answer to so many different stimuli and act as a coordinator of the growth/differentiation balance.

In this thesis, I will use the fly model to investigate the role of Hippo in three different contexts.

During development, many pathways are repurposed to lead different cellular fates. This is the case for the Hippo pathway in the fly photoreceptors. I will define the contribution of the Hippo core kinase Warts in leading the R8 fate choice, by analysing the timing of its expression and localisation in the pupal eye.

The second part will focus on the Gaucher disease. Here I will show some preliminary data that describe a partial impairment of the pathway in *GBA* knockout and knockdown contexts, thus opening the possibility of its involvement in the more severe neuronopathic GD form.

The last part will instead shed light on the transcription factor Orthodenticle (Otd) as a possible co-factor of Yki in producing an overgrowth phenotype.

Overall, this thesis aims at adding some new knowledge to the complex mechanism of action of the Hippo pathway, both in normal growth and in a pathological context.

3. Materials and Methods

- Fly stocks, fly rearing and genetic manipulation

Fly stocks were as follows, those not held or produced in our lab or in Professor Harvey' lab are specified:

w^{1118}

Wts-venus/TM6b

$Gba1b^{KO}$ (L. Partidge)

$Gba1b^{KD}$ (Bloomington 38970)

Minute, en-Gal4, UAS-GFP

GMR-Gal4 (Bloomington 1104)

UAS-mCD8::GFP (Bloomington 5137)

repo-Gal4/TM6b

elav-Gal4 (Bloomington 8765)

Gba1b-Gal4 (Bloomington 78943)

UAS-lacZ (Bloomington 8529)

$UAS\text{-}luc^{KD}$ (Bloomington 35788)

UAS-Otd (Bloomington 5542)

$UAS\text{-}Otd^{KD}$ (Bloomington 29342)

UAS-Sd

$UAS\text{-}Sd^{KD}$ (VDRC 101497)

GMR-Gal4 UAS-Sd

GMR-Gal4 $UAS\text{-}Sd^{KD}$

GRM-Gal4, $UAS\text{-}yki^{S168}$

GMR-Gal4 UAS-Yki (D. Pan)

hs-flp; act::Gal4, UAS-GFP/TM6b

Each fly stock used was grown on the same medium at 25 °C. Few exceptions were for the *Minute, engrailed* flies, the glial *versus* neuron experiments, and the analysis of Otd alterations in *sd*-overexpressed or knockdown contexts, that were maintained at 29°C. The reason for modifying the temperature is to increase the effect of the UAS-Gal4 binary system mutuated from *S. cerevisiae*: Gal4 is the driver line, in which the transactivating protein GAL4 is placed under the control of a specific promoter, with its own spatial and temporal patterns, whereas the Upstream Activating Sequence (UAS) is localised upstream of the *locus* controlled by the UAS-Gal4. These two elements are not found in the fly genome, thus their introduction permits an extremely specific control of transgene expression. The increased temperature enhances the effect caused by the transgene in the tissue districts controlled by the chosen promoter (Brand and Perrimon, 1993).

The clonal experiments were performed using an actin-Gal4 flip-out promoter, that drove the overexpression of Otd and GFP. This was obtained through a heat shock of 10 min at 37°C at 48h after egg laying (AEL). This thermic shock provoked the activation of the flippase, responsible for the activation of the transgene, silenced since then. The tissue has been characterised by the presence of clonal areas, GFP positive, in which the transgene is expressed. After the heat shock, the larvae were moved from 25°C to 29°C and dissected on the 5^{th} day AEL.

- Dissection and Immunofluorescence

The dissection of each tissue analysed was performed through the use of thin forceps and following the instructions given in JoVE protocols (DeAngelis and Johnson, 2019; Kelly et al., 2017), all in a dissection dish filled with PBS 1% solution (Phosphate-buffered saline). Then the samples were fixed in PFA 4% (Paraformaldehyde) for 20 min. After that, the samples were cleaned from the fat bodies and tracheal tubes residues, in PBS 1% solution. At the end they were moved in a 1.5ml Eppendorf for the subsequent steps. The permeabilization of the membranes was performed by washing the sample with PBS Triton (PBST) 0.3% solution (3 times for 10 min), which is a detergent used also in tissue culture analysis. The incubation with the primary antibody was preceded by the block of aspecific sites through NGS (Normal

Goat Serum) or BSA (Bovine Serum Albumin) at the concentration of 5% with PBST, for 60 min. The primary antibody at the defined concentration was added to the blocking solution, and the vial was put at 4°C overnight. In this critical phase I facilitated the mixing, wherever possible, using nutator and trying to ensure that the internal face of the tissues was exposed to solutions in the tube during the procedures. The day after, 3 washes in PBST favoured the elimination of the excess antibody, before the staining with the secondary antibody; the latter was put at 4°C overnight as the primary (exception for the fat bodies immunofluorescence where the secondary was put for two hours at room temperature). After 3-4 washes in PBST of 20 min each (in the second DAPI was added to counterstain cell nuclei), the samples were mounted in Vectashield or glycerol. Regarding the pupal eyes, they were positioned with the internal face up using needles, and a bridge was created using the cover slip. The larval eyes and wing discs and the fat bodies did not need the "bridge" trick, as they are flat tissues.

Epitope	Species	Dilution	Supplier:
Dlg	mouse	1:50	DSHB
Rh6	rabbit	1:1000	C.Desplan
α-βGal	mouse	1:500	DSHB
α-CycE	rat	1:500	H.Richardson
α-Elav	mouse	1:50	DSHB
α-Repo	mouse	1:50	DSHB
Rh5	mouse	1:200	C.Desplan

Table 1. Antibody references and concentration used, listed as they appear in the Results chapter

- Pupal staging

The analysis of Wts and Rhodopsin expression at different time points required the staging of the pupae. Wts-venus individuals were placed at 25°C in a chosen proportion of females and males (1:2 for a total number of 24 females and 12 males). At 5 days after hatching the larvae start the pupariation; each few hours the new formed white pre-pupae were moved into a petri dish using a dampened brush, in this way they are easier to follow along

developmental stages. Then I selected the pupae for the 74h, 76h and 78h time points looking at some peculiar structures that differentiate in this time windows: the ocelli become visible at 74h, the head bristles differentiate at 76h and at 78h the wings appear light grey coloured. I proceeded with the dissection of them following the protocol described above.

- Confocal Microscopy

The images were captured under an Olympus FV3000 or a Leica TSC SP2 confocal microscopes. The images were acquired with the 20x air objective, the 30X silicon oil objective, or the 60X mineral oil objective. The magnifications used the Nyquist theorem to not exceed with the zoom and capture false signals. The Fluoview Software was used for acquisition, and the Fiji (ImageJ) for analysis.

- Wing Hair Count

The adult wings of *Minute, engrailed*>Gal4; UAS-GFP/UAS-GBA-RNAi (experiment population) and of *Minute, engrailed*>Gal4; UAS-GFP/UAS-RFP-RNAi (control population) were mounted in *Fluoromount* media on a slide and observed at the Nikon Eclipse 90i microscope. They were taken 8 images for each genotype using the 60X objective, always focusing on the distal vein area (Fig.12). For each picture, I traced two squares of defined dimensions (3X3 cm) and I counted the hairs included. I then calculated the average and compared the values obtained with the experiment population and the control one, using *GraphPad Prism 8*.

- Adult eye images

Adult flies were killed by freezing at -20°C. The fly is positioned on one side for lateral view, the head is then cut for the frontal acquisition. Images were taken using a SciTech Infinity 1 camera mounted on Olympus SZX7 microscope at 5X magnification, and using Infinity Capture 4.6 Software.

The fluorescent retinas were imaged using a Nikon Eclipse 90i microscope instead, taking advantage of the zed stack acquisition tool present in the Nis-element Software.

- Motility assay

The larval motility assay was performed on an agar plate prepared as follows: 200 ml of warm water and 8 gr of agar were heated over low heat for 4-5 minutes until boiling occurs, stirring slowly. Then 5 ml of 10% ethanol-Nipagin solution are added and, when dissolved, the mixture poured into the petri dish and allowed to solidify.
3rd instar larvae were carefully selected: they must have the front spiracles golden in colour but not exposed, to avoid the wondering larvae that are too near to pupariate to be used. An equal number of female and male larvae were used in each assay, selected by looking at the presence of male gonads. After washing them in PBS1X to remove food, they were placed, one at a time, on the agar plate. After one minute, to permit the larvae to adapt to the new condition, the whole contractions of the larval body were observed and counted for one minute, looking through a monitor connected to the optical microscope. In order to have a good statistical significance, the total of individuals subjected to the larval motility assay was about 60, with three independent experiments.

- Climbing assay

The flies for the climbing assays were separated at birth into males and females and kept in vials 25 each at 25°C, in the GBA-KO experiment, or 29° C, in the case of the UAS-Gal4 system. The date of birth was marked on each vial, so the climbing could be performed correctly on the third, seventh, fifteenth and twentieth days. For the experiment, an equal number of female and male flies were placed inside a 50 ml transparent glass cylinder. Once inside, they must acclimate to the environment, undisturbed, for 15-20 minutes; afterwards, it is necessary to tap down the cylinder, hard enough to knock all the flies down to the bottom; after 10 seconds it was counted the number of flies at the three pre-established levels: - below 5 cm - between 5-7.5 cm - above 10 cm. 10 sec is the time commonly used for this

type of analysis, and it is twice the time that a wild-type fly takes to reach the top of the tube. The rank is necessary for coding the flies' capability to climb. The protocol was repeated 10 times at 5-minute intervals. In order to have a good statistical significance, the number of flies used for the climbing assay was around 130, coming from three different crosses to have, again, three independent biological replicates.

- RNA extraction

Working on ice I removed the PBS 1X and added 500 µl of TRI Reagent® (Sigma-Aldrich), responsible to maintain the integrity of nucleic acids and at the same time lysate the cell membranes. Using a potter to disintegrate tissues mechanically, I homogenised the tissues. After adding 200 µl of chloroform, I put the samples in the centrifuge for 20' at 12.000 rpm (at 4°C), which allows, thanks to chloroform, the separation of the mixture into three distinct phases. Once transferred the supernatant, including the RNA, to another 1.5 ml eppendorf, I added an equal volume of isopropanol to make the RNA less soluble in water and allow it to precipitate after centrifuging. After placing samples at -20°C for 1h to favour the isopropanol action, I put them in the centrifuge for 12' at 12.000 rpm (at 4°C) in order to obtain the RNA pellet. The pellet was washed in ethanol 70% 3 times and then left under the hood to dry for about 30 min. The RNA was resuspended in the opportune quantity of RNase-free water and quantified at the Nanodrop.
To avoid genomic contamination, the RNA samples passed through a DNase step. DNase Buffer 10X, DNase enzyme, MilliQ water and the RNA to purify, in the correct quantities, were mixed into an eppendorf and put into the thermocycler at 37 °C for 30 min.

- Reverse transcription and Real-Time PCR

To obtain cDNA, 250 ng of DNase-free RNA underwent Reverse Transcription. Together with 4 µl of Supermix 5X and 11 µl of milliQ water (Sigma Bio-Rad), it entered the thermocycler and was processed as follows: 5 min x 25°C; 30 min x 42°C; 5 min x 85°C. The new-synthesised cDNA is now ready to be used in a Real-Time PCR.

The Real-Time PCR allows the quantification of a transcribed gene by monitoring the amplification of its cDNA. To objectively evaluate the differences existing between the samples, a housekeeping gene is used as a "reference", because its level of expression is presumably constant even between different samples. The housekeeping gene used in this work is Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), encoding a protein involved in the step 1 of the pathway that synthesises pyruvate from D-glyceraldehyde 3-phosphate. The experiments were conducted using "Syber Green" (SBYR); this molecule is an intercalant that emits fluorescence while binding the minor groove of DNA; the number of linked fluorescent molecules increases along with the number of copies of the amplicon, revealing the quantity of each target. It was used SYBR GreenER qPCR SuperMix (Invitrogen), which is a ready-to-use cocktail containing hot-start Taq DNA polymerase, SYBR GreenER fluorescent dye, 1 µM Fluorescein Reference Dye, $MgCl_2$, dNTPs and stabilisers. After having prepared the pre-dilution of the primers, this was added to the SuperMix, and MilliQ water in the right quantities and aliquoted for a final volume of 13µl each well. Then the samples followed the protocol suggested by Invitrogen kit (Invitrogen RT-PCR protocol). Because of the low quantity of some targets, I have performed the RT-PCR setting the melting temperature at 56°C for a time of 30 sec, in this way favouring the amplification of molecules present in low concentrations.

Target Genes	Primer sequence 5'->3'
Rpl32	Fw_ATCGGTTACGGATCGAACAA
	Rv_GACAATCTCCTTGCGCTTCT
dGBA1b	Fw_GAACCAGAGCAATCCCTTCA
	Rv_TCATCGAGAGTCACGTCCAC
myc	Fw_AGCAAATCAGCAGGGAGCTT
	Rv_GGTTGAGCACATCGAGGACA
dally like	Fw_AGCAAAACAATCGCGACG
	Rv_GCCATTTGAGCTGTTTGC
dally	Fw_ATTTGCGGCGGAAACTG
	Rv_TGGCCATTGCTGTTCGTA
dIAP	Fw_TCTCGGCCCGTATAGACACA
	Rv_CTGAAGTCGAAACTTGACGGC
cyc-E	Fw_TTCTACCAGCAGCACTGAGC
	Rv_CGAGTTCAGGGTCCTCGTTT

Table 2. Target genes and Primer sequences used for RT-Pcr analysis. Rpl32 is used as housekeeping gene.

