Systemic effects of Chromosomal Instability induced Tumorigenesis:

A role of JAK/STAT and cytokine secretion in coupling inflammation to maturation defects in *Drosophila*

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pela tua força inalcançável, pela tua dedicação imensa

e pelo teu amor incondicional

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ABSTRACT

Alterations in developmental transitions are common among animals to compensate for growth disturbances. These variations are usually a consequence of altered steroidal hormone production resulting in puberty delays. Inflammation and high cytokine release seem to be behind these altercations, although in humans no concrete model has been put forward.

Here we use a *Drosophila* epithelial model of Chromosomal Instability-driven malignant transformation to unravel a role of the Upd3 cytokine and JAK/STAT signaling in coupling the development of these tumors with a delay in metamorphosis. We present evidence that Upd3, produced by malignant and adjacent wild type cell populations, signals to the prothoracic gland, an endocrine tissue primarily dedicated steroid hormone production, to activate JAK/STAT and the bantam miRNA and to delay metamorphosis. These results identify a new regulatory network impacting on ecdysone biosynthesis and provide experimental evidence that malignant tissues can have a direct effect on steroidal hormone regulation.

RESUMEN

Las alteraciones durante las transiciones del desarrollo son comunes en los animales como compensación ante perturbaciones en el crecimiento. Dichos cambios, son generalmente una consecuencia de afectar la producción de hormonas esteroides, que puede ocasionar retrasos de la pubertad. Tanto la inflamación, como la alta liberación de citoquinas, parecen ser responsables de dichas variaciones, sin embargo, para estudios humanos no se ha presentado ningún modelo concreto que verifique este hecho.

En este trabajo, utilizamos un modelo epitelial de transformación maligna impulsada por inestabilidad cromosómica en *Drosophila* para revelar el papel de la citoquina Upd3 y de la señalización de JAK/STAT en el retraso en la metamorfosis durante el desarrollo tumoral. Presentamos evidencia de que Upd3, producida por poblaciones malignas y por tejidos adyacentes de células normales, es capaz de señalar a la glándula protorácica, responsable por la producción de hormonas esteroideas, para activar JAK/STAT y el miRNA de Bantam, retrasando así la metamorfosis. A la luz de estos resultados, identificamos una nueva red reguladora que afecta a la biosíntesis de ecdysona y proporcionamos la evidencia experimental de que los tejidos malignos pueden tener un efecto directo en la regulación de la hormona esteroidea.

PROLOGUE

Puberty delays in humans have been reported as a consequence of the development of certain disorders, such as inflamed bowel disease, cystic fibrosis or CNS tumors. Although high levels of inflammation are the common feature of these disorders, drawing an inflammation-based puberty delay model from studies in human patients is highly complex. This complexity arises from the presence of many uncontrollable elements or stimuli, that difficult the task of defining non-confounding variables or gather specific information. Therefore, model organisms such as *Drosophila,* characterized by their strong capacity to adjust maturation programs with growth alterations, offer a good opportunity to identify the systemic signals coupling inflammation to maturation defects.

In the case of the fruit flies, this compensation usually occurs by blocking steroidal hormone ecdysone, in this way altering the normal timing of their life cycle whilst ensuring a normal size and pattern in the adult. Ecdysone production occurs in the prothoracic gland (PG) located in the ring gland, an endocrine organ closely connected to the brain and similar to the pituitary gland in humans. Two pathways mainly regulate its biosynthesis: one that is dependent of the release and action of the prothoracic hormone (PTTH) and one that is dependent on insulin-signaling (IIS). The tight regulation of ecdysone biosynthesis helps in the coordination of systemic growth when a disturbance takes place. Inflammation processes that generate a high number of cytokines, such as bacterial infections, have been described as one of these disturbances capable of inducing systemic delays.

In this work, we analyze the impact of the highly inflammatory epithelial model of Chromosomal Instability-driven malignant transformation in developmental timing regulation and steroidal hormone production. In this model, we observe that the defects in developmental timing are not a consequence of misregulation of PTTH hormone and Dilp8 release, as it has been previously described. Instead, we show that the delay is a consequence of JAK/STAT signaling activation in the ring gland leading to an increase in the expression of bantam microRNA signaling. The activation of this major signaling pathway is the consequence of the high cytokine release, mainly unpaired-3, from both the tumor site and other sources, culminating in a major systemic response to prevent larvae to pupa transition.

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INTRODUCTION

1. The intricate pathways of Tumorigenesis: General notions about tumor development, microenvironment and systemic impact

Tumorigenesis is defined as the gain of malignant properties in normal cells. This includes not only primarily dedifferentiation and increased proliferation, but also evasion of apoptosis, increased inflammation and altered immune responses. These malignant features of cancer cells are caused by the enormous amounts of somatic gene mutations in both oncogenes and tumor suppressor genes that alter both expression levels and/or activities that could lead to neoplastic transformation in normal cells (Stratton, Campbell, and Futreal 2009).

However, solid tumors are not just clones of cancer cells, they are masses composed of multiple cell types and extracellular matrix. That being said, it is easier to think about these solid tumors as organs although structurally and functionally abnormal. They are able to develop complex interactions between the elements that compose them using processes often similar to those used by developing organs. Tumors are also able to interact with the rest of the organism, similarly to normal organs. Nevertheless, normal organs usually perform activities and function in a way that supports the survival of the organism, whereas tumors systemic effects are often the main responsible for the poor outcome of a patient treatment or survival (Egeblad, Nakasone, and Werb 2011)

1.1 The hallmarks of Cancer

Cancer is a heterogeneous disease; however, the majority of cancer cells share many molecular, biochemical and cellular features (Hanahan and Weinberg 2000). Tumors are more than just masses of proliferative cancer cells, they are complex tissues composed by multiple cell types that are able to interact with each other. Moreover, tumors have an associated stroma formed by different cell types, from mesenchymal supporting cells to immune cells, which are active participants in the tumorigenesis process. Together, both tumors and stromal cells contribute to the development and expression of certain features called the hallmarks of cancer (Figure 1).

The hallmarks of cancer started by comprising six biological capabilities acquired during tumor development. They were basically defined by the ability of cells to resist cell death and evade growth suppressors, the capacity of sustaining proliferation and enable replicative immortality, and their aptitude for activating invasion and metastasis as well as inducing angiogenesis (Hanahan and Weinberg 2011). Cancer cells acquire these functional features mainly for two reasons: the inflammatory state, determined by lesions caused by the sustained activity of the immune system; and the genomic instability, which creates random mutations, acting from elevated rates of base pair mutation to chromosomal rearrangements (Hanahan and Weinberg 2000, 2011). Aside from these two important enablers, in the last decade, emerging hallmarks have increasingly considered abilities not only comprised in the cancer cells themselves, but also their impact in the surrounding

tissues. Tumors contain a repertoire of abilities: from being able to create a specific microenvironment that helps them develop and progress, to having the capacity of impacting tissues and organs outside the initial tumor site.

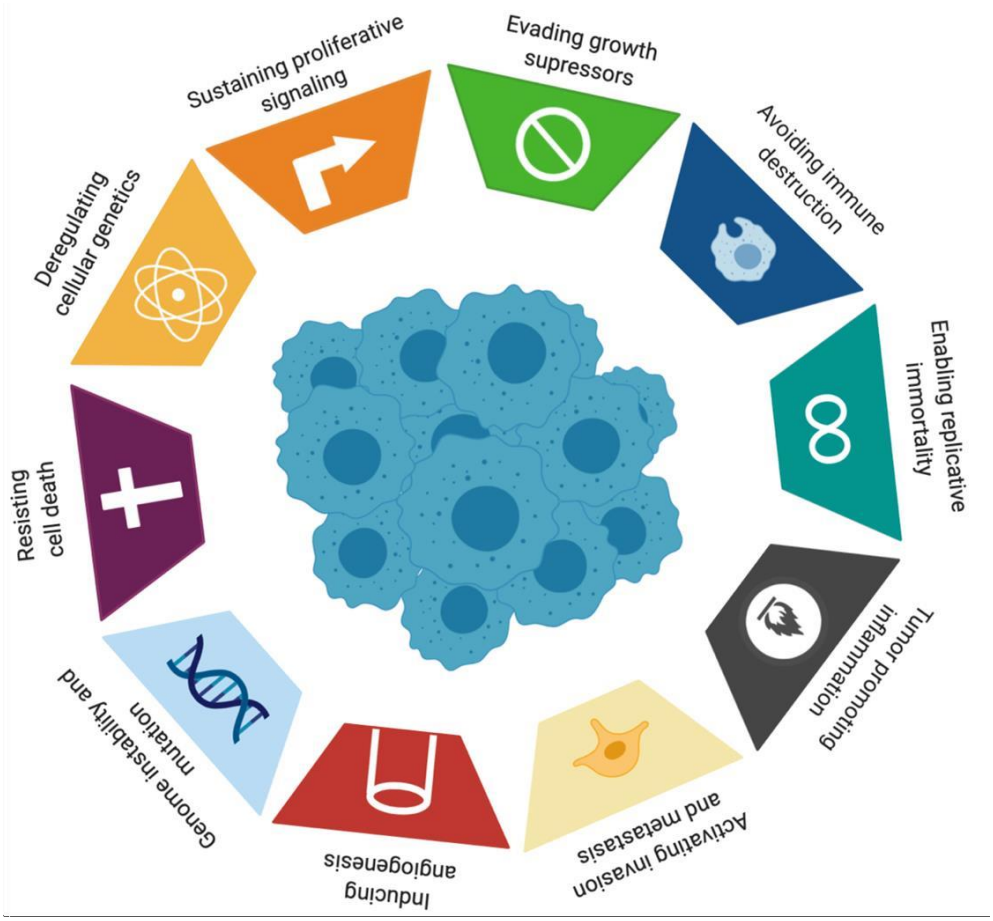

Figure 1: Hallmarks of Cancer

This illustration includes all the well-characterized hallmarks of cancer as well as the emerging ones proposed by Hanahan and Weinberg in 2011

1.2 The interplay between Tumor Development and Systemic Effects

Tumor Organization and Microenvironments

During the progression of tumorigenesis, tumors acquire a certain architecture that resembles earlier or less differentiated states of normal tissues. For instance, the multilayered epithelium with polarity defects seen in early mammary tumors matches the proliferative and invasive epithelium of the terminal end buds of the developing mammary gland (Ewald et al. 2008). Within the same line, the transcription factor GATA 3 not only maintains epithelial differentiation, organization and survival in the developing and adult mammary glands, but also plays a similar role in early breast carcinomas (Kouros-Mehr et al. 2008). However, as these breast carcinomas become less differentiated and transit to later stages, there is a progressive selection for GATA 3-negative progenitor-like cells. Therefore, reintroducing GATA 3 in cancer cells of later stages, leads to the acquisition of a higher differentiated state, which in turn leads to less metastatic tumors. This outcome seems to be due to an increase in tissue organization that brings the tumor architecture closer to the adult mammary gland pattern (Kouros-Mehr et al. 2008).

Aside from the fact that tumor organization seems to resemble different states of developing tissues, the tumor stroma or their microenvironment have also been increasingly observed to help characterize tumor development and progression. Extracellular matrix (ECM) deposition and immune cells infiltration, especially

leukocytes, are a good example of changes found in the tumor-stroma border that might be implicated in how the tumor progresses. In fact, most pathologists consider not only the morphology of the cancer cells, but also how this changes tissue organization happen, in order to identify and categorize neoplasias. For example, increased epithelial proliferation coupled with normal tissue organization usually represents a benign lesion (Muthuswamy 2009); and the presence of CD8+ cytotoxic T cells within cancer cell nests in colorectal cancer patients usually correlates with a better survival rate when compared with patients that only have T cells at the tumor margins (Naito et al. 1998).

Taking these examples into account, tumor microenvironments and their characterization has been considered of increasing importance since they can have a direct impact in the outcome of a patient's survival.

In normal tissues, the stem and progenitor cells that generate the final organ reside in specific environments called stem cell niches. Tumors also have specialized microenvironments or niches that confer distinct function to the cancer cells. These cancer cell niches are most probably able to sustain interactions with the ECM and supporting cells. The niches may prevent or drive cancer cell progression, or the cancer cells may hijack them and generate signals that lead to the generation of new niches (Plaks, Kong, and Werb 2015).

Some stem cell-like niches stimulate the development of epithelial tumors, such as in gastrointestinal tumors. In these intestinal stem

cells, Wnt signaling is required for self-renewal. Activation of the Wnt pathway, by inactivating mutations in the APC gene (a Wnt signaling inhibitor), drives both familial and sporadic colorectal cancers (Miller, Lavker, and Sun 2005). Moreover, germline mutations in SMAD4, a tumor suppressor gene that acts downstream of TGF-β, increases proliferation on the stromal cell population leading to the formation of gastrointestinal polyps and consequently epithelial cancer. Similarly, deletion of TGF-β receptors in stromal cells results in gastrointestinal epithelial malignancies in mice (Bhowmick et al. 2004). These results suggest that the alteration of the normal microenvironment has a deep impact in tissue homeostasis, which eventually might act to promote tumorigenesis.

However, just as there are tumor-promoting microenvironments, certain microenvironments can also restrict tumor progression. For instance, morphologically normal epithelium with the same gross chromosomal changes as adjacent breast carcinomas is found in about 25% of examined cases (Deng et al. 1996). While it is true that cancer cells might have acquired additional point mutations, this observation suggests that the microenvironment in the areas with morphologically normal epithelium may keep the epithelium from developing into carcinomas.

Oncogenes and tumor suppressors can regulate or increase the probability of loss of cell polarity. The activation of c-Myc does not transform or induce proliferation of quiescent mammary acinar structures unless an altered ECM or a silenced LKB1 disrupts the normal epithelial organization (a tumor suppressor also responsible for cell polarity maintenance) (Partanen et al. 2007). Within the same

line of thought, loss or mislocalization of Scribble, another cell polarity protein, acts with c-Myc to transform epithelial cells and induce tumors by blocking apoptosis in mouse models (Zhan et al. 2008). These results suggest that tissue architecture can provide a barrier to tumor development and that organisms have mechanisms in place that preserve the normal tissue structure, which must be overcome when tumors form.

Systemic effects derived from Tumorigenesis

Whilst we can logically infer that tumors affect their immediate surroundings, evidence is showing that tumors also present dramatic systemic effects. Indeed, their systemic impact is not just limited to metastatic behaviors, but also includes effects on immunity, coagulation and metabolism (Figure 2). Furthermore, it has been increasingly suggested that these major systemic changes are the main responsible for cancer patient's deaths, rather than effects of the direct overgrowth of the primary tumor or their metastasis. As a classical example, we have the metabolic syndrome cachexia. Cachexia is a metabolic disorder characterized by chronic wasting. Patients diagnosed with this metabolic disorder suffer from severe fatigue and weight loss, due to loss of adipose tissue and muscle mass. Cachexia is induced by factors secreted by tumors and may account for nearly a third of cancer cell deaths (Acharyya et al. 2004; Skipworth et al. 2007).

Introduction

Immune suppression

Tumors are known to suppress the immune system, increasing the poor outcome of a patient's treatment and, consequently, their mortality rate. Infection rates are mostly common in hematological cancers, but patients with solid tumors are also at increased risk of developing infections. Although immune system alterations are not common when diagnosis is performed, as the tumor progresses, immune deficiencies develop and are further amplified by certain treatments, such as chemotherapies (Hadden 2003).

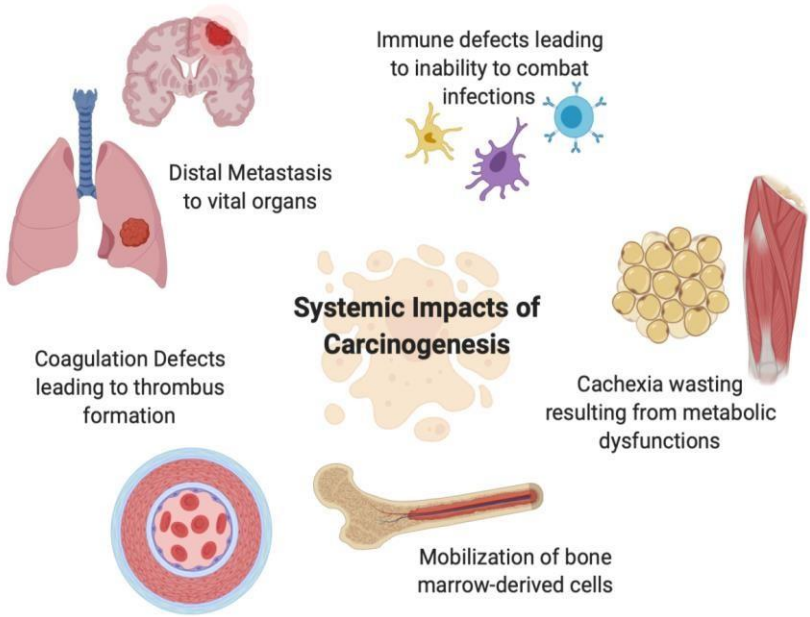

Figure 2: Systemic Impacts of Carcinogenesis

This illustration includes the main systemic effects observed in cancer patients

Tumor-induced increases in myeloid-derived suppressor cells (MDSCs) are also thought to be a crucial factor in the immune

dysfunction observed in cancer patients (Gabrilovich and Nagaraj 2009). The percentage of MDSCs in the spleen of normal mice increases from a 2-4% to a 20-40% in a tumor situation. Similarly, a ten-fold increase in the number of the same population of cells is observed in the blood of human patients (Gabrilovich and Nagaraj, 2009). The adaptive immune system is also affected by tumorigenesis: T cells are unable to perform their functions as regulatory T cells; a subpopulation of T cells that mainly acts as an immunosuppressive downregulating the induction and proliferation of effector T cells accumulate (De Visser, Eichten, and Coussens 2006).

Moreover, tumor-bearing mice of mammary carcinomas have also shown a diminished ability to produce antibodies to fight against foreign antigens (Danna et al. 2004).

Coagulation Abnormalities

Aside from the tumor's ability to affect the correct function of the immune system, 50% of all cancer patients, and 90% of those with metastasis, have some form of coagulation abnormality (De Cicco 2004). Consequently, thromboembolism is estimated to be the second most common cause of cancer-related deaths (Caine et al. 2002). The pro-thrombotic state in cancer patients originates from an abnormal hemodynamic system present in tumors with direct interaction between cancer cells and endothelial cells, platelets or monocytes. Aside from this, there is also an imbalance in pro-coagulatory and fibrinolytic (anti-coagulatory) factors induced by the tumor. Endothelial cells, macrophages and cancer cells in tumors

express tissue factor (the primary initiator of blood coagulation) and cancer pro-coagulant, which activates the coagulation cascade (Caine et al. 2002; De Cicco 2004; Rickles and Falanga 2001). Cancer induced changes in the coagulation system are linked to tumor angiogenesis. For instance, VEGF is produced on one hand to stimulate angiogenesis and the recruitment of blood vessels to the tumor site, but on the other, it also helps stimulate the secretion of tissue factors. In mouse models, inhibitors of coagulation can inhibit metastasis formation; whereas platelet activation and release of fibrin enhance hematogenous metastasis. The activation of both platelet and fibrin promotes the incorporation of cancer cells into mini-thrombi that protect the cancer cells from physical shear and attach from immune cells while they are travelling throughout the bloodstream (Camerer et al. 2004; Hejna, Raderer, and Zielinski 1999; Nieswandt et al. 1999; Palumbo et al. 2000). Consequently, the activated platelets may unknowingly facilitate extravasation of cancer cells to secondary sites where metastasis will occur (Figure 3).

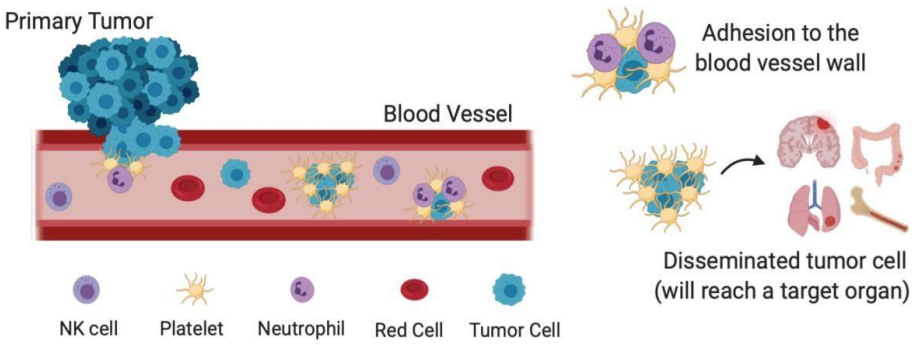

Figure 3: Dissimulated migration of tumoral cells through active platelet activity

This illustration includes the two main consequences tumoral cells suffer from interactions with active platelets

Endocrine and Metabolic Syndromes

Metabolic syndromes include various disorders from abdominal obesity, hypertension, dyslipidemia and hyperglycemia, thus being often linked to insulin resistance and diabetes development, as well as to nonalcoholic fatty liver disease. Many of these metabolic syndromes are highly prevalent or rising worldwide, and they are now being linked to the etiology and progression of certain cancer types and worse prognosis. Obesity and diabetes, for instance, have been associated with breast, endometrial, colorectal, pancreatic, hepatic and renal cancer (Table1). Dyslipidemia is characterized as an abnormal amount of lipids in the blood and hyperglycemia is characterized as an increasing concentration of sugar in the bloodstream - these metabolic syndromes are also considered relevant risk factors for cancer development (Hirano 2018).

The potential causal factors behind these syndromes and cancer development are inflammation and insulin resistance potentially caused by adipose tissue hypoxemia. The adipose tissue of obese patients shows inflammation, characterized by elevated inflammatory cytokines present in the plasma and inside the tissue, as well as macrophage infiltration and activation.

Inflammation, mainly due to TNF-α activation, is able to contribute to insulin resistance by intervening in the intracellular signaling cascade of insulin (Uysal et al. 1997; Ye 2009). Moreover, systemically elevated free fatty acids and decreased adiponectin levels found in the blood stream of obese individuals might also contribute to aggravate insulin resistance together with the increase number of inflammatory cytokines present, such as interleukins 1 and 6.

Table 1: Cancer types associated with different metabolic syndromes

Cancer Types	Obesity	Obesity and Type 2 Diabetes Mellitus	Dyslipidemia
Gastrointestinal	Cardia cancer Esophageal Adenocarcinoma Cholangiocarcinoma	Colorectal	
Gynecologic		Pancreatic Breast Endometrial	Breast
Hematologic	Cervical Adenocarcinoma B cell lymphoma Multiple Myeloma		
Renal		Renal cell	
Urological			Prostate
Lung			Lung

Interleukin 6 (IL-6) and TNF-α are also known to promote angiogenesis. Enhanced levels of IL-6 are commonly found in breast cancer patients, but the number rises when patients also show insulin resistance. However, even higher levels of this interleukin are present in estrogen receptor-positive breast cancer. In prostate cancer, IL-6 levels are significantly higher in hormone-resistant tumors, compared to hormone-dependent cancers. IL-6 was additionally shown to be necessary for the differentiation of immature plasmablasts into mature antibody producing plasma cells, which might explain the association of B cell lymphoma and multiple myeloma which are increased in obese patients (Gallagher and LeRoith 2010).

Despite being increasingly linked to cancer mechanisms, metabolic and endocrine syndromes are mostly presented as a risk factor,

rather than a consequence of tumor formation. However, due to the increased knowledge that tumors can alter their surroundings by secreting several long-range signaling molecules many of these syndromes might be revisited as a consequence of tumor formation, rather than just risk factors.

1.3 Drosophila as a model to understand epithelial tumors

Due to the high conservation of core biological systems across different species and the genetic code, model organisms can be used in order to address complex biological questions.

One of the most complex biological questions of our time is related to cancer development and their systemic impact. Cancer, as mentioned previously, is a multistep heterogeneous disease driven by the activation of certain oncogenic pathways and loss of tumor suppressor activity. Most of these pathways are highly conserved in *Drosophila melanogaster*, and the ability to easily manipulate its genome has made the fruit fly an incredibly useful organism to study cancer biology.

Drosophila genome is 60% homologous to that of humans and about 75% of all the genes, related to human diseases have homologs in flies (Ugur, Chen, and Bellen 2016). Aside from this, its short life span, low cost maintenance and the availability of an enormous genetic tool kit allows the fruit fly to become an eligible model to study complex pathways relevant in biomedical research (Bilder and

Irvine 2017; Kaufman 2017). In fact, ten Nobel prizes for six crucial groundbreaking research works (Figure 4) have acknowledged studies performed with the fruit fly.

In healthy organisms, development is a complex process that needs to be tightly regulated in order to generate a healthy individual. In cancer situations, this regulation is lost, which in turn leads to tissue overgrowth and other dysfunctional regulations known as the hallmarks of cancer (Hariharan and Bilder 2006). Many of the cancer hallmarks are well conserved in the fruit fly. Even back in 1918 Mary Stark was able to demonstrate that larvae of the lethal (1)7 strain developed intense black spots on their bodies and died at pre-adult stages because of these cellular growths. Aside from describing exhaustively these structures, she also tried to prolong animal survival by removing the black masses. She also was a pioneer in the attempt at tumor transplantation in *Drosophila* as she performed tumor transfers to healthy larvae using small needles, in order to assess if the mortality rate was due to the spreading of the cancer cells. However, the high lethality of the technique itself did not allow her to gather any convincing conclusions. Nonetheless, she did provide an answer to this question by dissolving tumors and injecting the suspension into healthy animals. By means of this experiment, she ended up observing that the tumor suspension was responsible for fly death, since control flies injected with a control solution survived. Mary Stark was also able to demonstrate the genetic origin of tumors (Stark 1918; Villegas 2019).

Aside from her many contributions, cancer studies in flies continued to flourish. Many pathways involved in cancer, including Notch, Hedgehog, Hippo, Wingless, and Toll, were also firstly described in flies. Moreover, tumor models for development, proliferation and cell invasion have been effectively established in this fly model (Gonzalez 2013; Richardson and Portela 2018). *Drosophila* has also been used as a pharmacological screening platform to identify cancer therapeutics and elucidate their mechanisms of action (Dar et al., 2012; Markstein et al., 2014.; Willoughby et al., 2013). In fact, some drugs currently used for the treatment of certain cancer types were primarily discovered in the fruit fly, such as the modulator of JAK/STAT signaling methotrexate. Methotrexate is a suppressor of STAT activation, reducing significantly STAT5 phosphorylation in human cells (Thomas et al. 2015).

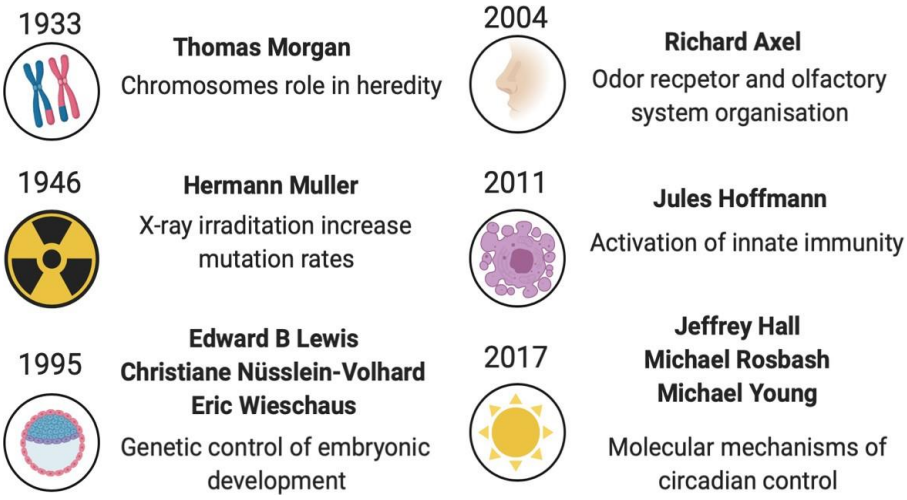

Figure 4: Nobel Prizes attributed to works conducted in *Drosophila*

This illustration includes the six Nobel Prizes and the ten award-winning researchers behind those projects

Introduction

Considering that, 90% of human cancers are of epithelial origin, fruit flies have also been used as a model organism to study epithelial tumors (Hanahan and Weinberg 2000). Epithelial tissues are characterized by a specific cell architecture composed of junctions and apical and baso-lateral membrane domains crucial for the maintenance of cellular functions. Loss of cell adhesion and polarity, coupled with an increase in cell motility and invasiveness, are common characteristics to many early cancer traits (Mirzoyan et al. 2019).

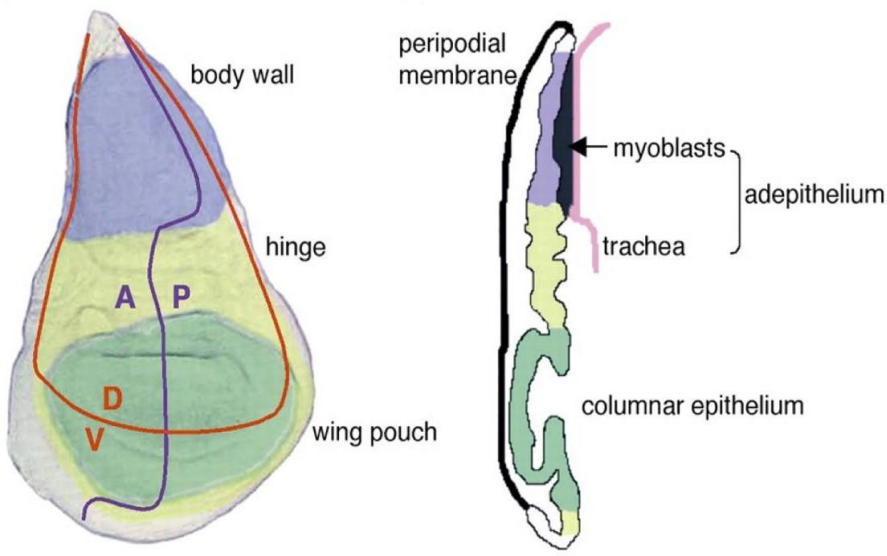

Figure 5: *Drosophila* wing imaginal disc structures and regions

This illustration includes the schematic representation of the wing epithelial tissues. Orthogonal view shows the pseudostratified nature of the wing epithelium. Extracted from (Butler et al 2003)

The fruit fly and its imaginal discs represent a monolayer epithelium limited apically by a squamous epithelium called the peripodial

membrane, and basally to the notum, by a layer of myoblasts embedded in ECM, constituting a perfect system in which to model the onset of epithelial cancer progression. These imaginal discs are morphological and biochemically comparable to the mammalian epithelia (Wodarz and Näthke 2007).