- Library for Transcriptome analysis

The RNA extraction followed the protocol previously described. The RNA was isolated from 60 heads for each of the three biological replicates, both for the GBA-KO and the wild type flies. The generation of the libraries of the template molecules and their loading in the chip wells was performed following the instructions described in the guide of the Illumina (TruSeq Stranded mRNA - Illumina).

4. Results

4.1 The Hippo pathway in normal development

Warts, together with the other core components Hpo, Sav and Yki, is responsible for cell growth and proliferation during the larval phases of development, while it restricts proliferation and promotes apoptosis during pupal development. Eyes lacking core components are significantly larger than eyes lacking the non-core components of the pathway, such as *ft*, *ex* and *Mer* (Milton et al., 2009).

The following experiments take advantage of a stock characterised by the presence of an endogenous tag for Wts (*venus* tag). Wts is localised at the cellular junction, just below the peripodial membrane, both in the eye and wing discs of third instar larvae (Fig. 5).

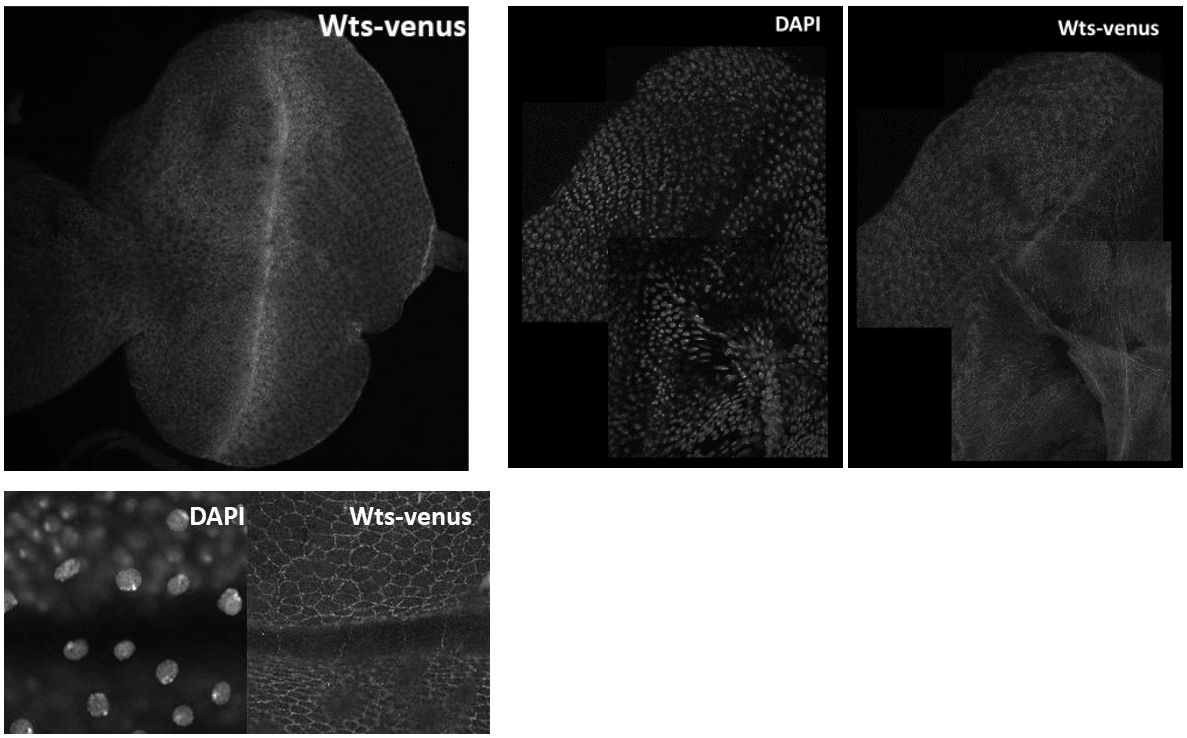

Fig 5. Wts is localised at the cellular junctions. The top three panels show two eye discs, complete on the top left, 60X zoom montage on the right. The bottom panel is a 60X magnification of the wing pouch region of the wing disc. It is shown the DAPI staining of the peripodial cell nuclei and the Wts-venus tag.

Verifying Wts localisation in the larval tissue was necessary to confirm the specificity of the signal. In the images above there is no staining for Wts and the fluorescence coming from the *venus* tag has been used as a positive control for Wts staining in all the following experiments.

4.1.1 Wts starts to be differentially expressed at 73h pupal development

Since now, Wts had been evaluated in relation to the expression of other factors such as Melted and the two Rhs, and the study of its ON/OFF status had been carried out in the adult retina (Jukam and Desplan, 2011; Jukam et al.,2013; Xie et al., 2019).

Therefore, I investigated what happens at the pupal stages. Previous data obtained in the laboratory of Professor Kieran Harvey revealed no Wts expression around 70h after pupariation. Additionally, Wts is expressed around the 78h development. The first aim was defining the timing at which Wts expression is differentiated among the R8 cells and I found out that the kinase is turned on around the 73h time point.

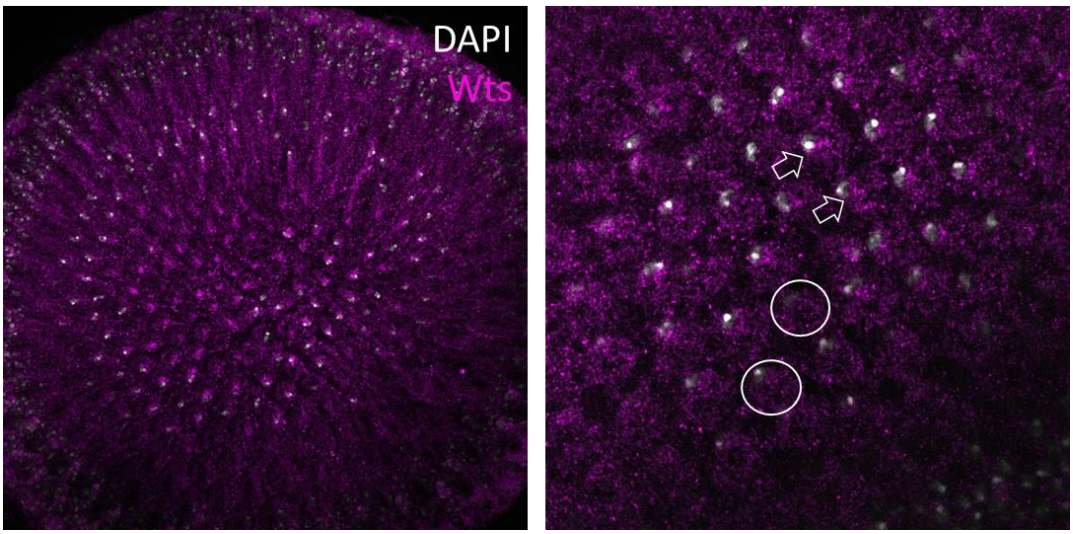

Fig. 6 Wts expression turns on at 73h of pupal development. In the figure, a pupal eye of 73h stained for DAPI (grey) and Wts (magenta). In the magnification on the right, the arrows indicate two R8 cells positive for Wts expression, whereas the circles indicate two negative examples.

In the above figure, each grey dot corresponds to the nuclei of the R8 cells. At 73h time point Wts (magenta) comes starts to be differentially expressed among the R8 cells, but not Rhodopsin (data not shown). The initial accumulation of the kinase can be seen although the expression is not high (it was used an elevated power laser value for imaging Wts). The expression is not uniform; it is absent in those cells that will become pale PRs (two examples are circled), and also expressed at lower level in cells that will become yellow PRs soon after this time point.

4.1.2 Wts expression increases dramatically in few hours

I have decided to look at Wts expression during the subsequent hours of development. Looking at the initial formation of the ocelli and at the appearance of the dorsal bristles, I have selected 74h and 76h old pupae respectively.

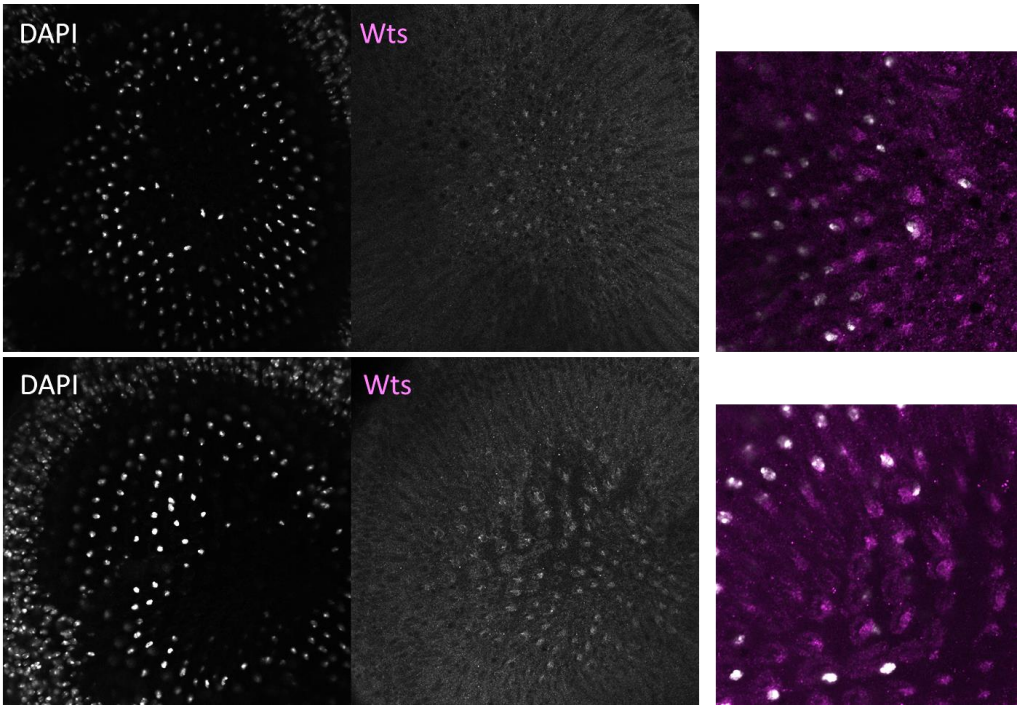

Fig. 7 Wts expression at 76h and 74h of pupal development. Pupal eyes dissected at different time points (top panel 76h; bottom panel 74h) and stained for DAPI (grey) and Wts (magenta). In the right panel the merged images are obtained with a 60X zoom.

Wts appeared more broadly expressed at 74h with respect to the 73h old pupae.
The intensity of the power laser for the images taken at 74h time point was twofold that one used for the acquisition at 76h; even if the power laser can't be used as an evidence of the increased Wts expression alone, this may suggest a higher expression of Wts at later time points in development and it is interesting to observe that it could happen in such a short time.
I also noticed a change in the localisation of Wts in the two time points. At 76h the kinase appeared to be diffused in the cell. The accumulation seemed to be perinuclear only at 76h, especially if we observe the plane of acquisition of the two 60X zoom images; in the top one, most of the nuclei are at the same plane as Wts, whereas at 74h, in the bottom image, most of the R8 nuclei are not visible, suggesting that Wts is occupying another position in the cell.

4.1.3 Wts localises along the membrane at earlier stages of development

It is well known that the functionality of the Hippo pathway is related to the position of its members within the cell. This is true especially for the kinase core components, which activation is tightly dependent on localisation. When Hpo and Wts are localised at the apical membrane of the cell, this results in an enhancement of Yki phosphorylation, which remains in the cytoplasm. This happens because Wts is recruited at the apical membrane by both Mer and Ex to facilitate the interaction with Hpo and Sav (Deng et al. 2013; Genevet et al., 2010; Sun et al., 2015a; Yin et al., 2013).
Because of that, I decided to repeat the analysis for Wts at 74h and 76h during pupal development, adding a marker of the basal-lateral membrane: Discs Large (Dlg). Together with Lgl and Scrib, Dlg it is a fundamental player in cell polarity determination and a member of the "neoplastic tumour suppressor genes" family, as mutant forms cause a deep impairment in tissue architecture that leads to a massive overgrowth phenotype (Froldi et al., 2008; Humbert et a., 2008; Grzeschik et al., 2010).

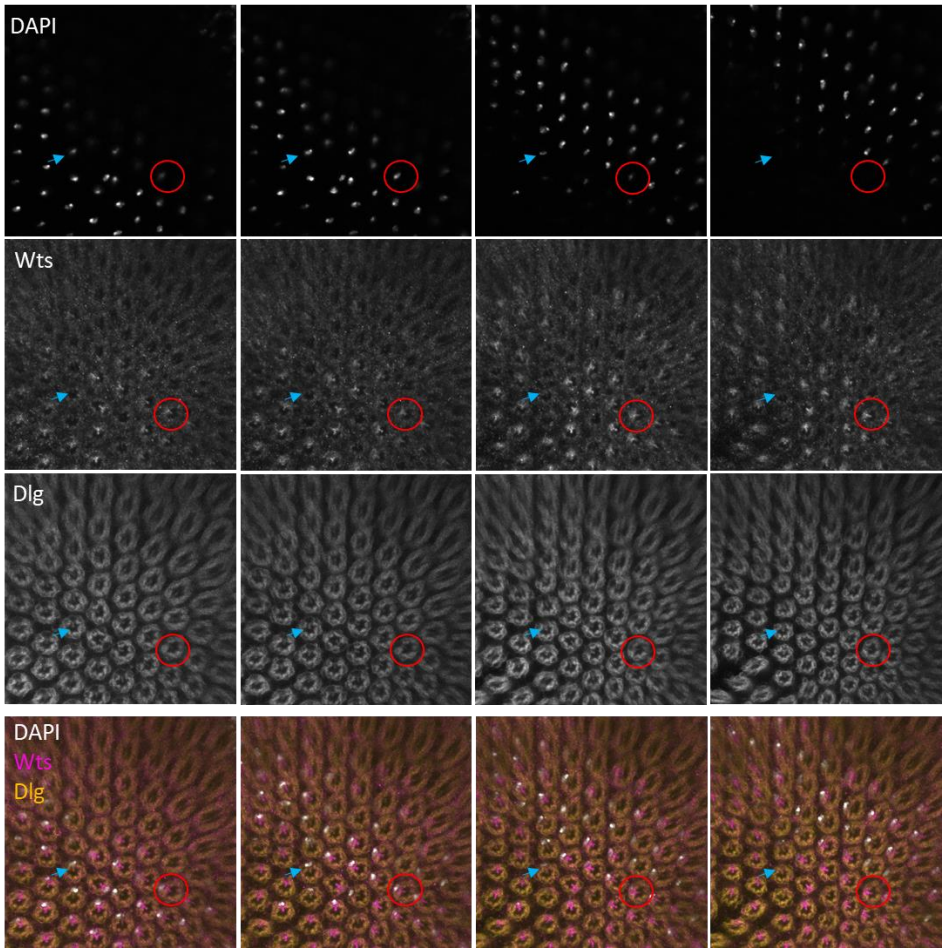

Fig. 8 Wts is localised along the lateral membrane at 74h. Z stack acquisition of a 74h old pupal eye with 60X objective. From the bottom to the top of the R8 cell (left →right), there are shown the single channels (DAPI for the nuclei, GFP for Wts and Far-red for Dlg), and the merge image indicating the nuclei (grey), Wts (magenta) and Dlg (yellow). The red circle indicates a Wts-positive R8 cell, whereas the blue arrow indicates a negative one.

Considering that the nuclei of the R8 cells are positioned at the bottom of the cell, the accumulation of Wts seems to be dislocated from them. Moving away from the bottom of the cell, the amount of Wts increases and appears to be localised along the lateral membrane. Indeed, the co-localisation of Dlg and Wts increases as we move far from the nuclei, acquiring a specific shape within the cell. This data is fundamental because it suggests an enhancement of the activation of Wts in this precise time point during development.

4.1.4 Rh6 follows the expression of Wts

After having defined that Wts expression changes in the pupal eye, I investigate Rh6 expression. It is known from the literature that Rh6 is expressed in those R8 cells positive for Wts (Jukam et al., 2013; Xie et al., 2019). Few evidences have pointed out that the Rhodopsin are turned on around the 80h in development. The first are the Rh1, Rh3 and Rh5, giving rise to the pale PRs; then the Rh4 and Rh6, which lead to the yellow ones (Earl and Britt, 2006). I decided to evaluate Rh6 expression both in 74h and 78h old pupal eyes, as it can be reasonable think that Rh6 expression follow Wts turning on.

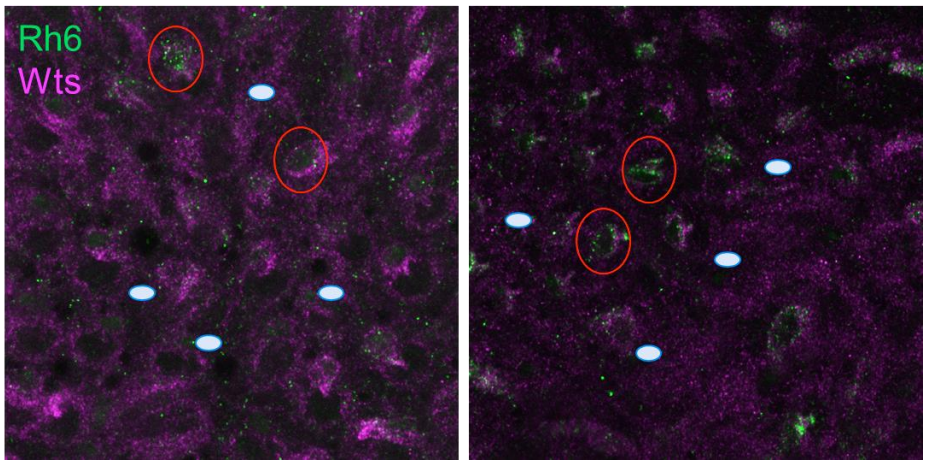

Fig. 9 Rh6 is expressed already at 74h and it increases dramatically at 78h. 60X magnification of a pupal eye of 74h (left) and 78h (right). The grey circles indicate the nuclei of R8 cells negative for Wts and Rh6. The red empty circles indicate two examples of Wts-Rh6 positive cells. DAPI staining is not shown.

This result is in contrast with what is shown in the literature. Interestingly Rh6 is already expressed at 74h, even if it is extremely low. Within 4 hours, Rh6 level is extremely augmented and correlates with Wts positive cells. Also, in this experiment the power laser was reduced at the 78h time point, suggesting an increase of the expression of both Rh6 and Wts. We can deduce from this analysis that Rh6 expression follows Wts activation in a rapid way.

4.2 Growth deficit in *GBA* KO/KD models: hyper-Hippo condition?

Besides mutations in the *GBA1* gene, alterations in cell trafficking, autophagy and inflammation have been shown to participate generically in the GD phenotype. Here I want to make a point of the growth defects underlying the GD neural phenotype by investigating the contribution of the Hippo pathway. The Hippo pathway is known to integrate myriad signals and to respond with the modulation of precise target genes in different tissues, and mutation/dysregulation of the upstream components may greatly compromise cell function. The aim of the second part of my thesis is to identify new *GBA1* genetic interactors/modifiers among the Hippo pathway components.

Why the Hippo pathway? Recent evidence demonstrated that YAP, the downstream effector of the Hippo signalling in mammals, is fundamental for the suppression of the JAK-STAT-mediated neuroinflammation and BBB dysfunction (Huang et al. 2016). It is thus reasonable to hypothesise that YAP is involved in neuronal cell death. This is only one of many examples where YAP and the other members of the Hippo signalling trigger an inflammatory response. Others examined the interactions with the JNK pathway that, depending on upstream ligands, mediates cell death or instead proliferation (Irvine et al. 2011; Codelia et al. 2014; Zhang et al. 2016). Recently, a "Hyper-Hippo" condition has been associated with neurodegenerative phenotypes. In the few studies so far available, hyper-activation of upstream components of the pathway has been found to cause YAP/Yki inactivation and to block growth signals. This resulted in neuroinflammation and neuronal cell death both in mammals and in *Drosophila*. (Calamita and Fanto, 2011; Reddy and Irvine, 2011; Fallahi et al., 2016; Wang et al., 2016; Dubei and Tapadia, 2017). Finally, a new form of cell death, namely Transcriptional Repression-Induced Atypical cell Death of neurons (TRIAD) has been found associated with a Hyper-Hippo condition in Hungtington's disease (Yamanishi et al. 2017). Near to that, it is well established that the Hippo pathway is involved in the control of trafficking, autophagy and growth: all of them proved to be altered in GD.

The chronic inflammatory response is a typical trait of the brains of nGD patients, which become more pronounced with disease progression. It was observed an oxidative damage

caused by the activation of macrophages and microglia, suggesting that once a critical threshold of glucosylceramide storage is reached in neurons, activated microglia performs a pro-inflammatory role, releasing pro-inflammatory cytokines that amplify the response, contributing to neuronal death (Vitner et al 2012).

Given the fact that the Hippo signalling covers a central role in the cell, interacting at multiple levels with all the mechanisms previously described, it may be reasonable that members of this important cascade play a role in the development of GD and in the diversification of the pathological spectrum. Once I have validated the model, I have produced some preliminary data suggestive of an impairment of the pathway in the *GBA*-KO context.

4.2.1 The *GBA1b* KO model shows climbing motility defects increasing with age

I obtained the *dGBA1b* Knock Out (GBA-KO) stock from the Prof. Linda Partridge Laboratory. The deletion eliminates the presumptive promoter and more than 3/4 of the amino terminal coding sequence of *dGBA1b*, resulting in a null allele. First of all I want to confirm the data regarding the climbing ability of the *GBA*-KO with respect to wild type flies, as this behavioural assay is commonly used to evaluate neurodegeneration patterns.

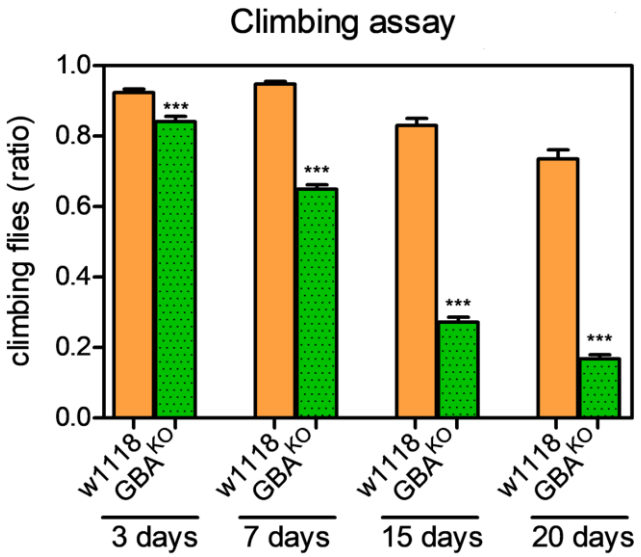

*Fig. 10 Climbing assay of wild type (w^{1118}) and dGBA1b KO flies, performed at 3, 7, 15 and 20 days from eclosion. *** indicates a value of P<0.001*

As can be seen in the graph, the locomotor course of individuals homozygous for the *GBA* mutation worsened dramatically over time; the biggest drop is observed at fifteen days after hatching. The result confirms the expectation, as *GBA*-KO provides a neurodegenerative model for GD. The measure of the climbing impairment is synonym of a dramatic neurological damage.

4.2.2 *GBA1b* RNAi retinas show decrease in immunofluorescence associated with ommatidia degeneration

Another method used in the fly community to evaluate the neurodegenerative phenotype is using a membrane-targeted Green Fluorescent Protein (mCD8-GFP) to monitor neurotoxic protein-dependent degeneration of *Drosophila* eyes (Burr et al., 2014). Taking advantage of this, I used flies expressing a shRNA against *GBA1b*, or luciferase (negative control), in the adult retina, and I have evaluated the fluorescence decrease in a *GBA* RNA interference model (*GBA*-RNAi /*GBA*-KD), along different time points: 7, 35 and 63 days after eclosion.

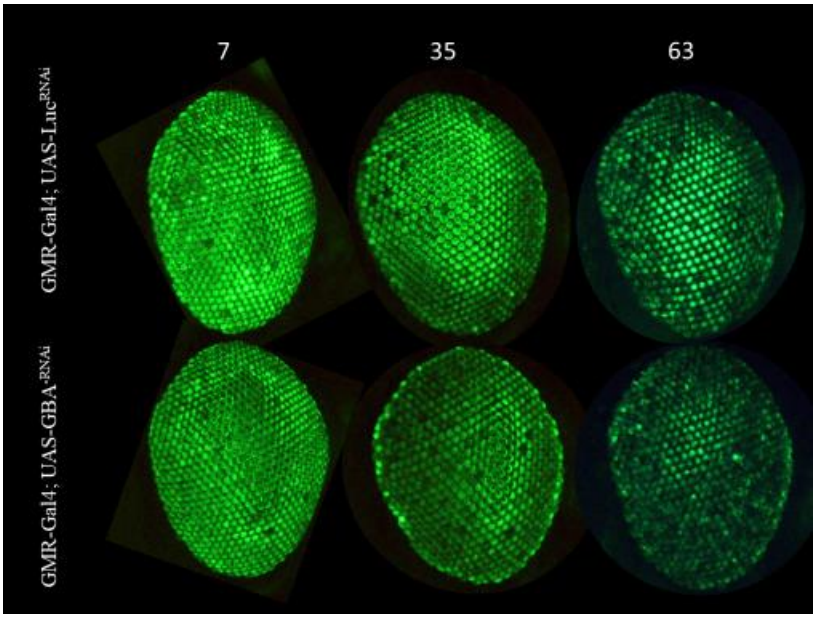

Fig. 11 GBA-RNAi flies show a decrease in fluorescence not only due to aging. Zed stack acquisition under the fluorescence stereoscope of adult eyes expressing luc-RNAi (top) or GBA1b-RNAi (bottom) under the retina-specific promoter GMR.

The neurodegenerative pattern is defined by a decrease in fluorescence, which is associated with the progressive death of photoreceptor neurons. The result shows how in the *GBA*-RNAi context the reduced fluorescence is not only related to the aging of the flies. Indeed, counting the "black ommatidia", the result show that at 35 days the luc-RNAi flies have only a 9% of degenerated ommatidia, with respect to the 31% present in the experimental population.

This observation, together with the behavioural impairment shown before, is suggestive of a neuronal damage arising from the lack, or at least the reduced quantity, of GCase.

4.2.3 *GBA1b* RNAi causes impairment of the adult veins

GBA KD does not cause extreme phenotypes also when flies are reared at 29°C, thus pushing the UAS-Gal4 temperature-dependent system. Therefore, I decided to evaluate the KD

phenotype in a *Minute* context. *Minute* (*M*) mutants show a characteristic prolongation of developmental time, caused by the slow-down of protein synthesis due to mutations in ribosomal protein genes (Ritossa and Atwood, 1966; Morata and Ripoll, 1975). This technique is commonly used to increase the size of a mutant clone, or to permit the survival of a mutant clone that would otherwise fail to grow (Blair, 2003). In my study I used a *Minute* background to worsen the phenotype caused by *GBA*-RNAi, combined with the *engrailed* (*en*) promoter, which is specific for the posterior compartments (Fig. 12) of *Drosophila* wing and permits an internal control.