Imaginal wing discs have been extensively used to modulate epithelial tumorigenesis. These structures symbolize the primordial structure of the adult wing and proliferate exponentially during the larval stage. The wing imaginal disc is a sac-like structure formed by a continuous epithelial monolayer, which comprises two opposing layers that surround the disc lumen. One side of the imaginal disc is formed by a columnar epithelium, pseudostratified epithelium, while the peripodial membrane, a squamous epithelium, composes the other side. The wing disc is divided into different regions defined in concordance to the expression of selector genes, which confer specific cell identity (Garcia Bellido 1975) consequently preventing these groups (Anterior, Posterior, Dorsal, Ventral) from ever mixing (Figure 5). Specific growth signals such as Hedgehog, Notch, Wingless and Decapentaplegic are responsible for defining the growth and patterning of these regions during wing development (Garcia-Bellido and Merriam 1971; Lawrence and Struhl 1996)

Aside from their structure and morphology, the main signaling pathways that regulate growth in humans are also conserved in *Drosophila*. During the last few years, the wing and eye imaginal discs have been successfully used to study tumor growth and invasion, to investigate and assess cancer gene functions and to perform chemical and genetic screenings (Tipping and Perrimon

2013). The imaginal discs also arise as a good model to analyze oncogenic cooperation. Thanks to the MARCM system (Lee and Luo 1999) it is possible to induce, simultaneously in single cells, mutations in tumor suppressor genes, such as mutations in cell polarity genes, and oncogenes. This system also allows to overexpress specific genes, such as RAS, Myc, Yorkie or EGFR, resulting in tissue overgrowth, alteration of the tissue architecture, disruption of the basement membrane and invasive behavior (Brumby and Richardson 2003; Pagliarini and Xu 2003; Wu, Pastor-Pareja, and Xu 2010).

In addition to the MARCM system, and in order to achieve gene regulation in a specific tissue of the fly, the epithelial transformation model depends on the Gal4/UAS transactivation system (Brand and Perrimon 1993). This system relies on the Gal4 component, a transcriptional activator of yeast expressed in a tissue-specific manner, and on a UAS component, an upstream activator sequence bound by Gal4 in order to drive expression of any transgene located further downstream. This way, tissue-specific expression of a given transgene can take place. In addition to the Gal4 system, the QF system and the LexA/LexAop system are analogous to the Gal4/UAS system. They are binary expression systems that allow the expression of reporters and/or effectors in a defined subpopulation of cells. Using a combination of these systems, due to the fact that they function independently of one another, means that they can be used simultaneously to express several reporters or genes of interest in a different subset of cells (Riabinina and Potter 2016).

1.4 Drosophila as a model for chromosomal instability-induced tumorigenesis

As seen previously, *Drosophila* has been an extensively used model organism to understand the underlying mechanisms of tumor development. Since epithelial tumors account for a majority of diagnosed solid tumors, in this work I will use one of the current established epithelial tumor models to unravel the interactions between tumor development and the systemic impacts that arise within the organisms, especially focusing on steroidal hormone production. In order to do so, I will use the chromosomal instability (CIN)-derived tumor model, also named the CIN model.

CIN is defined as the dynamic change in chromosome number or structure and it has been extensively characterized in human tumors. CIN is known to generate aneuploidy, one of the most widely studied genomic alterations in cancer (Rajagopalan and Lengauer 2004). These alterations help maintain the heterogeneity of the tumor, which in turn favors its evolution to higher malignant and invasive states.

Drosophila, having just four pairs of chromosomes carrying 15.882 genes, arises as a useful model to study CIN and aneuploidy in a tumor context because loss or gain of a single chromosome represents an enormous difference for the cell (Milán et al. 2014). In fact, studies in the fruit fly have already allowed for the gathering of important information for the aneuploidy field. For instance, works with the fruit fly demonstrated that: chromosome trisomies are lethal, while increasing the full genome three times was viable (Bridges

1921). Therefore, segmental aneuploidies present a reverse correlation between fragment size and viability (Lindsley et al. 1972; Patterson 1935; Ripoll 1980). In addition, it is also important to considered not just size itself, but also the amount of information, for instance, trisomies of major autosomes (the second and the third chromosomes) are lethal whilst flies with different copies of the X and the 4th chromosome are perfectly viable (Bridges 1921).

Most recently, experiments performed depleting the spindle assembly checkpoint (SAC) genes (*bub3, rod*), the correct chromatin condensation (*orc2*), the process of cytokinesis (*dia*) and the genes involved in the spindle assembly (*asp*) showed induction of CIN in the epithelial cells of the fruit fly (Dekanty et al. 2012; Dekanty and Milán 2013). CIN-induced aneuploidy in these cells leads to the loss of apical-basal polarity, consequent cell delamination, and apoptosis. Aneuploid cells are removed by the activation of the JNK-caspase mediated apoptosis, which relies on the activation of the effector caspases DrICE and Dcp1 (Figure 6 A). This apoptotic response differs from those used by the mammalian system which is p53-dependent (Thompson and Compton 2010).

The activation of JNK-caspase mediated apoptosis in the face of CIN happens in order to eliminate these aneuploid cells from the tissue, this way, reassuring tissue homeostasis.

CIN is insufficient to drive tumor development in *Drosophila* stem cells (Castellanos, Dominguez, and Gonzalez 2008) or in SAC mutant mice (Holland and Cleveland 2009). However, if additional mutations in caretaker genes happen, such as mutations in the p53 gene, that regulates cell cycle functions and tumor suppression,

apart from the initial SAC mutations, mice are able to now develop tumors (M. Li et al. 2010). The same can be observed in the fruit fly: if apoptosis is blocked at different levels of the caspase cascade and aneuploidies are maintained by the continuous activation of the JNK pathway, the prevalence of aneuploid cells in the tissue results in tumorigenesis (Dekanty et al. 2012; Dekanty and Milán 2013).

Tumor development taking place as a result of CIN-induced aneuploidy and apoptosis blockage presents several interesting characteristics such as: DE-cadherin delocalization, basement membrane degradation and neoplastic growth (Dekanty et al. 2012). Moreover, in this model, cell delamination is not a consequence of the apoptotic response, neither it is dependent on the continuous activation of the stress response JNK pathway. Cell delamination is a consequence of the misplacement of DE-cadherin and blocking JNK does not rescue this behavior although it can rescue cell death (Dekanty et al. 2012).

Continuous activation of JNK, though, is essential for the development of the tumoral behavior. JNK is a stress response pathway present in *Drosophila* tissues whose original role is to remove aneuploid cells throughout apoptosis. Nonetheless, in a tumor situation, blockage of apoptosis leads to a continuous activation of this response pathway activating the JNK-dependent transcriptional program which promotes tumorigenesis (Benhra et al. 2018; Clemente-Ruiz et al. 2016; Dekanty et al. 2012; Muzzopappa, Murcia, and Milán 2017). Within this transcriptional program, we have an increase in the expression of the mitogen Wingless (Wg) (Pérez-Garijo, Shlevkov, and Morata 2009; Ryoo, Gorenc, and

Steller 2004; Smith-Bolton et al. 2009) and metalloproteases such as the Matrix Metalloproteinase 1 (MMP1) (Uhlirova and Bohmann 2006).

Aside from the JNK-induced transcriptional program, CIN derived tumorigenesis allows for the division of the epithelial tissue in two main populations: the growing epithelium with low levels of aneuploidy, and the delaminated population, with high levels of aneuploidy. The delaminated population has high levels of JNK activation and high secretory capability. This population also presents senescence-like features, such as increased lysosomal activity, alterations in the cellular morphology and cell-cycle arrest.

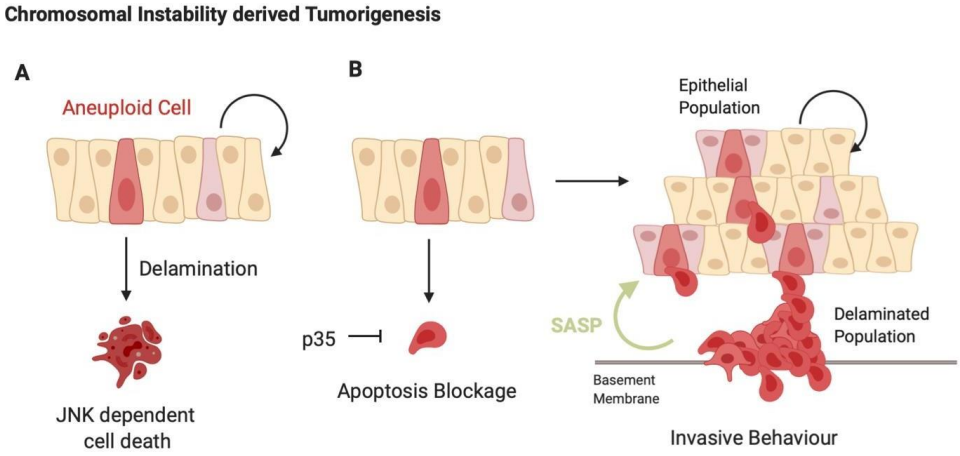

Figure 6: CIN derived tumorigenesis in *Drosophila* epithelial model

A – Aneuploid cells generated by the induction of CIN (through SAC depletion), delaminate from the epithelium and enter into JNK dependent apoptosis.

B – Delaminated cells with and additional blockage of the apoptotic response via p35 generate a second population of cells that is able to activate the senescent associated secretory phenotype (SASP) and promote the overgrowth of the epithelium population (through the release of mitogens) as well as degradation of the basement membrane (through the release of MMP1) to promote tumorigenesis.

The mitogens secreted by the delaminated population reach the low aneuploid growing epithelium and induce tissue overgrowth. This increased overgrowth leads to an increase in cell errors that, in consequence, leads them on a path to become more aneuploid and, eventually, delaminate. Therefore, the cross-feeding interaction between these two populations of cells drives the uncontrolled growth characteristic of CIN derived tumors (Muzzopappa, Murcia, and Milán 2017) (Figure 6 B).

Taking into account the relevance of the characteristics found in the delaminated population of cells in order to study the systemic effect of CIN derived tumorigenesis, the secretome of this population gains special interest. Remarkably, by analyzing some of the long-range signaling molecules secreted by this population of cells, not only do we find the aforementioned mitogens, but also metabolic regulators such as the lactate dehydrogenases known as ImpL3, and the insulin growth factor binding protein (IGFBP), known in flies as ImpL2. Both have either been linked with the Warburg effect metabolic shift (ImpL3) (Eichenlaub et al., 2018; Wang et al., 2016), or with the development of cancer associated cachexia (ImpL2) (Figueroa-Clarevega and Bilder 2015). In the secretome of the delaminated cells we can also find other important molecules such as cytokines, known in flies as the Upds (Upd1-3), that have functions regarding the immune system (Upd3), patterning (Upd1) and insulin secretion (Upd2) (Trivedi and Starz-Gaiano 2018a).

Characterizing the secretome of the delaminated cell population might shed a light onto the possible systemic impact and malignancy

of tumor development, giving rise to a better understanding of a complex disease in the context of complex organisms.

2. Developmental Timing Control: The central role of ecdysone steroidal hormone and their master regulators

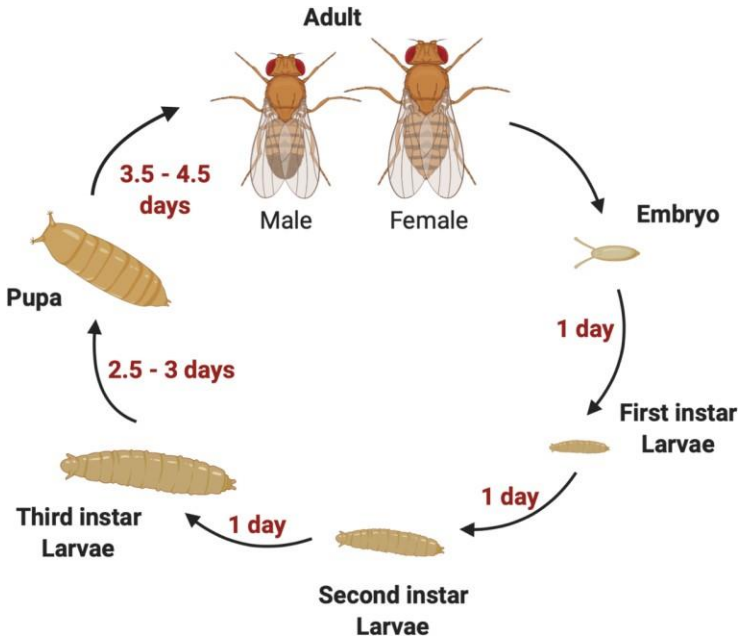

Figure 7: *Drosophila* life cycle

This illustration includes all the well-characterized phases of the *Drosophila* life cycle based on (Ong et al; 2015)

Most developing animals are capable of adjusting their growth and maturation programs in order to compensate for growth disturbances or alterations, such as injuries or tumor formation (A. Garelli et al. 2012). In the case of fruit flies, this compensation usually occurs by blocking the production, preventing the release or the activation of

the steroidal hormone ecdysone, in this way altering the normal timing of their life cycle whilst ensuring a normal size and pattern in the adult.