The wing, and especially the larval precursor wing disc, is commonly used to study growth and development regulation as well as cell competition mechanisms and cancer evolution (Cho et al., 2006; De La Cova and Johnston, 2006; Harvey et al., 2003; Moreno and Basler, 2004; Neto-Silva et al., 2009; Tapon et al., 2002). The wing disc contains two different territories that will give rise either to the adult wing blade, or to the hinge and part of the notum. During larval stages, the monolayered epithelium forming the wing disc exhibits A-P and D-V compartment boundaries, oriented cell division, and tissue stretching by different division rates, obtained through a coordinated signal exchange between the Wingless, Decapentaplegic, EGFR and Notch pathways (Blair et al., 1994; Casares and Mann, 2000; Kim et al., 1996; Ng et al., 1996; Paul et al. 2013; Rafel and Milan, 2008; Wang et al., 2000). At the beginning of metamorphosis, and as a result of eversion of the wing pouch, the wing blade consists of two epithelial layers facing each other at their basal surfaces.

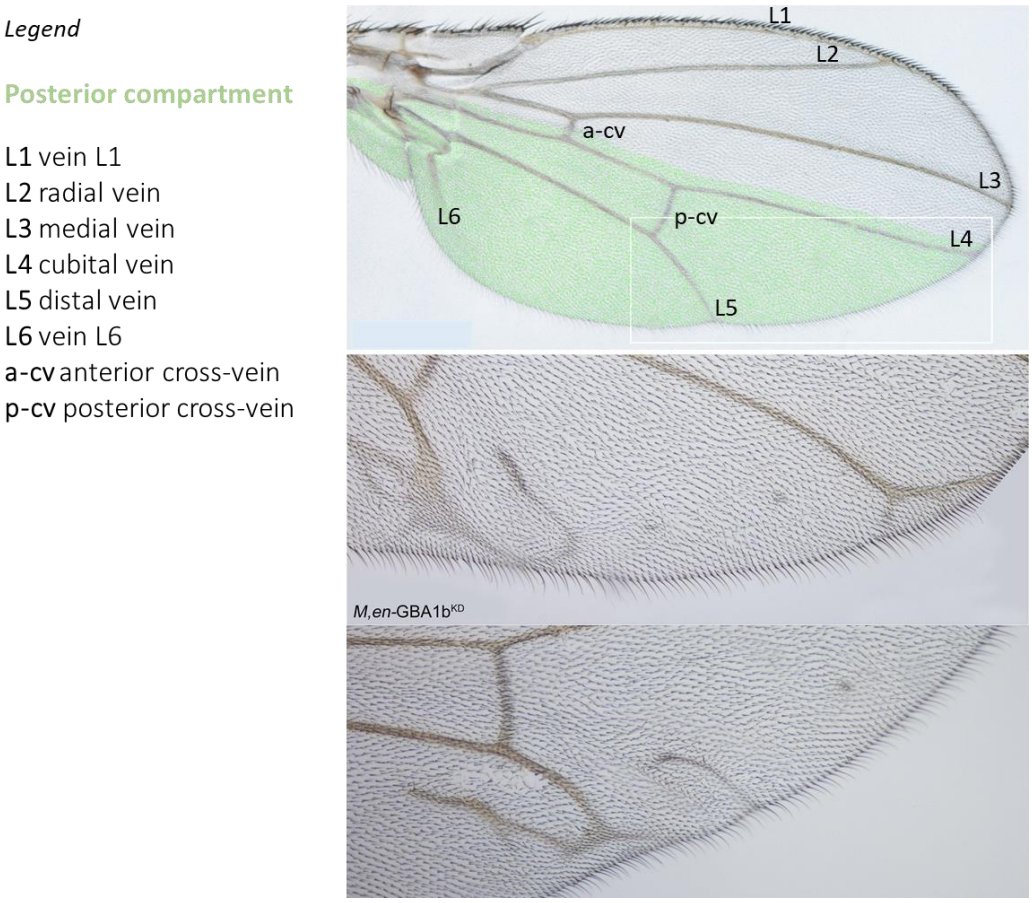

Fig. 12 The GBA-RNAi wing shows venation defects in the posterior compartment. The top panel shows the engrailed-positive area (in green). The bottom panels are two examples of Minute-engrailed GBA-RNAi adult wings showing impairments of the correct development of the veins.

Few hours after pupariation, the developing wing undergoes expansion, elongation, separation and re-apposition of both epithelial sheets. Contraction of the wing hinge induces oriented cell division and cell rearrangement to re-shape the wing to its 'definitive stage'. This happens around the 40 hours of pupal development; at the same time are formed veins and hairs. After hatching of the adult fly, the folded wing spreads out due to fluids that fill the veins and the intervein epidermis, which are soon after removed generating a cuticle-made adult wing (Requena et al., 2017).

The damage appears obvious in the adult wing (Fig. 12). The normal pattern of the posterior veins is altered, resulting ramified at the intersection with the wing border in more than 80%

of individuals. The impairment of the wing veins came up also in other studies that evaluated the overexpression or the down regulation of some pathway involved in growth regulation: Notch, Epidermal Growth Factor (EGF), Insulin Receptor, Bone Morphogenetic Pathway (BMP) and the Hippo pathway. Ectopic veins, like the ones present in the GBA-RNAi, can be formed due to the gains in EGFR, BMP, or *Wingless* signalling or by a loss in *Notch* signalling function (Blair, 2007).

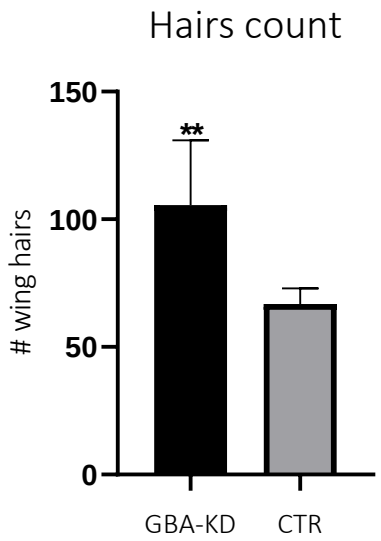

*Fig. 13 Wing hair unbalance in GBA-RNAi mode. Count of the wing cells of multiple areas of 8 individuals (each) representative of the GBA-RNAi progeny and the control (CTR). ** indicates a value of p<0.01.*

The count of the wing hairs, each representing one cell, indicate a strong impairment of the proliferation/growth balance of this tissue, resulting in a significant increase of cell number (and consistent decrease in cell size) in the posterior compartment of the *GBA*-RNAi wings. Even if it is a speculation, this result can be assumed as the first evidence of a growth impairment following *GBA* reduction.

4.2.4 Yorkie targets are strongly downregulated in *dGBA1b*-RNAi fat bodies

To analyse a possible impairment of the Hippo pathway, I started analysing the targets of Yki. Taking advantage of the same *M, en* system used before, I have decided to perform an immunofluorescence against Expanded (Ex) and Cyclin E (Cyc E) in the fat bodies, because *dGBA1b* is mainly expressed in this tissue and in the brain (Davis et al., 2016; Kinghorn et al., 2016).

The *Drosophila* larval fat body originates from segmentally repeated clusters of mesodermal cells that are formed during early embryogenesis (Hoshizaki et al., 1994). The genesis is due to the dialogue between the Engrailed and Wingless pathways (Riechmann et al., 1998). The total number of fat cells is around 2200 and remains constant from the embryonic stage; indeed, their change during development is due to an increase in cell volume, not in cell number (Britton and Edgar, 1998; Butterworth et al., 1988). Even if the fat bodies are composed of adipocytes, it is described that these have different destinies depending on the part of the body occupied (Bond et al., 2011; Larsen, 1976; Nelliot et al., 2006). They constitute an optimum model to study cell components, as they are composed of giant cells that form a monolayered tissue (Zheng et al., 2016).

en is a promoter for the posterior compartments, but it is also active in scattered cells of the fat bodies. Figure 14 shows that the *en-GFP* positive cells, that have a reduced production of the glucocerebrosidase, are characterised by a reduced amount of *Ex* and Cyc E, compared to the wild-type neighbouring cells. Both are targets of Yki, and *ex* is commonly used in literature as a readout of the functionality of the pathway because of its role as a regulator of Yki itself in a feed-back loop mechanism.
This result suggests a downregulation of the Yki transcriptional potential in the *GBA*-KD context.

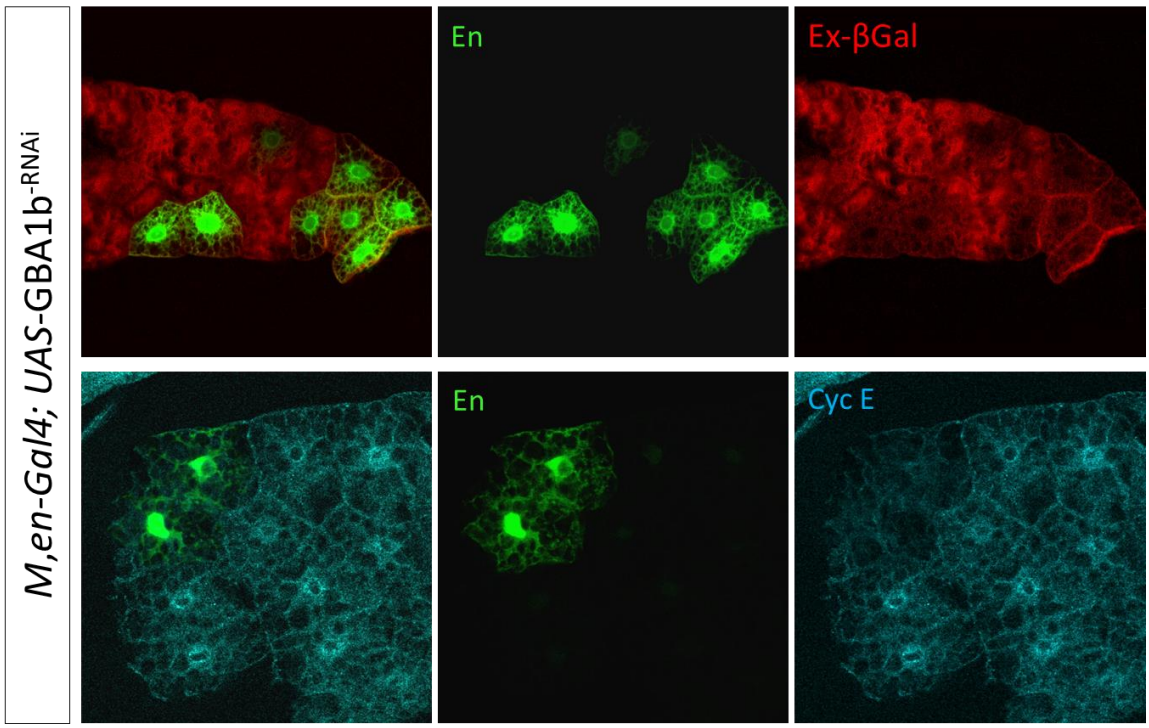

Fig. 14 Ex and Cyc E are strongly reduced in a GBA-KD context. Fat bodies of Minute individuals where GBA-RNAi is expressed under the engrailed promoter. Merged images are presented on the left; the middle images show the GFP-positive clonal areas; Ex-LacZ (red) and CycE (cyan) are shown in single channels on the right.

4.2.5 *GBA1b* is mainly expressed in the glial cells of the adult brain

Because I want to focus on the neurological impairment in GD, I have evaluated *GBA1b* expression in the adult brain.

The adult central nervous system (CNS) of *Drosophila* is composed of a central brain, two optic lobes and a ventral ganglion. The central brain (CB), in a medial position, is responsible for receiving sensory inputs and processing them, whereas the optic lobes (OL), localised in a lateral position, are necessary for the processing of the visual inputs coming from the fly compound eyes. The CNS is connected to the ventral ganglion (VG), also called ventral nerve cord (VNC), localized in the inner part of the thorax and absolving the same function as the human spinal cord. The same regions are present also in the larvae, even if in a different

shape. The CNS originates from progenitor cells called neuroblasts (NBs), which are similar to the neural stem cells of vertebrates in their ability to self-renew and to produce many different types of neurons and glial cells. The adult CNS consists of approximately 100.000 neurons and 700 glial cells, which neuroanatomy is quite complicated, and many aspects are still poorly understood. It is relatively small, but it is sufficiently complex to provide a proper model; indeed, despite the evolutionary distance between flies and humans, a strong conservation of genes, pathways and regulatory molecular networks has been demonstrated (Bellen et al., 2010). Because of that, the adult brain of *Drosophila* is commonly used to study syndromes like the Alzheimer disease or Parkinsonism.

Taking advantage of the newly generated *GBA1b*-Gal4 (Lee et al., 2018), I examined *GBA1b* expression pattern in the adult brain.

As it can be appreciated in Figure 15, the glial cells appear positive to *GBA1b* expression. This makes sense if we take in mind that glial cells absolve similar functions in the brain as that played by the macrophages in the rest of the body, and that glucocerebrosidase is necessary for lysosome functionality.

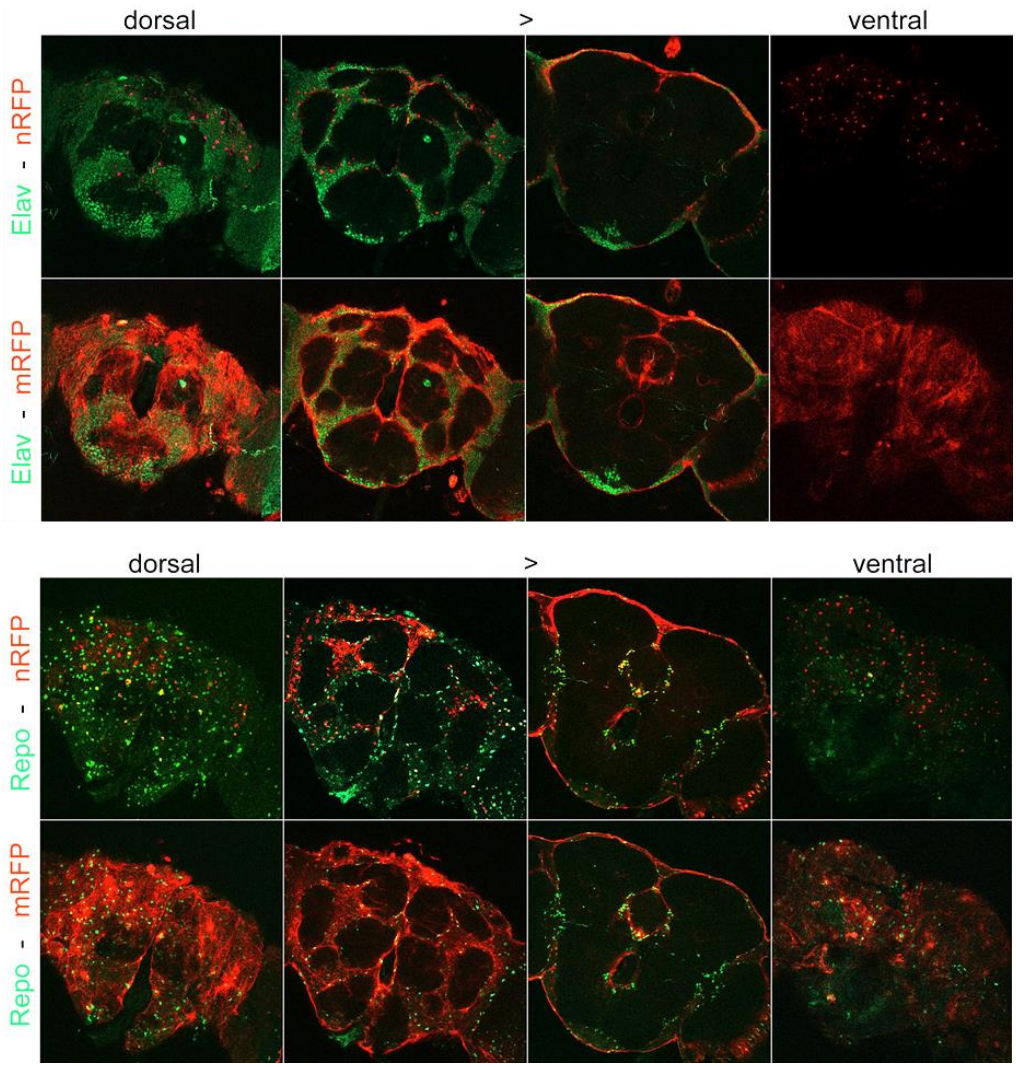

Fig. 15 GBA1b expression in the adult brain. GBA1b-Gal4 drives the expression of a nuclear Red Fluorescent Protein (nRFP) or a membrane one (mRFP). Staining for Elav (top panel) and Repo (bottom panel) evidences the neuronal and the glial components respectively (green)

4.2.6 Yki targets are downregulated in GBA-KO brains

Given that GBA is abundantly expressed in the brain, I have moved the analysis of the Yki targets to the adult brain. This time I have looked at the mRNA content of some targets such as myc, *cycE*, *dIAP* and also two other molecules, *dally* and *dally like* (*dlp*), in the *GBA*-KO flies.

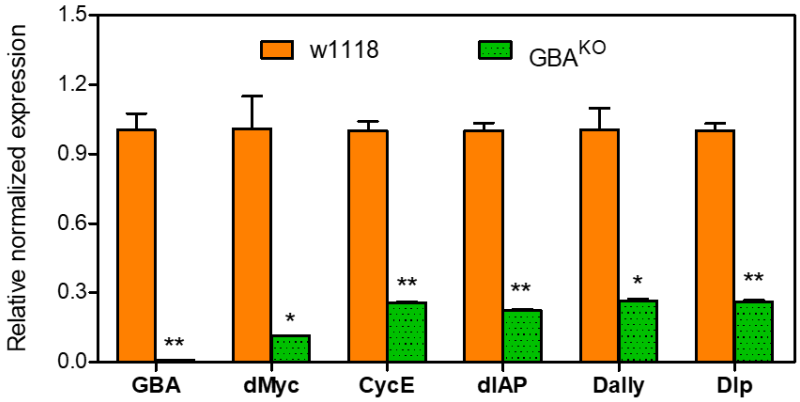

*Fig. 16 In the GBA-KO heads the expression level of Yki targets and Dally and Dlp results compromised. RT-PCR performed on the RNA extracted from wild type (w^{1118} in orange) and GBA-KO (in green) heads. * p value> 0.05; ** p value> 0.01*

As can be deduced by the graph, the *GBA*-KO flies show a strong impairment of the expression of Yki targets. dMyc, Cyc E and dIAP are well-known master regulators of growth and cell proliferation. This supports the hypothesis of a hyper-activation of the Hippo pathway and a damage of the mechanisms of growth regulation.

Furthermore, also *dally* and *dlp* result greatly down-regulated. The encoded proteins represent the *Drosophila* glypicans, which are surface proteins, anchored to the cell membranes, that allow a proper distribution and activity of some morphogens such as Wingless, Hedgehog and Decapentaplegic, during organ development. Their regulation also depends on Fat cadherin, but independently of its role in the Hippo pathway (Baena-Lopez et al., 2008). The morphogens control the correct differentiation of neuronal cells and the production of synapses. Their low expression level may explain, at least in part, the neurodegenerative phenotype found in these *GBA*-KO flies.

4.2.7 *GBA1b* KD in glial cells induces a significant impairment of crawling and climbing activities

Taking into consideration that *GBA1b* expression is more evident in the glial cells and that *dally* and *dlp mRNAs* are strongly down-regulated, being the encoded proteins fundamental in the communication between different cell populations in the brain I have evaluated the damage arising from the KD of *GBA* in the glial cells or in the neurons. The crawling and the climbing assays analyse the motility ability at both the larval and the adult phase.

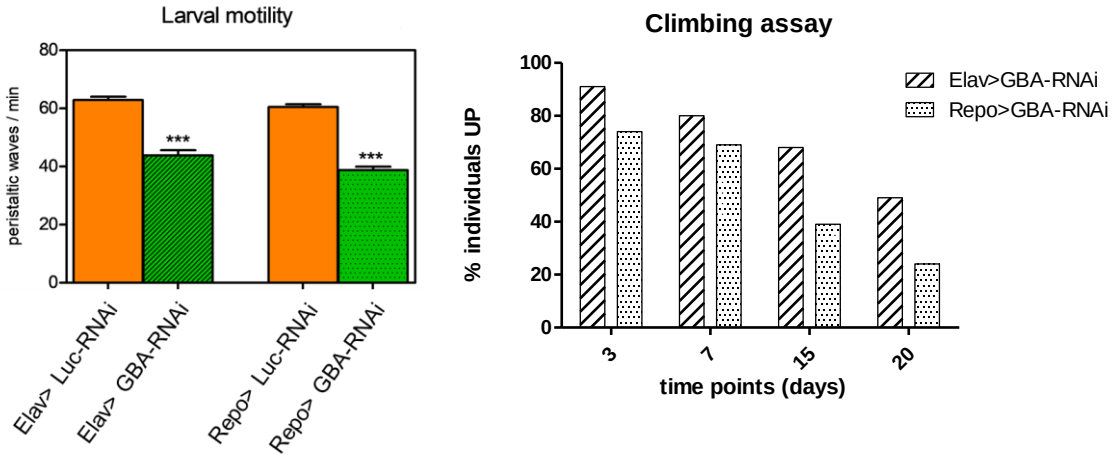

*Fig. 17 The GBA-RNAi induces a stronger impairment of the locomotor ability when induced in the glial cells. The left graph shows the crawling ability of L3 larvae, when GBA-KD is induced in the neurons (Elav promoter, orange column) or in the glial cells (Repo promoter, green column). luc-RNAi is used as control. *** indicates a value of P<0.001. The right graph shows the climbing assay performed using the same promoters. On the Y axis are indicated the percentage of individuals that haven't lost the climbing ability; on the X axis are indicated the time points.*

As can be seen in the larval motility graph, the reduction of *GBA* impaired larval locomotor ability in the two different experimental sets, suggesting that both the neuronal and glial populations contribute to the phenotypic manifestations of *GBA*-KD. Although, it was also interesting to note that the larvae with reduced *GBA* in the glia showed a strong disability in coordination, visible as a less constant and unbalanced rolling locomotion, compared to the

elav>*GBA*-RNAi larvae (qualitative data not shown). The same genotypes were subjected to a climbing assay. Again, even if the lower quantity of *GBA* led to a motility impairment in both cases, it is when the *GBA*-RNAi is induced in the glia that it is visible a faster damage of the motor ability. The deficit produced is comparable to what resulted from the climbing assay performed on the *GBA*-KO individuals. This is suggestive of a major role for the glial component in contributing to the neuropathic GD. The reason might be related to the function that the glia plays in the brain and maybe to the role absolved by the glucocerebrosidase in this particular cell population.

4.2.8 The transcriptome analysis describes the pathway signature of the *GBA* KO model

The last experiment performed is a transcriptome analysis of the *GBA*-KO fly heads compared to wild type ones. The RNA is extracted at 3 days and at 15 days after hatching, as this second time was found to be a critical one from the previous analysis performed. Three biological replicates were analysed for each time point, and a first evaluation pointed out that wild type and *GBA*-KO samples give rise to different groups (Fig. 18).
This is a synonym of the different transcriptome pattern of each sample, that also show changes due to the age of the flies.

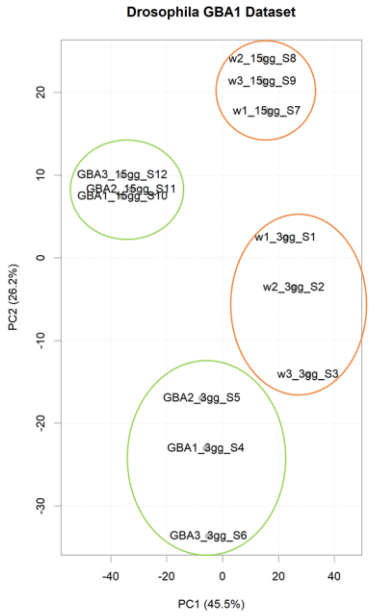

Fig. 18 Principal component Analysis (PCA) of wild type and GBA-KO at 3 and 15 days shows excellent replicate concordance.

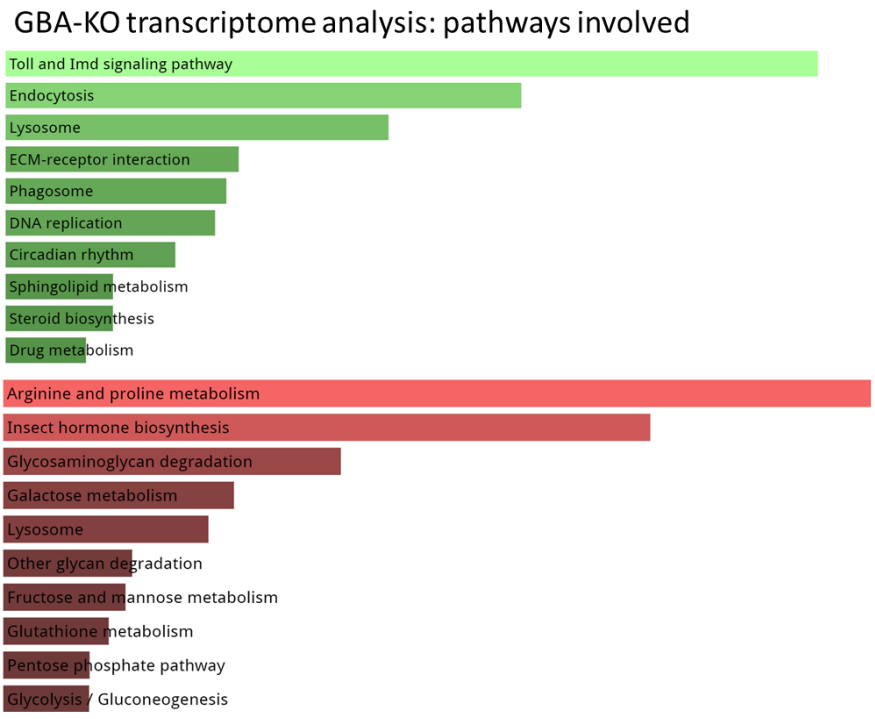

Fig. 19 Pathways hyper and down regulated in the GBA-KO model at 15 days of development. The scheme shows the hyper-activated pathways (in green) and the down-regulated one (in red) resulting from a Kegg-pathways analysis of transcriptome signature. All of them are statistically significance with a value of at least P<0.01.