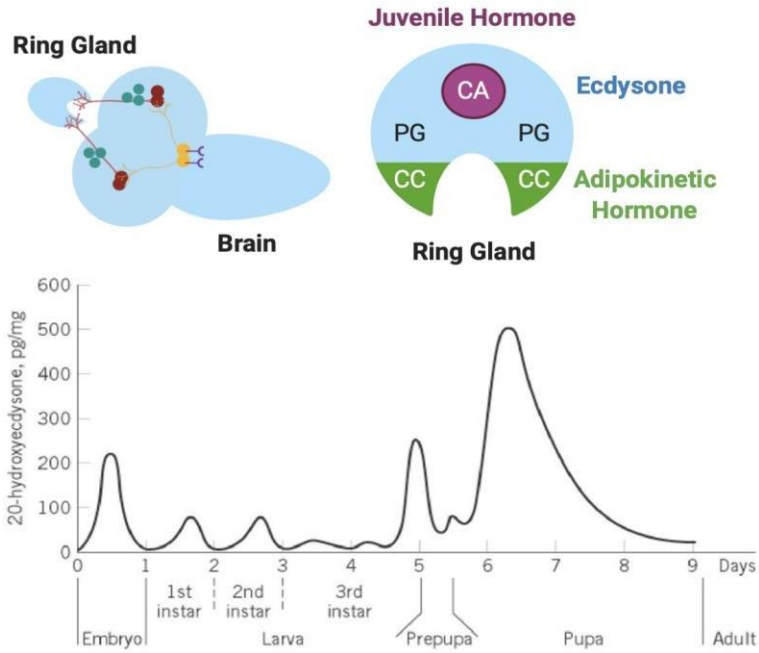

Figure 8: *Drosophila* ecdysone peaks during different life cycle stages

This illustration includes a schematic representation of the brain and ring gland, divided in the three main regions that compose it: the *corpora cardiaca* (CC), the *corpus allatum* (CA) and the prothoracic gland (PG). All three regions are color coded and represented next to the hormone they produce. The graph shows a representation of the ecdysone peaks during the different life cycle stages (adapted from Ou and King-Jones, 2013)

The life cycle of *Drosophila* is separated into four stages (Figure 7). The first stage refers to embryonic development, in which case the body axes are first established, followed by cellularization, segmentation and complex morphogenetic events leading up to larval hatching. Larval phases are divided into three instars before

metamorphosis initiation. The molting of the cuticle (ecdysis), which happens in order to accommodate animal growth, separates the larval stages. Larval tissues grow thanks to successive rounds of genome duplication leading to an increase in cell size without cell division.

The consequent increase in body mass observed during larval instars is mainly due to the growth of these endoreplicative tissues. At the end of the third instar, larvae enter into metamorphosis and transform into a pupa. This phase is marked by intense tissue remodeling and changes in the body structure culminating in the hatching of the adult.

In the fruit fly these transitions are clearly punctuated by pulses of ecdysone secretion. Ecdysone is a steroidal hormone that regulates insects' developmental transitions. It is secreted by the prothoracic gland (PG), an endocrine tissue located in the ring gland and connected to the larval brain. The ring gland, situated between the two brain lobes in the larvae, is composed by three main regions: the PG, the *corpora allata* (CA) and the *corpora cardiaca* (CC) (McBrayer et al. 2007a; Žitňan et al. 2007).

Ecdysone release is controlled by a complex combination of upstream factors, including peptide hormones and neuropeptide signals. The PG is able to incorporate several signals from the organism in order to coordinate ecdysone biosynthesis.

The peaks of ecdysone (Figure 8) at the end of the first (L1) and second (L2) larval instars trigger molting of the larval cuticle, to

accommodate increased animal size. A series of low-titer hormone pulses follow during the third instar (L3) preparing the animal for metamorphosis by inducing key behavioral and developmental changes (Warren et al. 2006). These changes include cessation of feeding, initiation of the wandering behavior, glue protein synthesis in the salivary gland, and the initiation of fat body autophagy (Rusten et al. 2004; Scott, Schuldiner, and Neufeld 2004). At the end of L3, a high titer ecdysone peak triggers pupariation, growth arrest and the onset of metamorphosis (C. K. Mirth et al. 2014). The larva stops moving, its spiracle evert, and the larval cuticle hardens into a pupal case that surrounds the organism for the time of its metamorphosis. At this point, the imaginal discs evert to form the basic outline of the adult body, but the head is still tucked within the body cavity. Soon after that, a brief pulse of ecdysone triggers the eversion of the head from the thorax and the transition from prepupa to pupa. The final peak of ecdysone brings the adult differentiation prior to hatching.

Ecdysone acts by binding to its nuclear receptor, EcR, and initiates several gene expression cascades responsible for tissue-specific responses (Yamanaka, Rewitz, and O'Connor 2013)

Ecdysone biosynthesis requirements for metamorphosis are extremely well regulated; therefore, its production will only take place if two main criteria are met: firstly, critical weight must be achieved and secondly the imaginal discs need to reach a mature state. Different regulatory networks extensively supervise these steps and they ensure that the transition from larva to pupa goes smoothly. Nevertheless, in case of growth disturbances, these regulatory networks can induce some changes in ecdysone biosynthesis, preventing this hormone from being released ahead of time and giving the damaged tissues time to recover (A. Garelli et al. 2012).

2.1 Ecdysone Signaling and Biosynthesis

Just like vertebrate steroids, ecdysone binds to members of the nuclear receptor superfamily, which function as ligand-dependent transcription factors (King-Jones and Thummel 2005). Ecdysone acts through a heterodimer of the ecdysone receptor, EcR, and Ultraspiracle, USP, nuclear receptors (Thomas et al., 1993; Yao et al., 1993). When ecdysone is absent, the heterodimer functions as a transcriptional repressor to prevent precocious activation of metamorphosis (Schubiger & Truman, 2000; Schubiger et al., 2005) (Figure 9).

The ecdysone receptor gene encodes three protein isoforms due to its two promoters and alternative splicing, which are denominated EcR-A, EcR-B1 and EcR-B2 (Talbot, Swyryd, and Hogness 1993). These three isoforms seem to have different ways to stimulate transcription when in the presence of ecdysone (Spindler et al. 2009) and they are also expressed in a tissue- and time-specific manner performing different functions throughout development (Cherbas et al. 2003). Mutations affecting the common region to all isoforms of EcR are embryonic lethal (Kozlova and Thummel 2003). EcR-A seems to be required during late development, since by mutating it arrests metamorphosis progression in late pupa. This isoform of the receptor is expressed mainly in the ring land and in imaginal discs, and it is sufficient to support development of the wing disc margins (Cherbas et al. 2003; Davis et al. 2005). EcR-B1 is predominantly expressed in larval tissues that don't contribute to adult structures, especially the midgut and salivary glands. Loss of function of EcR-B1 blocks ecdysone response in these tissues resulting in a failure to properly complete metamorphosis (Bender et al. 1997). EcR-B2

is the only isoform that supports ecdysone signaling in the larval epidermis and in the border cells of the developing egg chamber (Cherbas et al. 2003).

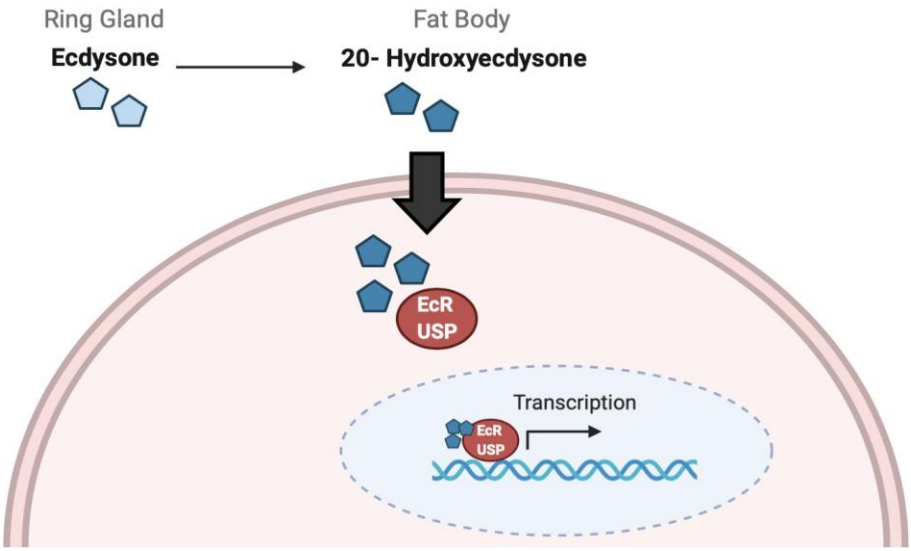

Figure 9: *Drosophila* ecdysone interaction with EcR and USP

This illustration includes a schematic representation of how ecdysone interacts with its receptors in order to promote transcription.

All three EcR isoforms are able to interact with USP, the fly homolog of the vertebrate retinoic X receptor (RXR) (Oro, Mckeown, and Evans 1992). The expression of the EcR is positively regulated by the presence of ecdysone hormone. The microRNA *miR-14* targets EcR mRNA for degradation and is repressed by EcR (Varghese and Cohen 2007). When ecdysone levels are high, there is a requirement for higher EcR activity, inhibiting *miR-14* and allowing for increased EcR expression. This positive autoregulatory loop sensitizes target tissues to ecdysone pulses. The EcR/USP heterodimer triggers

transcription of primary and secondary response genes in ecdysone target tissues.

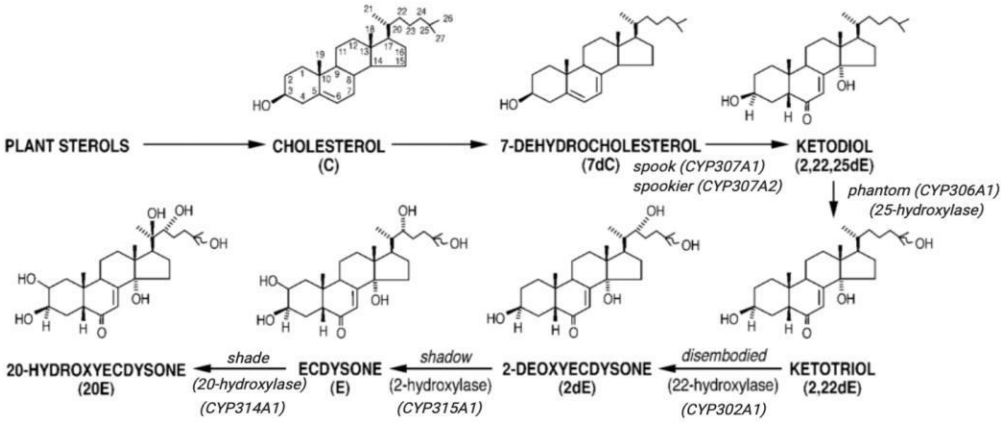

Figure 10: Ecdysone biosynthesis in *Drosophila*

This illustration includes a schematic representation of the synthesis of ecdysone from cholesterol relying on the activity of genes coding for Cytochrome P450 enzymes that catalyze the different reactions of the pathway. These genes are called Halloween genes and they are six: *spook* (*spo*), *spookier* (*spok*), *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*). The first five are produced in the PG and regulate ecdysone production. *Shade* is expressed in non-endocrine tissues where it converts ecdysone into 20-hydroxyecdysone, the active form of the hormone. (adapted from Warren et al 2002)

In order to properly interact with its receptor and form heterodimers with USP to trigger a transcriptional response, ecdysone hormone needs to be produced and activated.

During larval stages, the steroid hormone is produced primarily in the prothoracic gland (PG) by a series of enzymatic steps that converts cholesterol into ecdysone (Figure 10).

The Halloween genes encode cytochrome P450 enzymes that catalyze the majority of the successive reactions of the ecdysone

biosynthesis (L. I. Gilbert 2004; Huang, Warren, and Gilbert 2008). To date, six genes have been described as members of this family: *spook* (*spo*), *spookier* (*spok*), *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*). These genes were characterized by an embryonic lethality associated with a failure to form differentiated first instar cuticle (Jürgens et al. 1984; Nüsslein-Volhard, Wieschaus, and Kluding 1984; Wieschaus, Nüsslein-Volhard, and Jürgens 1984). The functional characterization of the enzymatic properties of these genes was determined by using *Drosophila* S2 cells and known substrates (Warren et al. 2002, 2004). The coding region of each gene was inserted into an expression vector under control of an actin promoter and transfected into the cells which were then incubated with different radiolabeled intermediates in the biosynthetic pathway. The radiolabeled products were identified, and the enzymes kinetics were established. *spook* (*spo*), *spookier* (*spok*), *phantom* (*phm*), *disembodied* (*dib*) and *shadow* (*sad*) are expressed specifically in the embryonic and larval PG before each peak of ecdysone secretion. Once the hormone is released into the hemolymph, ecdysone is taken up by several peripheral tissues including the gut, fat body and Malpighian tubes, where it will be converted into its active form 20-hydroxyecdysone (20E), by the enzyme P450 monooxygenase, a product of the *shade* gene (Petryk et al. 2003).

2.2 Ecdysone Regulation and Release

In order to regulate the timing of ecdysone production, the PG has to acquire information from different inputs coming from the central

nervous system and systemic cues that bring information from both the peripheral tissues and the surrounding environment.

The main actor in controlling ecdysone biosynthesis is the neuropeptide PTTH. However, other players are known to intervene in the process, revealing that a network of complex regulatory mechanisms tightly regulates steroidal hormone production.

The PTTH-dependent ecdysone regulation

The prothoracic hormone (PTTH) is the primary player in stimulating ecdysone production in the prothoracic gland. PTTH is released from two pairs of neurosecretory cells in the brain in order to generate ecdysone pulses during development. Its release is of special importance to allow for the transition of larvae to pupa (Kawakami et al., 1990; McBrayer et al., 2007). Originally purified from *Bombyx mori* brain extracts, PTTH was initially denominated the brain hormone, responsible for stimulating ecdysone production in the prothoracic glands (Kawakami et al. 1990). In Lepidoptera, PTTH is produced primarily in a pair of bilateral neurosecretory neurons whose axons terminate innervating the *corpus allatum*, a secretory gland of the neuroendocrine system (Agui et al. 1979; Dai, Mizoguchi, and Gilbert 1994; Mizoguchi et al. 1990). In *Drosophila*, PTTH is produced by a pair of bilateral neurosecretory cells in the brain which directly innervate the PG (McBrayer et al., 2007). Ablating the PTTH by expressing a pro-apoptotic gene using a *ptth-gal4* driver strongly delays development and, at the same time, allows for bigger adults. The delay generated is of several extra

days, which the larvae spend in the third instar. However, they eventually enter into metamorphosis, suggesting that the PTTH hormone is not absolutely required for the onset of metamorphosis but that it controls final body size by regulating timing of metamorphosis. PTTH signaling promotes ecdysone biosynthesis by tampering with the expression of several Halloween genes, as their expression is also strongly delayed in ablated animals (McBrayer et al., 2007).

PTTH signals through the receptor tyrosine kinase (RTK) Torso located in the PG (Figure 11) (Rewitz et al. 2009). Torso signaling is known to regulate terminal patterning in the embryo through binding to its ligand Trunk, which is structurally very similar to the PTTH hormone. Their similarity is so remarkable that expression of PTTH in the embryo can rescue terminal differentiation in *trunk* mutants. Torso/PTTH signaling relies on the activation of the canonical MAPK signaling pathway leading to the phosphorylation of ERK (extracellular signal-regulated kinase). The depletion of either *torso* or *ERK* in the PG cells generates the same results as the ablation of the PTTH-neurons, meaning, higher body size and increased delay in developmental timing. These alterations can be rescued by feeding the larvae with an activated form of ecdysone (20E), confirming that the delay and increased body size are indeed caused by abnormal levels of this steroidal hormone.

These observations also provide an explanation for previous studies showing that Ras activity (a member of the MAPK signaling pathway that can be activated by Torso) in the PG cells is able to modulate

developmental timing by regulating ecdysone synthesis (Caldwell, Walkiewicz, and Stern 2005).

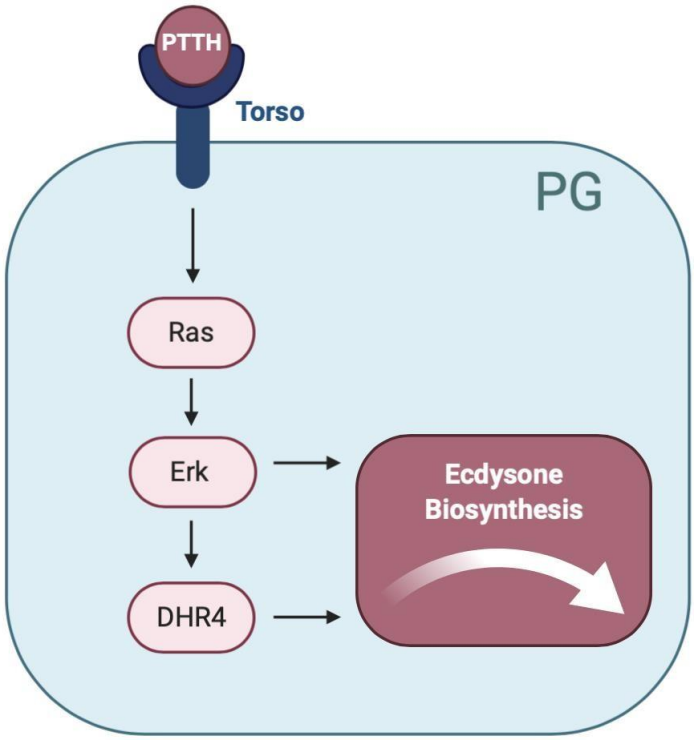

Figure 11: PTTH-dependent regulation of Ecdysone Biosynthesis

This illustration includes a schematic representation of PTTH hormone activating its receptor Torso and, consequently, the MAPK signaling pathway leading to ERK phosphorylation.

Loss of PTTH is able to cause developmental timing delay in the transition from third instar larvae to pupa. However, losing PTTH affects only slightly (in a matter of a few hours) the transition between larval molting stages, suggesting a minimal role played in these transitions. Though larval transitions do not seem to be affected by PTTH signaling, PTTH is involved in the generation of the low titer peaks of ecdysone at the very beginning of the third larval instar

through the regulation of the nuclear receptor DHR4. A study performed in 2011 unraveled that the nucleocytoplasmic trafficking of DHR4 in the PG is required for the formation of these low titer peaks inducing changes in the behavior when faced with the impossibility of doing so (Ou, Magico, and King-Jones 2011). When *DHR4* expression is impaired in the PG cells, larvae stop feeding and enter into early metamorphosis supported by a faster increase in ecdysone levels. DHR4 protein oscillates between the cytoplasm and the nucleus of L3 instar larvae. Its nuclear translocation marks the termination of the ecdysone pulses. DHR4 remains localized in the nucleus in the absence of PTTH, while constitutive activation of this hormone maintains the protein in the cytoplasm. Therefore, PTTH not only has a strong role in metamorphosis transitions but also in the promotion of ecdysone production in early L3, by preventing DHR4 from entering the nucleus of PG cells too soon.

As it happens for other insects, PTTH release in *Drosophila* seems to be regulated by the photoperiod (McBrayer et al., 2007). The expression of *ptth* is not uniform but shows a cyclic pattern with an eight hours' time period until it strongly increases twelve hours before pupariation. In animals mutated for *pdf*, a gene known to regulate circadian rhythms, the oscillations of the cyclic pattern are altered and *ptth* mRNA levels are, consequently, upregulated. Moreover, PDF-producing neurons directly innervate on PTTH neurons, thus reinforcing the idea that the circadian clock contributes to the periodicity of PTTH production and negatively regulates its expression. The levels of PTTH are also modulated by two developmental checkpoints that monitor animal size (the critical weight) and the development of the future adult structures. This

leads us to the concept that not only is it necessary to control PTTH hormone peaks, but also to assure correct nutritional availability for the animal to withstand the metamorphosis process.

TOR and Insulin dependent ecdysone regulation

The critical weight and the minimal viable weight are two nutrition-related concepts that are also necessary to allow the transition to metamorphosis. The minimal viable weight is defined as the state where the animal has enough amount of nutrients stored to assure survival during the metamorphosis timing. The critical weight is defined as the minimal size obtained where a condition of starvation will no longer delay larvae to pupa transition. The critical weight is also defined as the moment where there is a decline in juvenile hormone release and an increase in the release of PTTH.

In order to coordinate the responses to allow for the entering in metamorphosis, the PG must be able to coordinate the information coming from the neuropeptides, such as the PTTH, but also incorporate information coming from the organism itself. Meaning, specifically information regarding nutrient availability- such as the one arriving through the signaling of the TOR and Insulin (IIS) pathways.

IIS/TOR signaling plays a major role in increasing larval body mass and postnatal body size in fruit flies and mammals, respectively, with an intimate connection to developmental maturation processes.

As larvae prepare to mature, by initiating the formation of the puparium, the activity of the steroidal hormone ecdysone increases

with the deceleration of body growth, thereby finalizing the size of the adult body at the end of larval development. In mammals, exponential body growth occurs mainly from birth to puberty. The entering into adulthood is accompanied by an increase in sex steroidal hormones activity that coincides with the deceleration of body growth. In order to do so, IIS and TOR act as nutrient-sensing pathways, transducing nutritional conditions to control cellular and systemic metabolism and growth.

Insulin and insulin-like peptides (ILPs or dILPs in *Drosophila*) secreted from insulin-producing cells, called the IPCs, function in an endocrine manner by binding to the insulin receptor (InRs) on the membrane of target cells. This ligand-binding action triggers the phosphorylation cascade that comprises the insulin receptor substrate (IRS or CHICO in the fruit fly), phosphoinositide-3 kinase (PI3K) and Akt (Oldham and Hafen 2003). The PI3K complex consists of a catalytic subunit, called p110, and a regulatory subunit, named p85a. These subunits allow PI3K to catalyze the conversion of phosphatidylinositol 4,5-biphosphate (PIP_2) into phosphatidylinositol 3,4,5-triphosphate (PIP_3) in the membrane, an action that can be reversed by action of the phosphatase PTEN (Lee et al., 1999). Accumulation of PIP_3 in the cell membrane recruits proteins such as PDK1 and Akt (Mora et al. 2004; Oldham and Hafen 2003). Once Akt is activated by phosphorylation by PDK1, there is the subsequent phosphorylation of several downstream effector proteins, such as the transcription factor Forkhead box O (FOXO). FOXO is an inhibitor of insulin activity, which upon phosphorylation is prevented from going to the nucleus, thus suppressing its transcriptional activity (Arden 2008). Aside from FOXO, the activity

of the insulin signaling pathway can also be regulated in extracellular spaces by binding proteins called IGFBPs (insulin-like growth factor binding proteins). These binding proteins are able to connect with the IGFs and both prolong their half-lives and modulate their availability and activity (Smith et al. 1997)

A protein complex containing the TOR kinase (TORC1) is activated in a cell-autonomous manner either by the signaling through insulin/PI3K/Akt or in response to the availability of extracellular nutrients. Rag GTPases mediate this activation of TOR through cell autonomous sensing of nutrient availability (Kim et al. 2008). Activation of TOR through the insulin pathway functions through the suppressing of the complex formed by TSC2 (tuberous sclerosis complex), or through the phosphorylation of PRAS40 by Akt, both of which are TOR inhibitors. Therefore, repressing TSC2 and PRAS40 allows the activation of TOR in an IIS-induced manner (Haar et al. 2007; Sancak et al. 2007)

The activation of TOR kinase stimulates cell growth by increasing protein translation with enhanced ribosome biogenesis. These processes occur because there is phosphorylation of 4EBP (translation initiation factor 4E-binding protein), which increases translation, and S6K (ribosomal protein S6 kinase), which increases ribosome biogenesis (Bruce A. Hay, Huh, and Guo 2004). On its end, S6K is able to phosphorylate IRS in order to inhibit IIS activity, which contributes to the prevention of the overactivation of the insulin pathway by an excess of nutrient stimuli (Bhaskar and Hay 2007). The activity of Akt can also be promoted by TOR (Hietakangas and Cohen 2007), and the expression of InR is induced by FOXO, a

transcription factor repressed by Akt, which may potentiate the effects of ILPs on the IIS pathway upon starvation (Puig and Tjian 2005). These examples are a clear proof that there is an extensive crosstalk between both the IIS and the TOR pathways. Such crosstalk exists in order to control both growth and metabolism in response to conditions of nutrient fluctuation (Hyun 2018).

As mentioned previously, the interaction between these two pathways helps the fruit fly correctly assess and control its body size, namely through the attainment of the critical weight checkpoint which coincides with the onset of three serial low-titer pulses of ecdysone in the early third instar larvae stage. The PG plays a critical role in determining final body size by regulating the attainment of the critical weight and the duration of the larval growth period. The suppression of ecdysone production by inactivation of either PI3K, Ras or Raf in the PG leads to increased final adult size (Caldwell, Walkiewicz, and Stern 2005; C. Mirth, Truman, and Riddiford 2005). This happens because their inactivation causes the larval growth period to be extended, and even though the growth rate of the larvae is not altered, the extra time they spend in the food causes the larvae to increase in size. Moreover, preventing EcR signaling in peripheral tissues also increases final adult size, stimulates the activities of PI3K and Akt, and blocks FOXO nuclear localization (Julien Colombani et al. 2005).

This brings us to the conclusion that ecdysone might affect final adult size in one of two ways: 1) by altering the duration of the larval growth period (delay in pupariation) or 2) by changing the rate of body growth through the modulation of peripheral IIS. Ecdysone is

also responsible for the attenuation of insulin activity in peripheral tissues during the larval growth period. Studies regarding the effect of ecdysone and its receptor showed that ecdysone mainly acts on EcR in the cells of the fat body, which suppresses the activity of Myc (transcription factor that promotes growth) and consequently represses IIS and body growth at a systemic level (Rénald Delanoue, Slaidina, and Léopold 2010).

microRNAs (miRs), in the fly, are also involved in the ecdysone mediated growth control processes. miR-8, for instance, promotes IIS in both flies and humans by suppressing a common target gene named *u-shaper (ush)* (Hyun et al. 2009). miR-8 promoter region has a number of binding sites for proteins encoded by ecdysone's early response genes, a sign that this microRNA is repressed upon ecdysone signaling. Increased levels of ecdysone in the hemolymph were proposed to suppress miR-8 expression, increasing Ush expression and attenuating insulin signaling in the peripheral tissues (Hyun et al. 2009).

Aside from miR-8, bantam, an insect-specific microRNA, is also well-known for its cell autonomous mitogenic effects in the imaginal epithelial discs in *Drosophila* larvae (Boulan, Martín, and Milán 2013; Brennecke et al. 2003). bantam can regulate growth not only at a local level, but also by regulating systemic body growth. The expression of this microRNA in the PG suppresses the biogenesis of ecdysone, which reduces the basal ecdysone level released into the hemolymph, thereby increasing peripheral IIS activity and the rate of larval body growth. In turn, when the insulin activity increases in the PG, it acts as a suppressor of bantam, thereby connecting the systemic response and ecdysone biosynthesis (Boulan, Martín, and Milán 2013).

Recent studies showed that Warts (Wts) signaling mediate ecdysone-synthesizing effects of dILPs and PTTH. Wts apparently downregulates the activity of bantam and Yorki (Yki), controlling ecdysone biosynthesis and larval growth (Moeller et al. 2017). The gene targets of bantam responsible for the control of ecdysone generation are still, to this day, not identified (Figure 12).

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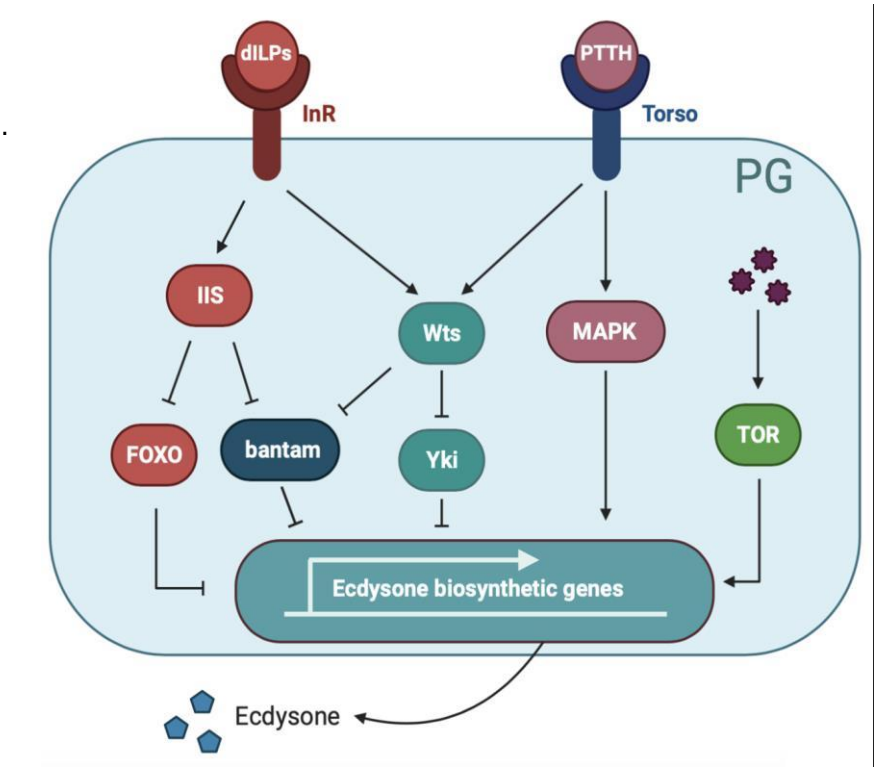

Figure 12: IIS and TOR-dependent regulation of Ecdysone Biosynthesis

This illustration includes a schematic representation of the molecular pathways taking place in the PG implicated in ecdysone biosynthesis.

2.3 Steroidal Hormone Alterations: From correct Nutrient-Sensing to Injury responses

Nutrient-sensing Responses and the Fat body as a Nutrient-sensing Organ

Attaining the critical weight (CW) is important for the larvae because it is believed that the storage of the nutrients gathered will allow the larvae to undergo further development without further nutrient feeding. Therefore, nutrient-sensing is critical for the developing larvae to know if they should stop feeding and enter into a wandering stage, or if they should delay the timing of puparium formation and extend the feeding stage. Whether or not the starvation of the developing larvae ends up delaying pupariation timing depends on when the starvation happens relative to the CW (Mirth & Riddiford, 2007). For instance, when larvae are starved before they attain the CW checkpoint, they pause their developmental progression until normal nutritional condition is restored; but when larvae experience starvation after the CW checkpoint, they continue transitioning into the pupal stage without any delay taking place. Indeed, it has been shown that starvation after critical size was obtained was even capable of accelerating developmental timing (Stieper et al. 2008). This acceleration was deeply associated with the larvae weight at the time of pupariation: larvae showing weights lower then 0.8mg showed higher variations in pupariation timing then the ones suffering starvation whilst weighing more than 0.8mg. Mostly, these results are related to the fact that starvation processes prone larvae to gradually lose mass as they continue developmental processes. Therefore, at a given time point, it is better for the animal to transit to the next stage of metamorphic development and compromise in

adult size, than to continue starving. In order to control cessation of larval feeding and transition into puparium formation, nutrient sensing and larval body growth control is of extreme importance.