Preliminary observations obtained through the use of bioinformatic databases like David, FlyEnrichr and Kegg, reveals that most of the genes altered belong to metabolic pathways and endocytosis signalling. It appears clear both the stress due to the accumulation of the substrates, and also the activation of the inflammatory response (Toll and Imd signalling), in our *GBA*-KO model. This is only partially in accordance with what shown by Horowitz and collaborators. Their model consists of a mutant protein obtained by a *Minos* insertion that creates a truncated coding sequence. This one seems to closer represent the molecular alteration found in GD patients; indeed, in their transcriptome analysis the activation of the Unfolded Protein Response (UPR) and the inflammation response is greatly up-regulated (Cabasso et al., 2019). From this preliminary observation, it can be concluded that in our study, the *GBA*-KO phenotype arises mainly from a damage of the vesicle trafficking combined with a strong impairment of the metabolic signalling, that may cause, in the end, neuronal cell death. Still statistically significant are the alteration of some pathways like FOXO (Forkhead box protein O), known to be implicated in many cellular processes such as cell cycle, metabolism, apoptosis and autophagy, and Hippo. They are not presented in the graph because they show a lower *fold change* ($\log2<1.5$). The transcriptome analysis of the whole brain does not take into consideration the differentiated expression of each cell type within this tissue; first of all, not all the cells express *GBA* (Fig. 15). This may cause an underestimation of the higher or lower expression condition of some pathways, thus causing a low log2 value.

A goal of a much deeper analysis will be identifying key molecules that considerably change their expression status within the *GBA* positive cells, and study their role in our *GBA*-KO.

4.3 Overgrowth: Orthodenticle as a possible partner of Yorkie

Orthodenticle is a transcription factor essential for the correct development of the rostral head and forebrain in many different species, including mammals and humans (Acampora et al., 2005; Finkelstein and Boncinelli, 1994; Plouhinec et al., 2003; Reichert, 2005; Simeone and Acampora, 2001; Tallafuss and Bally-Cuif, 2002). In *Drosophila*, Otd is critical for embryo patterning. Together with Bicoid (Bcd), another K50 homeodomain protein, it coordinates brain segmentation and axon guidance. More recently, it was found important for the lineage-specification and survival of some dopaminergic neuron clusters. My initial interest in Otd was due to its involvement in governing the visual system (Blanco et al., 2011; Datta et al., 2018; Finkelstein and Perrimon, 1990; Leuzinger et al., 1998; McDonald et al., 2010; Ranade et al., 2008; Royet and Finkelstein, 1995; Sprecher et al., 2007; Tahayato et al., 2003). In the *Drosophila* eye, it is responsible for the terminal differentiation of the PRs and the proper morphogenesis of the rhabdomeres (Mishra et al., 2010; Tahayato et al., 2003; Vandendries et al., 1996). In particular, Otd is fundamental for the Rhodopsin specification in each PR, especially it appears to be responsible for Rh5 expression (Jukam et al., 2013; McDonald et al., 2010). However, its overexpression is not sufficient to activate the Rh5 in the larval eye.

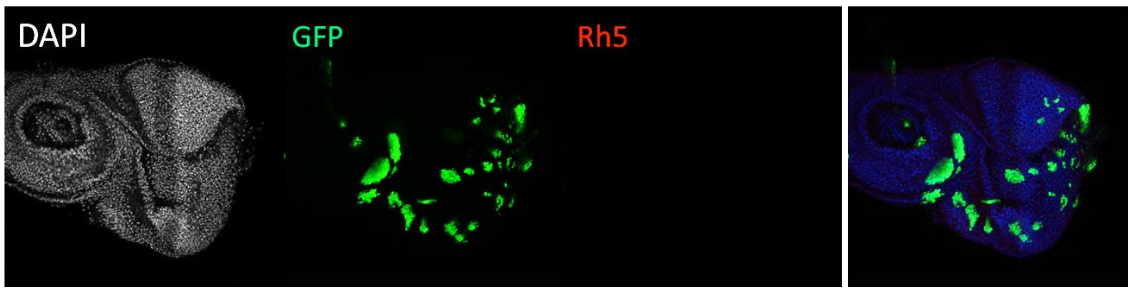

Fig 20. Over-expression of Otd does not induce Rh5 turn on in larval stages. Actin flp-out system that induces a clonal over-expression of Otd (GFP positive clonal area). Rh5 staining does not show any differences between the clonal areas and the wild type neighbour cells. The right panel represents the merged image.

otd overexpression is induced in the larval eye through a clonal strategy using the actin promoter. In the GFP-positive clonal areas there is no increase in Rh5.

4.3.1 Otd as a possible Yki co-factor

It is known that R8 fate decision requires both Yki and Otd. Indeed, *otd* mutants show a massive Rh6 expression and no Rh5 mRNA is detectable (Tahayato et al., 2003). The same result was obtained in a Yki loss-of-function context, whereas in the *sd* KD there is still around 50% of the Rh5 mRNA, compared to the *otd* mutant eye (Xie et al., 2019). These observations made me think about a possible relationship between Yki and Otd. Even if there is no evidence about a physical interaction between Otd and Yki, also in other species like mammals, I decided to evaluate their possible cooperation in the adult eye.

Taking Sd as a positive control, as the phenotypes induced by its knock down and overexpression are well known, I have looked at first at the phenotype produced by Otd alteration under the GMR promoter in the adult eye.

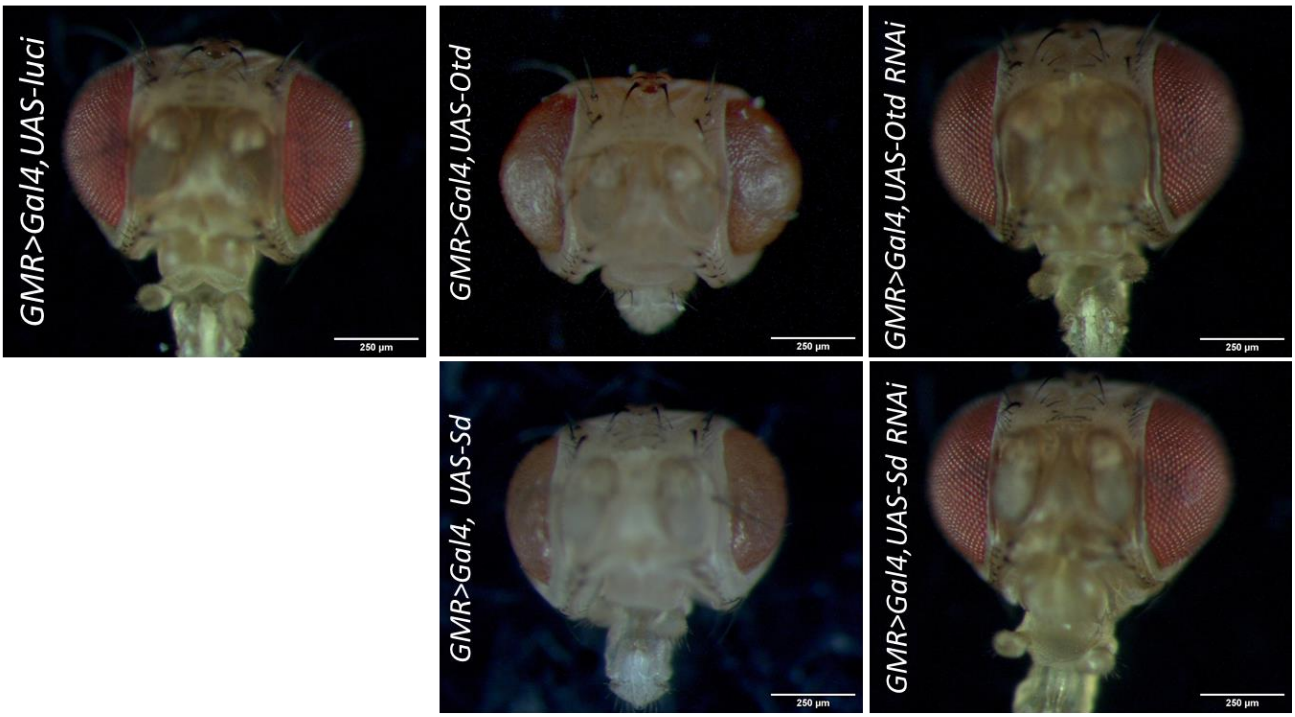

Fig. 21 Otd overexpression alters the normal phenotype in the adult eye. The left panel shows the negative control. The middle panels show the phenotype induced by the overexpression of Otd (top) or Sd (bottom). The right panels illustrate instead the KD. Scale bar 250um.

otd overexpression altered the normal morphology of the eye, making it a bit bulky and glazed. The phenotype is totally different from what can be observed following *sd* overexpression; here the eye resulted much smaller compared to the control one. It is evident that the two transcription factors play different roles. Following the model suggested by the scientific community, Sd normally acts as a *default repressor* when it is not associated with Yki; therefore, it makes sense that blocking the Yki targets leads to a reduced organ dimensions (Koontz et al., 2013). To clarify the role of Otd I repeated the experiments in a *yki* mutant context, where the final effector of the Hippo pathway is constantly active.

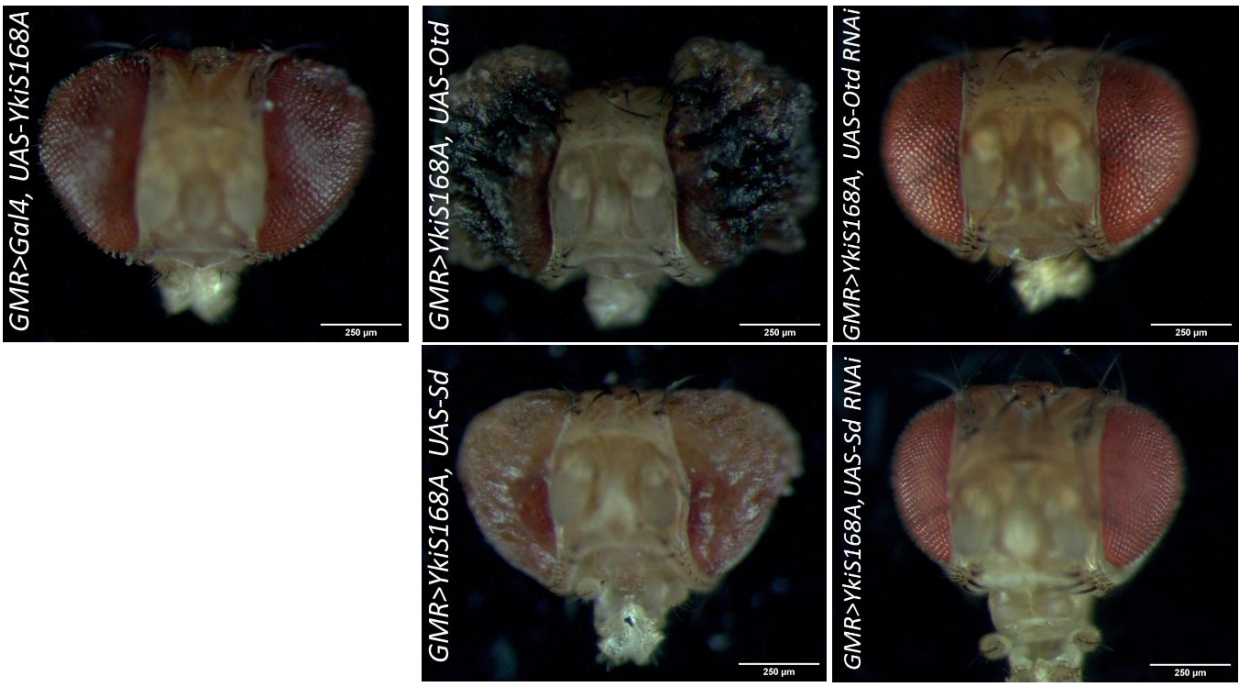

Fig. 22 Otd overexpression in Yki mutant context causes massive overgrowth. The left panel shows the negative control. The middle and right panels show the phenotype caused by the overexpression or KD, respectively, of Otd (top) or Sd (bottom). Scale bar 250um.

YkiS^{168A} is a mutant form of Yki which cannot be phosphorylated by Wts, resulting constantly active within the nuclei. This hyper-activation is able to produce an abnormal phenotype by itself. What is surprising is the massive overgrown obtained from the combination of this mutant form with the overexpression of *otd*. The progeny showed a multi-folded eye which has lost pigmentation; only few individuals died at the pupal stage. The eyes are much bigger than those obtained following *sd* overexpression in the same context. Looking at the KD genotypes, *sd*-KD leads to a total rescue, whereas *otd*-KD only partially recovers the overgrowth, leading to a bigger eye but with a normal morphology. This suggests that Sd is still the elective partner of Yki. Neither *sd*-KD nor *otd*-KD produce an altered phenotype in the normal context. Therefore, it is reasonable thinking that Yki must have other partners to maintain physiological growth, like Homothotax and Teashirt (Peng et al., 2009) that in the eye guarantee proliferation.

I have performed the experiments also using a *yki* transgene that induces overexpression of the wild-type form.