In *Drosophila,* the fat body is the metabolic organ with functions similar to the mammalian liver and adipose tissues, and it is thought to have a nutrient-sensing ability. This tissue monitors the nutritional condition, being responsible for the balance between storing or mobilizing energy resources in the forms of glycogen or lipids. Evidence also shows that the fat body can play an endocrine role by producing some hormonal peptides and releasing them in the hemolymph, regulating systemic metabolism and growth capacities.

For instance, suppressing TOR signaling in the fat body by inactivation of the cationic amino acid transporter *slimfast*, causes the deceleration of growth on a systemic level (Julien Colombani et al. 2003). This particular transporter is known to be involved in amino acid processing. Amino acid deprivation generates a growth defect even when normal feeding and digestive tract processing are taking place. In this scenario, the fat body senses a.a. deprivation and activates TOR signaling, leading to a systemic response which involves remobilization of lipid vesicles, reduction of cell size and reduction of endoreduplication levels. This fat body mediated systemic modulation is induced by a downregulation of PI3K response in the peripheral tissues, and a reduced expression of both *dilp3* and *dilp5* in the m-NSCs neurons. This indicates that the secretion factors coming from the fat body are able to diffuse into the peripheral tissues and modulate their ability to respond to insulin signaling, thereby controlling the rate of body growth.

Many other humoral peptides that may have the capacity of modifying growth at a systemic level have also been reported as being expressed in the fat body - ALS (acid-labile subunit), for example, is a binding partner of IGF-1 in mammals, and it is responsible for stabilizing and restraining its activity. In *Drosophila*, ALS has been shown to be expressed in the fat body and form a complex with the dILPs in a manner similar to what happens in mammals (Arquier et al. 2008). Other binding proteins, such as ImpL2, are also able to block dILP activity, specifically by forming a ternary complex with dILP2 and dALS (Alic et al. 2011; Honegger et al. 2008). Consistently, the blocking of ImpL2 activity leads to an increase in adult size (Honegger et al. 2008).

Neural Lazarillo (NLaz), a lipocalin family member, homologous of the mammalian retinol-binding protein 4, has also been shown to be secreted from the fat body and having the capacity of attenuating insulin signaling responses (Hull-Thompson et al. 2009). When NLaz is mutated, flies are bigger in size and exhibit an increase in the peripheral insulin signaling response. Although it is known that this lipocalin causes insulin resistance in the peripheral tissues, the mechanism by which NLaz modulates IIS activity is still unknown.

Aside from the already mentioned secreted factor, additional humoral factors released from the fat body can modulate systemic IIS in different ways. By employing an ex vivo co-culture of both fat body tissue and larvae brains containing IPCs, it was observed an increase in dILP secretion from the IPCs, promoted by the humoral factors of fat body origin (Géminard, Rulifson, and Léopold 2009). Some of these humoral factors have been identified over the years, such as upd2. Unpaired 2 (Upd2) is a cytokine that was shown to be secreted from the fat body of well-nourished larvae. Upd2 is capable

of activating JAK/STAT signaling in the GABAergic neurons located close to the IPCs and responsible for their secretory inhibition. The JAK/STAT signaling activation in these neurosecretory neurons is able to release their repression on the IPCs, causing these cells to release dILPs into the hemolymph (Rajan and Perrimon 2012).

Other cytokines are also known to be involved in IIS regulation, such as Eiger, the fly homolog of the TNF-α, a proinflammatory cytokine. Eiger was found to be released into the hemolymph upon cleavage by the TACE (TNF-α converting enzyme) enzyme found active in the fat body of low-protein feeding larvae. Eiger then travels and binds to its receptor Grindelwald present on the IPCs, leading to a JNK-dependent inhibition of dILP production (Agrawal et al. 2016).

Lastly, two growth-blocking peptides (GBPs), called GBP1 and GBP2, have also been shown to be expressed in the larval fat body in response to amino acids and TOR signaling. These two GBPs are secreted from the fat body and can stimulate dILP secretion from the IPCs, although their direct targets of action are still unknown (Koyama and Mirth 2016).

Interestingly, expression of humoral protein from the fat body and their impact in dILP production and secretion from the IPCs appears to depend specifically on different macronutrients. For instance, Upd2 expression appears to be induced by fat and sugar rather than by amino acids, while Eiger and GBPs appear to respond to the availability of dietary proteins.

This ability of the fat body to coordinate metabolism and growth in response to systemic nutritional cues through an endocrine response reinforces the theory that IIS/TOR signaling in the fat body

plays a central role in controlling larval body growth and adult size determination. Although it is not completely understood all the situations where IIS and TOR act, in parallel or together, within the fat body, the suppression of either one can cause a decrease in body size.

Inactivation of an amino acid transporter, Slimfast (Slif), in the fat body is able to suppress TOR's activity giving rise to smaller adults (Julien Colombani et al. 2003). The suppression of IIS in the fat body by inactivation of InR or PI3K activity also attenuates growth (Britton et al. 2002; Hyun et al. 2009). On the contrary, activation of Akt or InR in the fat body rescues the small body size induced by immune responses or Torso knockdown, which in the fat body is responsible for activation of both TOR and IIS signaling (DiAngelo et al. 2009; Jun et al. 2016).

Mostly because of their impact on a systemic level, TOR and IIS interactions studies continue to be of deep interest for the scientific community. For instance, it has been recently identified that a member of the secretin-incretin receptor subfamily, called Methuselah (Mth), generated a similar response to the one observed in larvae fed with low amino acid diets, a response generally associated with TOR signaling (Delanoue et al., 2016). This receptor responded to two ligands, identified as SunA and SunB, and was required in the IPCs for the correct secretion of Dilps. Indeed, in this study, the authors demonstrated that reduced TOR levels in FB cells actually controlled Sun ligand secretion into the hemolymph. Consequently, low levels of circulating Sun eventually impeded its receptor from being activated in the IPCs, preventing Dilps from being secreted. Low levels of circulating Dilps, as previously

mentioned, led to a systemic response of reduced larvae growth (Delanoue et al., 2016).

Aside from the Sun peptide, inter-organ communication via fat body derived signals (FBS) has also been described for other players, such as Upd2 and CCHa2, known to be released in order to stimulate IPC activity after sugar and/or lipid intakes. Aside from these signals, GBPs (GBP1-2), growth factors produced by the fat body cells, have also been described as modulators of Dilp secretion in response to alterations in amino acid signals. Indeed, GBPs are released from fat cells and act as long range hormonal agonists of EGFR signaling in a set of neurons called the IPC-connecting neurons (ICNs) (Meschi, Léopold, and Delanoue 2019). These sets of bilateral neurons make direct synaptic connections to the IPCs and, in a normal situation, are usually blocked, allowing Dilps to be released and promoting animal growth. This is controlled by the capacity of GBPs to activate EGFR signaling in these neurons, relieving their inhibitory effects on the IPCs and resulting in Dilp secretion. However, when larvae are submitted to low-protein diets, ICNs are able to inhibit Dilp2 secretion through modulation of the referred neural activity (Meschi, Léopold, and Delanoue 2019). These results revealing that metabolic hormones, such as EGF-like signals acting on EGFR, are able to act as critical modulators of EGFR dependent neural activity, coupling insulin secretion to the nutritional status.

All of these studies share two things in common. First, the idea that both TOR and the IIS signaling pathways cannot be visualized as two completely independent mechanisms, but as synergetic ones that can act individually or not depending on the situation at hand. And second, that inter-organ communication is extremely relevant

for the correct sensing of nutrient status, especially when concerning signals derived from the fat body cells.

Developmental delays – Lessons from Tumor and Injury models

From the previous chapters it is clear that both developmental and environmental signals are important in the determination of ecdysone pulses' timing that dictates the duration of larval growth and entering into metamorphosis.

However, aside from these natural cues, it is known since the 70's that metamorphosis can be delayed when imaginal discs suffer damage that induces cell death (Russell, 1974; Simpson et al., 1980; Pat Simpson & Schneiderman, 1975). Delays also happen when imaginal discs suffer irradiation, that in turn slows down proliferation cell rates, or when ribosomal proteins are knocked-down which also has an impact in proliferation rates (Stieper et al. 2008). Larvae delay their developmental timing in these situations in order for the damaged tissues to have time to regenerate and reestablish both growth and pattern after the injury takes place. Elimination of an entire imaginal disc, however, does not affect metamorphosis timing (Simpson, Berreur, and Berreur-Bonnenfant 1980). Aside from tissue injury, mutants where discs can overgrow beyond their normal size also display developmental timing delays (Sehnal and Bryant 1993). That being said, both regeneration and overgrowth are capable of generating additional cell proliferation events, suggesting that dividing discs are able to produce signals that prevent ecdysone release or activation, preventing the entering into metamorphosis

stages. Therefore, there must be a direct relationship between growing and cell proliferation periods and the timing of pupariation.

Now, when damage is induced in the first or second instar larvar stages, the delay extension always takes place in the third instar while the other transitions between larval instars remain unaffected (Halme, Cheng, and Hariharan 2010). This happens, most probably, because there is a developmental checkpoint during the third-instar stage that delays the entry into puparium formation after tissue damage events. Irradiating animals at different developmental time-points, revealed that this checkpoint occurs around 24 hours after critical weight is obtained, consistent with previous observations that showed that irradiation delayed metamorphosis mainly by increasing the terminal growth period (the time that comprises the reaching of the critical weight size to the entering into pupariation) (Stieper et al. 2008).

Aside from irradiation, chronic expression of pro-apoptotic genes in the wing imaginal disc is equally capable to generate developmental delays. However, this chronic expression produces continued damage, causing pupariation to be strongly delayed. Eventually, this blockage is overcome, indicating that the delay mechanism has a defined threshold in which either death or progression must occur.

The developmental checkpoint taking place in the third instar larval stages regulates the ability of damaged discs to regenerate. Tissues damaged before this checkpoint induce a delay that allow the tissue to recover and grow to the appropriate size. After the checkpoint, the induction of lesion in imaginal discs no longer affects the timing of pupariation, giving rise to adults that exhibit a reduction in tissue size

as well as patterning defects. Before the checkpoint, imaginal discs are therefore able to produce a signal to inform the endocrine tissues involved in the regulation of pupariation about their growing status. Mutations in genes encoding components of the retinoid biosynthetic pathway partially rescue the delay induced by tissue damage. They do so by reducing the delay in *ptth* transcription, indicating that a retinoid or its metabolite could function as one of those signals (Halme, Cheng, and Hariharan 2010).

However, the better-known molecule identified as the main responsible for the interplay between tissue regeneration and developmental delay is Dilp8. Dilp8 is a relaxin-like protein that was initially characterized as a secreted factor related to the insulin-like peptide family. Nevertheless, Dilp8 is now known as the signal emanating from growing larval discs capable of inhibiting the entry into metamorphosis (Colombani et al., 2012; Garelli et al., 2012). A project developed by Colombani, Andersen and Léopold used a neoplastic growth condition of imaginal discs, known as the Avalanche Model, to carry out a genome-wide screen looking for RNAi lines able to rescue the delay observed in pupariation when co-expressed in the discs.

The Avalanche Model consists of using an interference RNA for the syntaxin avalanche, that functions in the early endocytic machinery, making up one of the two essential components for the vesicle trafficking machinery (syntaxin and Rab protein). Whenever one of these two components is mutated or is not functioning properly, there is an expansion of the membrane apical domain of the epithelium, causing polarity defects. Such polarity defects coupled with

increased proliferation leads to neoplastic growth, and consequently tumor formation. Of the positive scores obtained for the Avalanche Model, only one targeting *dilp8* rescued the delay caused by a transgene that slows down the growth rate of imaginal discs, the Rpl7 (that codifies a cytosolic large ribosomal subunit). Shortly after, Garelli, Gontijo and colleagues also published their work showing that *dilp8* could also rescue the developmental delay produced by eye tumors (the eyeful model). *dilp8* was found by the microarray technique to be the most enriched putative secreted peptide in eye tumors.

This secreted peptide was being produced in the imaginal disc cells and was found upregulated in response to disc damage or overgrowth (using the eyeful model and two other regeneration models: the Bx>rpr model were cell death is obtained using reaper, a pro-apoptotic gene, expression in the wing pouch area; and the EMS-feeding model were using these genotoxic dose-dependent component similar effects to irradiation or upregulation of caspases activity are recreated). Dilp8 upregulation seems to delay pupariation by inhibiting PTTH production but also by acting directly on the PG cells and ecdysone release (Figure 13).

During normal development, Dilp8 is also produced and expressed, however the levels of *dilp8* mRNA drop in mid-third instar. This reduction is highly likely to be a prerequisite for the initiation of pupariation, since overexpressing *dilp8* in imaginal discs delays pupariation and gives rise to bigger adults. *dilp8* loss of function, on the other hand, does not lead to early metamorphosis entering neither to smaller sized adults. This particular loss of function constitutes a permissive event for the onset of metamorphosis, but the actual entering into pupariation requires additional signals. In *ex*

vivo cultures of discs, brains and ring glands, incubation of a full-length Dilp8 expressing discs inhibits the expression of ecdysone-responsive genes when compared to incubation with Dilp8 expressing either a truncated or non-secretable form of Dilp8. Indicating, in this way, that Dilp8 produced by the disc remotely acts on the complex formed by the brain and the ring gland to delay ecdysone production and activity.

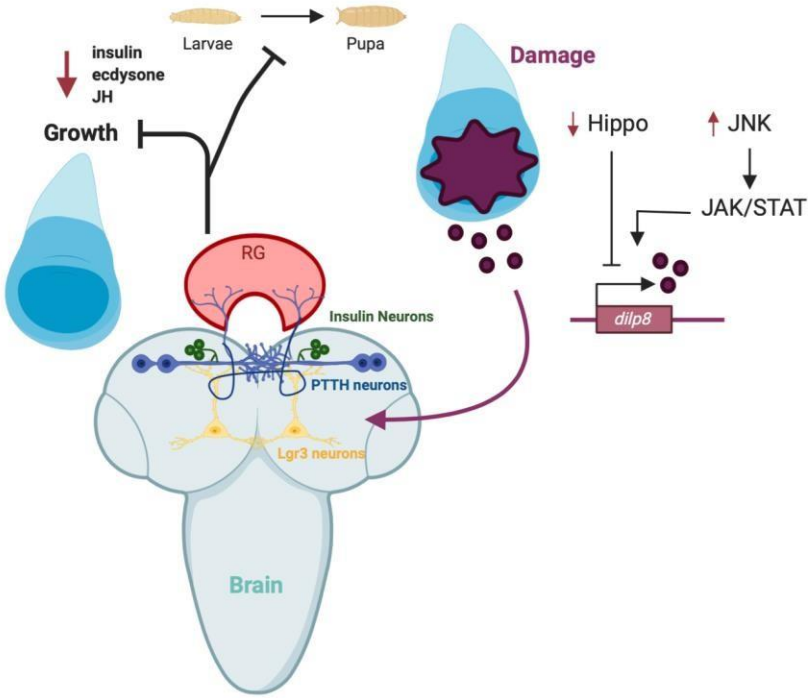

Figure 13: Dilp8 release and action upon Ecdysone Biosynthesis

This illustration includes a schematic representation of the action mechanism of Dilp8 in the production of ecdysone hormone. Dilp8 (purple) is released from the damages tissues and it is able to connect to its receptor Lgr3 in the brain. The Lgr3 neurons (yellow) are activated and can signal the PTTH neurons, preventing the release of PTTH hormone into the ring gland and blocking ecdysone production an causing a developmental delay. During the developmental delay healthy tissues also need to slow down growth rates in order for the final patterning not to be affected.

When growth is impaired in a particular imaginal disc, the other intact discs have to deal with an extended period of time for growth before they can undergo metamorphosis. However, they do not differentiate into bigger organs or appendages (Simpson et al., 1980). This particularity leads us to the belief that during the prolonged growth period, an additional set of mechanisms take place to prevent undamaged tissues from overgrowing.

Measurements of imaginal discs where growth was affected, and comparison of that set of measurements to those obtained from undamaged discs, revealed that growth rate is tightly coordinated among developing organs. Undamaged discs show an approximately equal reduction in growth rate as their perturbed counterparts, thereby maintaining the correct proportions relative to one another throughout development (Parker and Shingleton 2011).

A recent paper published by Pierre Leopold's lab further elucidates about this phenomenon by inducing damage, through the targeting of ribosomal proteins, in growing tissues and analyzing both inter and intra organ coordination events. They found out that Dilp8 was required for growth coordination both within and between organs in a JNK and Hippo independent manner (Boulan et al. 2019). Instead, Dilp8 levels were fine-tuned by *xrp1*, a gene capable of rescuing efficiently the developmental delay induced by slow-growing discs and that has been described previously as a downstream target of p53 activation. In this work, instead of being described as a downstream target of p53, Xrp1 was suggested to be controlled by RpS12, the atypical ribosomal protein known to play a role in *Minute*-

induced cell competition (Kale et al. 2018). Knockdown experiments of *xrp1* in the damaged wing discs prevented non-autonomous growth inhibition from happening due to the prevention of *dilp8* induction. The overexpression of *xrp1*, by contrast, blocked tissue growth both autonomously and non-autonomously, showing, however, that only the non-autonomous response relied on Dilp8. This was justified by the fact that Xrp1 carried two independent functions when referring to growth control. On the one hand, Xrp1 autonomously inhibits tissue growth and triggers apoptosis, and on the other hand, activation of Xrp1 signaling in damaged discs remotely inhibits tissue growth in a Dilp8-dependent manner (Boulan et al. 2019).

Although Xrp1 was not described, in this study, as a p53 target, it does not mean that p53 it's not influencing intra and inter-organ responses during animal development. Indeed, p53 is known to regulate growth and proliferation in a non-autonomous manner, through the transcriptional induction of Eiger, the TNFα fly orthologue. Eiger induction via p53 leads to the cell-autonomous activation of JNK which consequently triggers two distinct signaling events that independently regulate tissue size and cell number. On the one hand, expressing Dilp8 acts to systematically reduce growth rates and tissue size, and on the other hand ROS production, activated as a consequence of apoptosis, acts in a non-autonomous manner to reduce cell proliferation rates (Sanchez et al. 2019). These two signaling events act, in this way, to assure both tissue size and patterning, as well as the formation of a well-proportioned and functional adult.

In most of the referenced studies, feeding these animals with an activated form of ecdysone suppresses the developmental delay and disrupts the coordination of growth among damaged and unaffected discs. Therefore, it is intuitive to think that growth-perturbed discs delay growth in other peripheral tissues by delaying the production of ecdysone. That being said, the modulation in the timing of ecdysone release is crucial not only for the adjustment of the growing period length in response to localized tissue damage, but also for the systemic coordination of growth between peripheral tissues.

3. JAK/STAT Signaling Pathway: A story about travelling cytokines

The Janus kinase (JAK) and Signal transducer and activator of transcription (STAT) signaling pathway is a major regulator pathway that has been shown to be involved in the regulation of several responses such as: the immune response, stem cell regulation and cell identity determination (Trivedi & Starz-Gaiano, 2018).

Extracellular cues trigger JAK/STAT signaling, which ultimately leads to the transcriptional activation of several target genes. JAK/STAT signaling is such a crucial life-support signaling pathway that its basic framework has been maintained across different species with some differences in terms of redundancy. For instance, the mammalian signaling system includes families of proteins with overlapping roles, whereas the fly has fewer components and, thus, less redundancy.

Introduction

In humans, a set of more than 40 interleukins and cytokines serves as activating cues, whilst in flies only three proteins hold this function: Unpaired (Upd) 1, Upd2 and Upd3 (Arbouzova and Zeidler 2006; Bromberg 2001). Aside from the multiple ligands, humans also have multiple cell-surface activators that can act singly or multimerize to respond to the different ligands. In the fruit fly, however, there is only one signaling receptor called Domeless (Dome) (Brown, Hu, and Hombría 2001) which can interact with the non-signaling receptor Eye transformer (Et, whose homolog in humans is the receptor GP130) (Fisher et al. 2016; Kallio et al. 2010; Makki et al. 2010). Receptor-ligand binding activates the Jak proteins docked to the cytoplasmic portion of the receptors. In humans there are four Jak proteins: Jak1, Jak2, Jak3 and Tyrosine kinase 2 (Tyk2), whilst in flies there is only one Jak protein, named hopscotch (hop), which presents most similarities with Jak2 in (Hanratty and Dearolf 1993; Perrimon and Mahowald 1986).

When Jak is activated via receptor-ligand binding, a second Jak associated within the same receptor dimer or multimer is targeted and a subsequent cascade of phosphorylation creates binding sites for cytoplasmic STAT proteins (Figure 14). There are seven STAT family members present in humans (STAT1-4, 5a, 5b and 6), but only one present in *Drosophila* named STAT92E, which resembles STAT5b in humans the most (Hou et al., 1996; Silver-Morse, 2013; Yan et al., 1996). Although some unphosphorylated STATs can have some roles in mammalian cells, such as cytoskeleton regulation, mitochondria and Golgi functions or even NF-kB signaling modulation, most roles associated with the STAT family of proteins are associated with their active form. Phosphorylated STATs are able to undergo dimerization, which promotes their translocation into

the nucleus where they can directly bind DNA and recruit transcriptional activators (Levy and Darnell 2002).

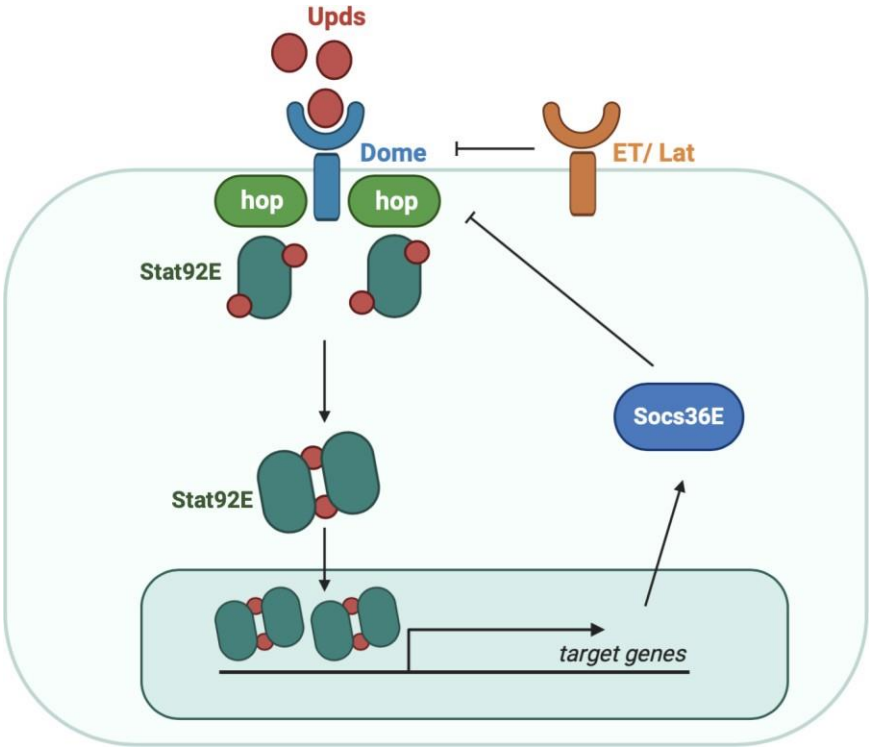

Figure 14: JAK/STAT signaling pathway in *Drosophila*

This illustration represents the several players of the JAK/STAT pathway such as the ligands, named Upds (in red). Upd activates the receptor Dome (blue), which results in the activation of hop (green), leading to tyrosine phosphorylation (small red circles) on Dome. Stat92E dimers (dark green) bind to the phosphorylated receptor. Once bound, Stat92E is phosphorylated, generates an active dimer that undergoes nuclear translocation where it binds to a consensus site and alters gene expression. Socs36E is a target gene that encodes a negative regulator (dark blue) of Dome/JAK activity. A second receptor ET/Lat (orange) inhibits JAK/STAT signaling.

Introduction

Since in humans the several STAT proteins can homo- or hetero-dimerize and be activated by an increased number of ligands and/or receptors, the combinatory outcomes for the activation and function of the JAK/STAT signaling pathway are several and complex. Therefore, the stripped-down and far simpler pathway that can be found in fruit flies is important to provide a tractable, but still highly relevant, system for characterization of this signaling cascade and its essential regulators (Trivedi & Starz-Gaiano, 2018).

3.1 JAK/STAT Signaling in Development and Adulthood

JAK/STAT signaling pathway is required for normal early development and its misregulation later in life might be detrimental. For instance, humans with inborn errors are usually found to be immunocompromised, since this pathway affects blood cell lineages. Moreover, abnormally high Jak and Stat activities in adults have also been described as closely related to the development of autoimmune diseases, cell overproliferation, blood disorders and cancer progression. Therefore, studies done regarding the importance of this pathway in development and adulthood might in turn give information relevant for the improvement or prevention of pathology development (Trivedi & Starz-Gaiano, 2018).

Mouse genetic studies have revealed that while some null JAK/STAT pathway mutations are lethal, some others can cause tissue-specific defects (Akira 1999; Levy and Darnell 2002). For example, mutants

for *Jak1* and J*ak3* in mice present severe immunodeficiency (SCID), and *Jak1* mutants also have neurological defects and poor survival rates past birth. Losing *Jak2,* on the other hand, is embryonic lethal, and mutations in *Tyk2* result in poor response to pathogens (Yamaoka et al. 2004). Moreover, mutant mice for *Stat1* present abnormal immune responses being increasingly more susceptible to infection (Akira 1999) and showing significant levels of neurodegeneration (Hennighausen and Robinson 2008). *Stat3* mutants die in early embryonic stages. Tissue-specific *Stat3* mutants, however, present alterations in their proliferation *versus* apoptosis balance in blood cells, decreased cell mobility capacity and inflammation (Akira 1999; Levy and Darnell 2002). Both *Stat5a* and *Stat5b* have similar roles in mammary gland and ovary development, as well as important functions in cell proliferation and cytotoxic activity. If female mice are mutated for both these Stat5 proteins, they will be sterile (Hennighausen and Robinson 2008).

In *Drosophila*, mutations that block JAK/STAT signaling cascade result in early lethality, most probably due to the lack of redundancy presented by the fruit fly. However, the already mentioned Gal4/UAS system or clonal mosaic analysis allows researchers to experimentally overcome this issue and express or block this signaling pathway in a tissue-specific manner. These strategies revealed JAK/STAT importance in sex determination, blood cell function, and its functions in wing precursors, eye progenitor cells, gut stem cells, adult testes stem cells, and adult ovary cell types. In many of these contexts, loss of signaling produces abnormal phenotypes, as abnormally high levels are also able to do so (Trivedi & Starz-Gaiano, 2018).

Since its functional disruption can cause defects in stem cell maintenance, cell survival, proliferative defects, cell fate specification and cell migration, JAK/STAT has become a signaling pathway of increasing interest in the tumor context.

3.2 JAK/STAT signaling in Epithelial Tumors

The JAK/STAT signaling pathway plays an important role during *Drosophila* development, particularly in imaginal discs.

In the wing and eye imaginal discs, JAK/STAT signaling is an essential regulator of growth and patterning. JAK/STAT activity is noticeable in all cells of early discs, and its signal is required in a cell autonomous manner to guarantee growth (Amoyel, Anderson, and Bach 2014). To maintain proper tissue size, the expression of Upd needs to be tightly regulated. Misexpression of Upd in the eye leads to a dramatic increase in tissue growth, whilst in the disc Upd expression is known to affect tissue size by promoting cell death.

JAK/STAT signaling has also been described as capable of accelerating cell cycle progression. However, direct molecular links between this signaling pathway and cell cycle progression and cellular growth have been poorly outlined. Nevertheless, it was described that Cyclin D transcripts are upregulated in Upd-overexpressing discs (Tsai and Sun 2004). Cyclin D is a member of the cyclin protein family involved in the regulation of cell cycle progression, where its synthesis is initiated during the G1 phase, driving the G1/S cell cycle transition.

Several screens in search of JAK/STAT signaling targets have been carried out over the years, especially for eye disc development projects. On one occasion JAK/STAT has been linked with BMP signaling (Bone Morphogenetic Protein), causing overgrowth in the eye disc (Bach et al. 2003).

In the wing disc, evidence has been presented stating that JAK/STAT signaling promotes the cycling and survival of Hedgehog-producing cells, allowing for a stable localization of the BMP/Dpp organizing center (Recasens-Alvarez, Ferreira, and Milán 2017).

In the developing wing primordia, JAK/STAT is required in a three-phase sequential manner to regulate both fate and growth activities of Wg, Hh and Dpp morphogens. The first phase comprises early development, where Unpaired expression and graded JAK/STAT activity along the proximal-distal axis restricts expression of downstream targets of the Vn/EGFR pathway to ensure wing fate specification mediated by Wingless. The second phase happens later in development, where JAK/STAT controls organ growth through the promotion of survival and cycling of Hedgehog producing cells, which allows stable expression of the Dpp morphogen in the center of the wing appendage. And finally, the third phase refers to the specification and building of the wing hinge area, a cell population that isolates the wing primordia from the surrounding body wall, which is maintained due to the activity of JAK/STAT and contributes to the delimiting of Dpp activity in the growing appendage (Recasens-Alvarez, Ferreira, and Milán 2017). In this way, it becomes clear that JAK/STAT is not only important in organ size and fate specification, but also involved in the regulation of both production and activity of morphogens as well as their spatial

distribution through the regulation and organization of their growth-promoting functions.