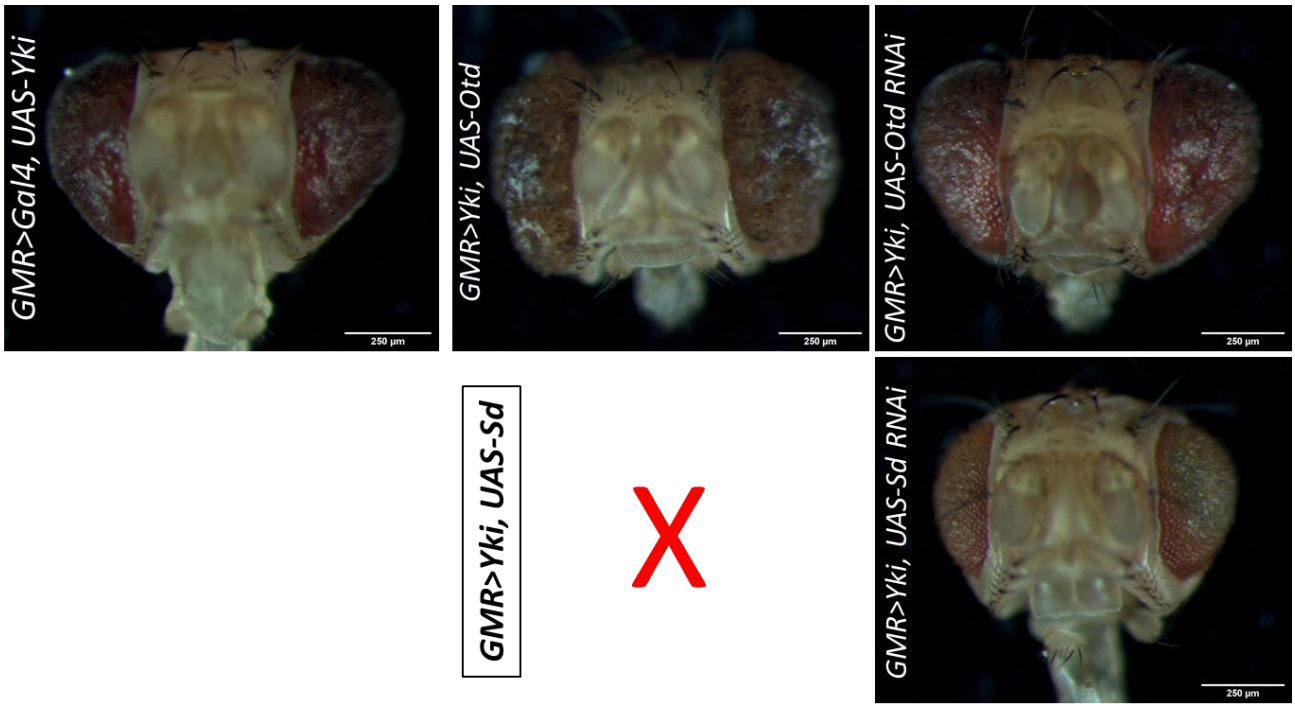

Fig 23. Otd overexpression in Yki overexpression context. The left panel shows the negative control. The middle and right panels show the phenotype caused by the overexpression or KD, respectively, of Otd (top) or Sd (bottom). The cross with GMR>Gal4, UAS-Yki with UAS-Sd did not produce live progeny. Scale bar 250um.

There are some differences. The overexpression of *yki* and *sd* together does not produce any progeny. Moreover, it seems that *sd*-KD is not able to induce a total rescue of the overgrown phenotype, as instead was shown for the *yki* mutant context. Regarding *otd*, instead, the overgrowth resulting from its overexpression is less severe, whereas with the KD the recovery to a normal morphology is nearly absent.

It can be concluded that overexpression of the mutant or the wild-type forms of Yki lead to different molecular responses.

4.3.2 *otd* and *sd* mutants show different phenotypes

The previous experiments demonstrate that Sd and Otd respond in different ways to Yki alterations. It would be interesting to know if they can compensate each other effects.

To follow this hypothesis, I have analysed their possible relation in a normal Yki context.

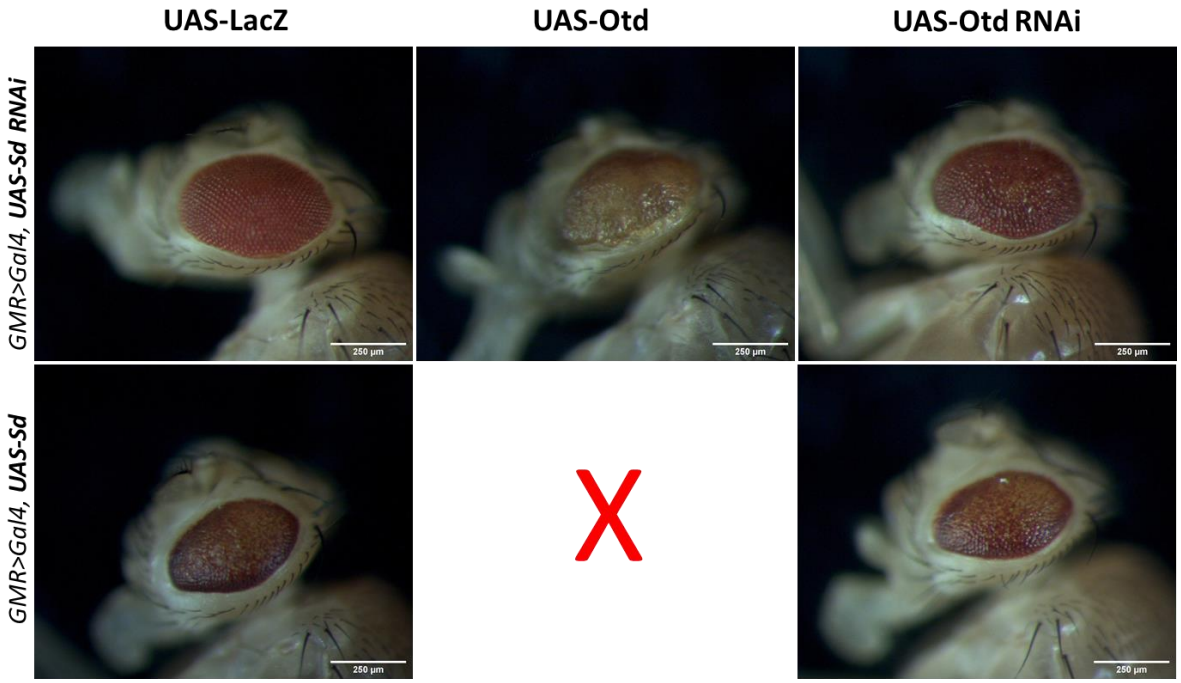

Fig. 24 Evaluation of Sd and Otd relation. In a Sd-KD (top panels) or Sd overexpression (bottom panels) context, it is evaluated the effect of the overexpression or KD of Otd. UAS-LacZ is used as a control.

The first observation is that the overexpression of both the transcription factors led to death of the progeny. On the other hand, double KD of *sd* and *otd* produced a rough eye, but with a conserved morphology. The up-regulation of *otd* in a *sd*-KD context produced a humpy eye, not overgrown but neither with a normal aspect. Instead, the overexpression of *sd* together with the KD of *otd* gave rise to the same smaller eye produced by *sd* alone. To conclude, *sd* seems to be epistatic, as its alteration cannot be rescued by *otd*. At the same time, *otd* has an additional effect, visible especially when in combination with the *yki* mutated form.

GENOTYPE	GROWTH LEVEL
GMR>Gal4,UAS-luci	0
GMR>Gal4,UAS-Otd	0
GMR>Gal4,UAS-OtdRNAi	0
GMR>Gal4,UAS-Sd	--
GMR>Gal4,UAS-SdRNAi	0
GMR>Gal4, UAS-Yki^{S168A}	++
GMR>Yki^{S168A}, UAS-Otd	+++
GMR>Yki^{S168A}, UAS-OtdRNAi	+
GMR>Yki^{S168A}, UAS-Sd	++
GMR>Yki^{S168A}, UAS-SdRNAi	0
GMR>Gal4, UAS-Yki	+
GMR>Yki, UAS-Otd	++
GMR>Yki, UAS-OtdRNAi	+
GMR>Yki, UAS-Sd	D
GMR>Yki, UAS-SdRNAi	-
GMR>Gal4,UAS-SdRNAi, UAS-LacZ	0
GMR>Gal4,UAS-SdRNAi, UAS-Otd	+
GMR>Gal4,UAS-SdRNAi, UAS-OtdRNAi	0
GMR>Gal4,UAS-Sd, UAS-LacZ	-
GMR>Gal4,UAS-Sd, UAS-Otd	D
GMR>Gal4,UAS-Sd, UAS-OtdRNAi	-

legend	
-	smaller
--	much smaller
0	normal
+	bigger
++	much bigger
+++	giant

Table 3. The table summarises the level of growth impairment, both in terms of deficit and overgrown, correspondent to each genotype analysed. The value are given on the basis of phenotype shown by 5 lateral and 5 front eye images representative of the whole population analysed, for each genotype.

5. Discussion and conclusions

The goal of this thesis was to better understand the role and the mechanism of action of the Hippo pathway in different contexts. It is proved that the Hippo pathway plays a central role within the cell, acting as a director of the cell outcome, funnelling and translating the multiple inputs coming from outside the membrane or from other signalling pathways, into one transcriptional response.
How can it do that? Taking advantage of the fruit fly, where this important signalling pathway was discovered, I have tried to add some new insights to the current knowledge.

Therefore, I have studied the Hippo kinase component Warts in the normal development of *Drosophila* eye; I have hypothesised and produced some results about the involvement of the Hippo pathway in the pathogenesis of the neurodegenerative Gaucher syndrome, and finally I have evaluated Orthodenticle as a co-factor of Yorkie in the overgrowth of the adult eye.

In the first part, I have focused on the role of the kinase Wts in guiding Rh6 expression in the developing pupal eye. Numerous studies have demonstrated that Wts is indirectly responsible for fate decision in the R8 cells, but they only delineated a sort of correlation between Wts and Rh6 expression. I firstly aimed at defining the timing of Wts turning on. I have dissected the pupal eyes at different time points and looked at Wts presence and I found out that Wts expression begins to differentiate among the R8 cells at 73h (Fig. 6). Knowing the starting point of Wts expression is an important step for the identification of the upstream molecules that trigger its activity. Indeed, it is known that the R7 cells, seated at the top of the R8 cells, are responsible for the initial choice of the Rh type. The dioxin receptor Spineless and the transcription factor Senseless are both responsible. On one side, Spineless is both necessary and sufficient for the formation of the ommatidial mosaic, as its mutant forms commit all R7 to the pale fate, while its overexpression induces the yellow fate. Therefore, it was suggested that the entire retinal mosaic required for colour vision is defined

by the stochastic expression of a single transcription factor, Spineless (Wernet et al., 2006). But it is not the only one responsible of PRs destiny. On the other hand, Senseless, together with Otd and in contrast to Prospero, another transcription factor, oppositely regulates R7 and R8 PR Rhodopsin gene expression (Xie et al. 2007). As a consequence of their activity, or silencing, the R7 cells express Rh4 or Rh3 respectively. Downstream of them, in the R8 cells, the growth regulator Melted is designated to respond to the Rh3 expression in the R7 cells, and antagonize Wts, thus leading to the pale fate (Jukam and Desplan, 2011; Mikeladze-Dvali et al., 2005). My results define a precise time point in which it is more likely to find the molecule/s responsible for mediating this process. Other indicators that some factors act above Wts are its changes in concentration and localisation. I found out that the amount of Wts seems to increase in only 5h (Fig. 7). Also, Wts changes localisation along this time period. In the earlier stages, it occupies a membrane position, visible thanks to the Z stack acquisitions, and co-staining for the basal-lateral marker Dlg used as reference point (Fig. 8). At 78h time point Wts appears to be localised around the nuclei. It is well known that the different position occupied by the kinase core components of the Hippo pathway influences enormously their activity. Indeed, as described above, if Hpo or Wts are localised next to the membrane, they are able to inhibit Yki with more efficacy (Deng et al. 2013; Sun et al., 2015a). The result obtained shows that Wts moves along the membrane leading to increased activity; indeed, Rh6 expression follows soon after the Wts turning on (Fig. 9). This can be caused by an upstream molecule acting on the localisation of Wts in order to modulate its activity. It remains unclear why the membrane proximity does not correspond to an increase of Wts, visible instead at 78h when the kinase occupies a perinuclear territory. It can be interesting to evaluate in the future, maybe also using a live-imaging time-course (already set up for other, not shown, experiments), the localisation of Wts around 78h, especially after it reaches a plateau. To conclude, the first part of my thesis has defined a critical time point in pupal eye development that can result to be key for evaluating other factors responsible for regulating Wts, and cell fate decision.

The second goal of my thesis was to evaluate the possible implication of the Hippo pathway in the neurodegenerative phenotype of the Gaucher disease (GD). The first observation to

be done regards the chosen *Drosophila* model. I have used both a *GBA*-RNA interference (*GBA*-KD) construct, that halves the quantity of enzyme respect to the wild type condition, and a *GBA*-KO fly. The *GBA*-KO model has been created in the laboratory of Professor Linda Partridge; this one results in the total absence of the acidic Glucocerebrosidase. This model is not the most appropriate to study the GD, as it shows some differences with the molecular basis evidenced in Gaucher patients. Indeed, in humans, mutations in the *GBA1* gene lead, in most cases, to a minor activity of the enzyme and to the accumulation of the unfolded protein in the endoplasmic reticulum. In the model I have used, as can be appreciated from the transcriptome analysis, the inflammatory response caused by stress mechanisms activated within the cell is not as high, as instead it is in the majority of patients affected by this pathology. The *GBA*-KO model is characterised by behavioural defects: climbing ability and survival are indeed deeply impaired (Fig. 10) (Davis et al. 2016; Kinghorn et al., 2016). At the same time, the *GBA*-KD model shows some defects only when the system is forced using a *Minute* background or higher temperatures. This can be related to the functionality of the enzyme in the fly that, even if conserving the same role played in humans, is probably less essential. Nonetheless, I have shown that the neurodegenerative pattern is also visible as a decrease in fluorescence in the adult eyes of *GBA*-KD flies, and it is not only due to aging but also to the degeneration of single ommatidia and to death of the associated optic nerve (Fig. 11). The unsolved question among the researchers interested in GD is why the same mutation in the *GBA* gene can either be mostly asymptomatic, or lead to a dramatic outcome. With the aim to identify possible new players in the neurodegenerative GD, I have decided to focus on the consequences deriving from the lack of the enzyme and investigate what molecules or signalling pathways are affected by this absence. For this reason, I have used the *GBA*-KO model. In this background I have followed the hypothesis of a growth deficit caused by alterations in the Hippo pathway. As described above, recent studies have shown that this signalling plays an important role in regulating growth and differentiation of many cell types within the brain (Irvine et al., 2011). Moreover, experiments performed in *Drosophila*, but also in mice models, revealed that the impairment of Yki/YAP is translated into a growth deficit that leads, in the end, to a neuroinflammatory response and to neuronal death (Calamita and Fanto, 2011; Dubei and Tapadia, 2017; Huang et al., 2016; Reddy and

Irvine, 2011). The first evaluations were performed in the *GBA*-KD context, as the *GBA*-KO was obtained only later during the PhD programme. The initial evidence of growth/differentiation mechanisms impairment is visible in the wing vein defects (Fig. 12). Looking at the literature, I found that the increased number of cross veins and the abnormal differentiation were traits peculiar of studies that investigated the impairment of several growth signalling pathway like Notch, EGFR and Hippo. Of note, the cells composing the *GBA1b*-KD regions were significantly smaller, supporting the "growth deficit" hypothesis (Fig. 13). To further confirm my hypothesis, I analysed some Yki targets. Both in the *GBA*-KD larval fat bodies and in the *GBA*-KO adult brain, I found a deep downregulation of some important target genes like *myc*, *dIAP* and *cycE* (Fig. 14 and Fig. 16). All encode well-known proliferation and apoptosis regulators. Even if they are activated also through other signalling pathways, it appears obvious that the lack of *GBA* impacts on some growth mechanisms. Moreover, the decrease in *expanded* levels (Fig. 14), commonly used as a readout of the activity of Yki, opens to the possibility that the growth defect is caused by a hyper-activation of the kinase core, that results in the impairment of Yki. In the next future, I aim at investigating the role of this transcriptional activator. It would be interesting to perform the same behavioural tests with the *GBA*-KO flies in a *hpo*-mutant or *yki*-overexpressing background. In agreement with my hypothesis, I expect that the mutation of the kinase or the extra-dosage of *yki* can rescue, at least in part, the neurodegenerative defects typical of the *GBA* mutant animals. In parallel to these analyses, I have noticed that the *GBA* promoter is differently activated in the neuronal and glial cells (Fig. 15). If we think about the glia as the macrophages of the brain, it makes sense seeing a higher activation of *GBA* in these cells, but glia absolves also other important functions. The most important for my study is its role in communication. The glial cells produce signals that are responsible for the balance between proliferation and differentiation of the neighbouring cells (Fernandes et al.,2017; Kanai et al., 2018; Lee et al., 2013; Spéder and Brand, 2018; Silies and Klambt, 2011). Because of that, I have decided to look at the phenotypic consequences of *GBA*-KD in the glial or in the neuronal populations. Both the larval motility and the adult climbing resulted more severely impaired when the knock down was promoted under the *repo* promoter, specific to the glial cells (Fig. 17). Of note, I have also tested animals in which *GBA*-KD was induced in both the cell types (data not

shown), but they did not reveal any additional phenotypic defect. In general, the reduced quantity of *GBA* led to locomotor deficits normally associated with neurodegeneration; it is interesting, although, that the damage is higher when only the glial compartment is involved. This damage can be due not only to the main function of GCase within the lysosomes, clearly more abundant in the glial cells, but also to the lack of communication between different neural cell populations. In accordance to that, I found deeply downregulated in the *GBA*-KO brain two important genes encoding the Dally and Dally-like Proteins, which are glypicans involved in the surfing of the morphogens. The low levels of these two sustain the hypothesis of an impairment caused by the lack of cues between glia and neurons. These results do not clarify the causes of the neurological damage typical of the neurodegenerative GD but offer two possible mechanisms of action. On the one hand, there could be a cell-autonomous hyper-Hippo condition which evolves into a growth defect responsible for neurodegeneration. On the other hand, the lack of *GBA* in the glial cells can damage the function of these cells and lead to the lack of signals that regulate neuronal homeostasis. Both mechanisms account on the impairment of endocytosis, which results altered in the transcriptome analysis (Fig. 19). Indeed, *GBA* is responsible for the production of ceramide, a fundamental component of cell membranes and vesicles. Moreover, endocytosis is also regulated by the Hippo pathway. To conclude, even if these are preliminary results, they open to an explanation of the mechanism that lay behind the neurodegenerative phenotypes of GD, and help find new possible therapeutics that can mitigate the severe conditions of the patients.

The last part of my thesis was focused on Orthodenticle and its possible interaction with Yki. I started investigating the role of this transcription factor in relation to Rh5 expression. The first experiments, conducted in the larval eyes, had the aim to define the potential of Otd in guiding Rhs expression. I found that the overexpression of Otd in not sufficient to lead to Rh5 turning on (Fig. 20). Knowing that both Yki and Otd are necessary for the pale fate induction, I have decided to over-express both and look at the phenotype produced in the adult. I was not able to look at the Rhodopsin expression pattern because the resulting organs were impossible to dissect, but what I found out is that Otd is implicated in eye overgrowth. The

overexpression of *otd* alone leads to a deformed eye, glazed and with some lumps, without affecting growth (Fig. 21). Otd is involved in the control of normal development of many tissues including some areas of the brain and the eyes; thus, it is reasonable thinking that the altered morphology is caused by its upregulation. The combined overexpression of *otd* and hyper-activated *yki* resulted in a massive eye overgrowth (Fig. 22). There is no evidence in the literature regarding the interaction of Otd with Yki, neither in humans between OTX2 and YAP. However, OTX2 is altered in both retinoblastoma and medulloblastoma where it is responsible for the over-proliferative and less differentiated pattern of the cells; indeed, it is under consideration as a therapeutic target (Bunt et al., 2011; Li et al., 2015; Stromecki et al., 2018). I have used Sd as a control. Sd is considered the elective partner of Yki, as their cooperation mediates the activation of many pro-proliferative and anti-apoptotic targets. Indeed, the overexpression of both leads to an overgrowth phenotype, less severe than the one induced by combined *otd* and *yki* (Fig. 22). On the contrary, *sd* overexpression alone led to a smaller eye, in agreement with the *default repression* model described in the literature, where Sd, when not conjugated with transcriptional activators such as Yki, acts as a transcriptional repressor (Fig. 21). These data suggest a different role for Otd and Sd. The differences were even more accentuated when I used another *yki* transgene. In the case above described, I induced *yki* hyper-activation through a mutation in the phosphorylation site, that leads to a constitutively active form; in the second case, we can observe the phenotypes induced by the simple overexpression of the wild-type form. The overexpression of both *otd* and *yki* leads to an overgrown eye, although smaller than the previous one generated by the *yki* mutant. Additionally, the combined overexpression of *sd* and *yki* did not produce any adult flies. What captured my attention was the result obtained with the RNAi constructs. *otd*-KD did not rescue the overgrowth phenotype produced by the overexpression of *yki*. This outcome contrasted what seen in the previous set of experiments: both the *yki* mutant and overexpression forms led to tissue overgrowth, but while *otd*-KD was able to recover, at least in part, the phenotype in the *yki*-hyperactive condition, it did not produce any effects in the *yki*-overexpression context (Fig. 22 and Fig. 23). What is the role of Otd? The knowledge about Otd is poor. I firstly supposed a role in chromatin remodelling: few evidences have indeed demonstrated that Otd is able to make chromatin

more accessible, thus enhancing transcription (Boulay et al., 2017; Yang et al., 2014). This hypothesis can explain the partial rescue obtained with the *yki* mutant; in quality of transcriptional enhancer, once Otd is removed the accessibility to DNA is lower, leading to a smaller eye, not still wild type, but neither characterised by an extreme overgrowth. On the contrary, the hypothesis does not explain the result obtained with *yki* overexpression. Assuming that the overdose of Yki is still able to enter nucleus and to conjugate with its partner, maybe Sd, and to activate transcription, DNA accessibility should be the same also in this case: enhanced by the overexpression of *otd* and reduced by its KD. Therefore, also in this case I should have observed a partial rescue of overgrowth, but instead I saw the same abnormal morphology caused by *yki* overexpression (Fig. 23). Given that, I formulated another hypothesis in which Otd does not act as a chromatin modifier, but instead binds to Yki in quality of transcription factor only when Yki is in the dephosphorylated, active conformation. It is well known that proteins change their shape as a consequence of phosphorylation. It can thus be reasonable thinking that de-phosphorylated Yki has more affinity for interactors like Otd. This may also happen in the cytoplasm, as there are few evidences that describe the mammalian orthologue of Otd, OTX2, outside the nucleus, shuttling between the two compartments (Baas et al., 2000). I will call this model "conformation-dependent interaction". This second hypothesis can better explain the different phenotypes obtained with hyper-activated or overexpressed *yki*. In the first case, Yki is already in the active conformation and can easily bind Otd, while in the second, the higher abundance of Yki does not increase the binding with Otd over the physiological threshold. Also, Sd produced different phenotypes depending on the context: a total rescue was visible when *sd*-KD was induced in the *yki* mutant background, but following overexpression of the *yki* wild-type form, the eye looked even smaller. The idea of a preferred binding conformation-dependent is not in conflict with Sd results. Indeed, Sd remains the elective partner of Yki and because of that its KD induces a total rescue. The proposed model is shown in Fig. 25.

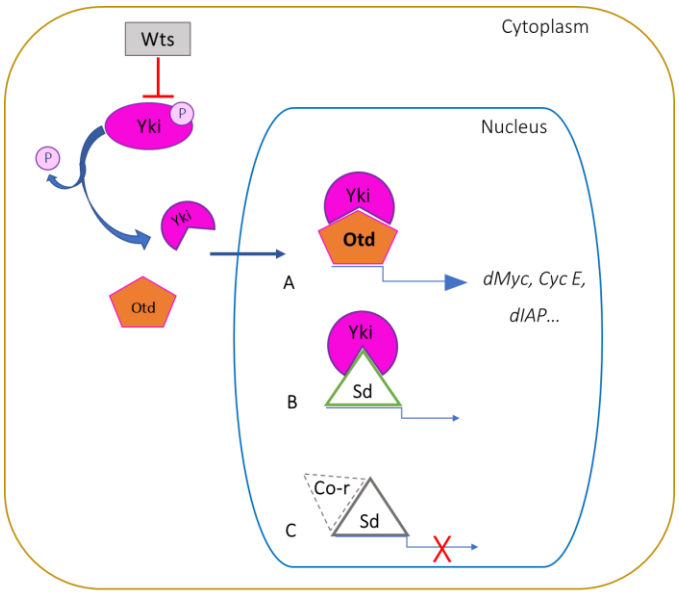

Fig. 25 The conformation-dependent interaction model. A. represents the case in which Yki is mutated in the phosphorylation site and, therefore, it can freely enter nucleus and associate with Otd; B. indicates what happens in normal development where Yki binds its partner Sd in order to regulate growth; C. describes the default repressor model, where Sd, when it is not binding Yki, is associated with co-repressors, like Tgi, and impede the transcription of the targets.

The last experiment wanted to evaluate the possible complementarity of Otd and Sd functions. As can be seen, overexpression of both leads to death, thus demonstrating an additional effect (Fig. 24). At the same time, double KD does not show a similar result, indicating that, even if the overexpression of the two transcriptional factors can lead to a dramatic result, maybe because of the *relationship* they have with Yki, thus hyper-activating too many target genes, at the same time they are not the only Yki partners, and the phenotype resulting from the double KD shows a rough eye that can still maintain the normal dimensions. The overexpression of *otd* in a *sd*-KD context does not lead to a normal eye, as well as the *otd*-KD does not modify the shape of the *sd*-overexpressing eye. It is deducible from these data that the two transcription factors do not compensate each other effects. To conclude this third part, Otd results involved in guiding the overgrowth phenotype. It is necessary to understand in which way: as a chromatin modifier or, more likely, as a Yki partner, or through other mechanisms still not identified. Even if there is no evidence regarding its direct interaction with Yki, it can be interesting to better define the role it plays,

also in relation to the position that this transcription factor occupies in some forms of cancer like medulloblastoma, where its oncogenic role is demonstrated.

In conclusion, my thesis has studied the Hippo pathway under different aspects. As can be noticed this signalling, more and more studied in the last decade, still surprises for the multiple roles played within the cell. The data I have produced amplify the already intricated *relations* that this signalling has with many molecules. This is the case of the Orthodenticle study; indeed, it is known that Yki interacts with many transcription activators, and maybe one, still uncovered, can be Otd. Noted for its involvement in growth mechanisms control, Otd can be an indirect or direct co-operator of Yki in leading to an altered proliferation in some brain cancers. Additionally, I have hypothesised and supported the possibility of a Hyper-Hippo condition as a background for the neuronopathic Gaucher. Although a molecular mechanism is still missing, I have shown that the dramatic neurological defects can arise from a growth deficit, possibly due to a hyper-activation of the kinase core that leads to a massive reduction in the growth targets of Yki. Finally, the study on Wts regulation in the pupal eye increases the knowledge regarding a relative new function of the Hippo pathway: cell fate decision. Understanding how Wts protein levels are modulated in such a quick way can shed light on the communication mechanisms between cells.

My work underlines the importance to keep on investigating the basis of the mechanisms of action of the Hippo pathway, as well of others, because it is from these studies that can be unveiled new functional molecular networks involved in the pathogenesis of a variety of human diseases.

6. Bibliography

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