Aside from its role in development, mutations in endocytic genes revealed a link between endocytosis, JAK/STAT signaling and tumor formation in epithelia. During endocytosis, cargo sorting and multi-vesicular formation require three large protein complexes called the Endosomal Sorting Complexes Required for Transport (ESCRTI, -II and -III) (Rusten, Vaccari, and Stenmark 2011). Mutations in most endosomal and ESCRT components lead to tumor formation in the fruit fly imaginal discs. In many cases, the mutant cells are eliminated from the tissue and overgrowth arises from the capacity of the neighboring wild type cells to proliferate. In *Drosophila,* cells mutant for *vps25* (that encodes an ESCRT-II component) and *erupted* (that encodes for an ESCRT-I and -II component) show dramatic overgrowth (Herz et al. 2006; Moberg et al. 2005; Vaccari et al. 2009). In these cases, ESCRT mutants trap the Notch receptor in endosomes, where it signals aberrantly and continues to induce transcription of *upd* ligand (Vaccari et al. 2010). Overgrowth, then, correlates with ectopic Upd expression. Reducing the genetic dose of *Stat92E* suppresses the non-autonomous overgrowth caused by the *vps25* and *ept* clones, because it prevents the action of the ligand.

Some endocytic mutants, however, present increased JAK/STAT signaling without presenting an increase in Notch signaling. The outcome of the aberrant JAK/STAT activation, nonetheless, remains the same: loss of epithelial structure and ectopic growth, both

hallmarks of the neoplastic transformation (Gilbert et al., 2009; Woodfield et al., 2013).

Experiments in imaginal discs composed by independent groups of cells almost entirely mutated for ESCRT, revealed that JAK/STAT signaling is also capable of presenting an autonomous activation in tumor cells. Within *ept* tumors, removing one copy of *Stat92E*, is sufficient to alter cell size and cell cycle dynamics. Without the extra copy of *Stat92E*, these cells are less capable of entering S-phase (Gilbert et al., 2009). Moreover, within endocytic tumors, reducing STAT92E activity significantly rescues the loss of epithelial polarity (Amoyel et al., 2014; Gilbert et al., 2009; Woodfield et al., 2013). These results suggest that JAK/STAT activation and signaling extends beyond simple regulation of proliferation but is also capable of affecting other cell behaviors and mechanisms. On that note JAK/STAT signaling has also been linked to defects in cell adhesion. One potential effector for this function is the apical determinant Crumbs (Crb): Crb de-regulation is sufficient to induce neoplastic overgrowth (Lu and Bilder 2005), and while *crb* was already described as a direct target of JAK/STAT signaling, this effects might be a secondary effect of impaired vesicle recycling more than a direct consequence of JAK/STAT signaling.

Aside from endocytic tumors, JAK/STAT signaling is also present in polarity-deficient tumors. Many neoplastic tumor suppressor genes encode regulators of epithelial polarity. A particularly famous case is the one of *scribbled* (*scrib*): a regulator of septate junctions that maintains the separation between apical and basal membranes. Loss of *scrib* in whole tissues leads to epithelial disorganization and

tumor formation, but clones of cells mutant for *scrib* in proximity to wild type cells are eliminated by cell competition (Bilder, Li, and Perrimon 2000; Brumby and Richardson 2003; Igaki, Pagliarini, and Xu 2006). However, when in cooperation with another oncogene such as the activated form of RAS, named RAS^{V12}, the tumorigenic potential of *scrib* mutants reveals itself and the mutant cells, now referred as RAS^{V12} *scrib*, are able to metastasize. In these tumors, cells display high JNK signaling, which can induce the expression of all three *upd* genes, leading to systemic activation of JAK/STAT. Activation of Stat92E coupled with RAS^{V12} causes massive overgrowth and metastasis (Brumby and Richardson 2003; Pagliarini and Xu 2003).

In a separate tumor model, RAS^{V12} combined with the loss of JAK/STAT inhibitors leads to metastatic tumor formation. The continuous activation of JAK/STAT together with a RAS^{V12} being able to cause tumorigenesis confirms the carcinogenic cooperativity of the two pathways. Within the same line, preventing JNK activation, thus preventing the release of Upd ligands, in *scrib* mutants prevents both STAT92E activation and neoplastic transformation. Preventing Sta92E activation in RAS^{V12} *scrib* suppresses both overproliferation and metastatic behavior in these tumors (Brumby and Richardson 2003; Herranz et al. 2012; Igaki, Pagliarini, and Xu 2006; Wu, Pastor-Pareja, and Xu 2010). These effects can be explained by the observation that expression of Upd ligands in damaged tissues is a mechanism for compensatory proliferation to restore tissue size. Indeed, the already mentioned Dilp8-mediated delay mechanisms is thought to be under the control of JAK/STAT, since Dilp8 is a direct target of the pathway (Katsuyama et al. 2015).

Another interesting aspect of the upregulation of Upd ligands in tumors is that it leads to the proliferation of circulating blood and immune cells called the hemocytes (Pastor-Pareja, Ming, and Tian 2008). These cells adhere to tumors and can also contribute to the reduction of their growth rates. These last results suggest that JAK/STAT signaling plays two types of systemic roles: an indirect one by altering tumor microenvironment by affecting hemocyte numbers; and a more direct one, being involved in the secretion of the long-range signaling molecules that might have a systemic impact on peripheral tissues.

3.3 JAK/STAT signaling, cytokine release and their impact in systemic timing alterations

In *Drosophila* flies the roles of JAK/STAT and cytokine release in tumor-related systemic effects are characterized as responsible for triggering inflammation processes and hemocyte recruitment, as well as potentiators of the secretome phenotype, releasing long-range signaling molecules that impact peripheral tissues, such as the relaxin-like protein Dilp8. As mentioned previously, it is the activation of JAK/STAT in the damaged tissues that allows for Dilp8 release and prevention of ecdysone steroidal hormone production through interaction with Lgr3 neurons that by interplay with the PTTH neurons prevent the release of the PTTH hormone. Aside from growth disturbances, other inflammation processes that generate the release of a high number of cytokines, such as bacterial infections, have also been described as capable of inducing systemic delays, systemic melanization and even patterning alterations (Olcott et al. 2010). Of course, these impacts are

microbial-charge dependent. And although it seems clear that a high number of circulating cytokines might be influencing steroidal hormone productions and developmental transitions, the connection between the two has not been properly established in flies.

In humans, on the other hand, the release of cytokines during inflammation processes is known to have deep systemic impacts in steroidal hormone control. Namely, it has been reported for some disorders such as obesity and inflammatory bowel disease the possibility that increased cytokine number might be in the base of alterations in puberty onset. Puberty marks the transition from non-reproductive states into reproductive ones and it is associated with different physical changes, from the development of pubic and axillary hair, adolescent growth spurt, an increase in fat and muscle tissue and, in females, breast development and increased hip width. The age at which the onset of puberty hits varies among individuals and between ethnic populations and socioeconomic status. In the clinical setting, the evaluation of puberty timing is assessed taking into account a set of stages for axillary and pubic hair development and breast and genital development proposed by Tanner and colleagues in the early 70's (Marshall and Tanner 1969, 1970). The hormonal mechanisms, however, for initiation of puberty in humans are not as well described as in *Drosophila*. Nonetheless, GnRH (gonadotropin-releasing hormone) is believed to be the initial signal. This hormone is synthesized by the nuclei of the hypothalamus and transported, via the hypophyseal portal capillaries, to the gonadotrophs of the anterior pituitary gland. Here it acts in order to stimulate the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH).

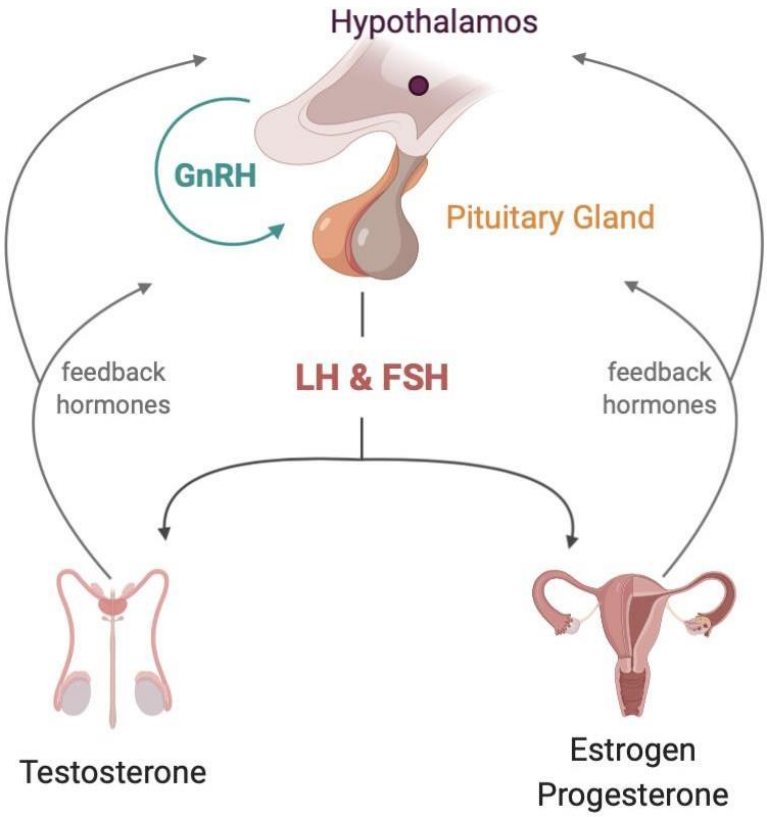

Figure 15: Hormones present in normal puberty regulation

This illustration represents the main hormones involved in the onset of puberty in humans and the intertalk between them. GnRH, the initial signal, synthesized by the nuclei of the hypothalamus, is then transported to the pituitary gland. Here it stimulates the synthesis and release of LH and FSH. These act in the testes to stimulate testosterone production. They also promote ovulation and stimulate secretion of the sex hormones estrogen and progesterone from the ovaries

In the testes, LH stimulates testosterone production from the interstitial or Leydig cells, which in turn acts locally to help spermatogenesis and systemically to produce male secondary sex

characteristics, anabolism and to maintain the libido. FSH, on its end, stimulates the Sertoli cells in the seminiferous tubules to produce mature sperm and the feedback hormone inhibin which decreases FSH secretion from the pituitary. In females, LH and FSH promote ovulation and stimulate secretion of the sex hormones estradiol (an estrogen) and progesterone from the ovaries (Ballinger, Savage, and Sanderson 2003) (Figure 15).

Different patterns of GnRH secretion are observed at different stages during puberty, but they usually obey a rhythm of pulsatile GnRH secretion. Before the onset of puberty both LH and FSH are visible in very small amounts but, as puberty approaches, the amplitude in their secretion pulses increases, increasing their secretion rates and circulating concentrations in the bloodstream, and the nocturnal rise in LH is amplified. The factors that act on the GnRH neurons to initiate puberty onset, though, have not been identified so far. Nonetheless, there are some hypotheses about changes that induce alterations in its secretion.

For instance, accordingly to a proposal put forward by Frisch and Revelle, a critical weight of 47.8kg has to be reached before menarche can occur (Frisch and Revelle 1971) and a body fat level of 17% of body weight is also considered to be crucial in the onset of this phenomenon. Moreover, 22% of the fat level should be maintained by females in order to maintain a regular menstrual cycle (Frisch and McArthur 1974). Nonetheless, some clinical reports suggest that these criteria are not so straight forward, although it emphasizes the importance of nutritional status during puberty development.

Similar to what has been previously mentioned for insects, in humans there is also an important protein capable of informing the body about the state of the adipose tissue mass. This protein is called leptin, and it gathers information about this state in order to inform the hypothalamic feeding centers (Zhang et al. 1994). Its function as the signal that relates adipose tissue mass to the onset of puberty has been suggested. However, there is some contradictory information coming from studies with rodents, where serum leptin concentrations do not show a significant increase until after the animal reaches adulthood. These same studies also reveal that the level of hypothalamic leptin receptor remains the same in juvenile, prepubertal and post-pubertal female rats, and treatment with leptin in fasted rats with developmental delays does not advance the timing of entering into puberty in the female mice compared (Cheung et al. 1997). Therefore, it seems that leptin does not act as a primary trigger in the onset of puberty but has a rather permissive role, in the sense that allows puberty to proceed (Urbanski 2001).

Other studies have also revealed that the hypothalamic neurotransmitter NPY might play a role in the onset of puberty, since its administration to prepubertal rats can delay sexual maturation though GnRH secretion inhibition (Pralong et al. 2000). Aside from this, the hormones IGF-I and dihydroxy-androstenedione might also play a part in puberty development. These hormones are secreted from the adrenal cortex and they are known to act on the pattern of sexual maturation, in the case of IGF-1, or on the maturation of the GnRH neurons, in the case of the dihydroxy-androstenedione.

As mentioned previously, entering into puberty is highly affected in some patients suffering from highly inflammatory disorders. A good

example is inflammatory bowel disease (IBD) patients suffering from Crohn's disease and ulcerative colitis.

Puberty is often delayed in patients with Crohn's disease, a type of inflammatory bowel disease (IBD) that may affect the digestive tract causing severe abdominal pain, diarrhea, fatigue, weight loss and malnutrition. Studies with young patients reveal that menarche occurs at a later stage (age 16 or higher) in 73% of female patients in whom the disease onset preceded puberty (Ferguson and Sedgwick 1994). Aside from that, IBD patients also presented a mean age of puberty onset of 12,6 in young females, compared with 11,1 in healthy control, and in young males, a mean age of 13.2 compared with 12.4 of their healthy counterparts (Brain and Savage 1994). The duration of puberty may also be prolonged, particularly in patients with frequent disease relapses during these childhood to puberty transition years.

Patients with IBD are frequently underweight and undernourished, and these have been considered to be the main reason for delayed puberty. The nutritional deficit results mainly from inadequate intake, rather than increased needs or losses. Disease-related anorexia may be severe and proinflammatory cytokines produced by the inflamed bowel are thought to be responsible. Experiments with rats with TNBS-induced colitis, a commonly used animal model that shares significant properties with human Crohn's disease, have implicated a role of interleukin IL-1β in the development of anorexia, although in other inflammatory models TNF-α, IL-1β and IL-6 have been related with induced anorexia. Receptors of proinflammatory cytokines are expressed in the central nervous system (CNS), and it seems highly likely that peripheral produced cytokines coming from inflammation areas induce CNS synthesis of a higher number of

cytokines. Consequently, these will interact with specific hypothalamic feeding pathways and, in turn, induce anorexia.

Children with Crohn's disease and growth failure usually receive nutritional supplementation which might help pushing the onset of puberty and increase growth velocity (Aiges et al. 1989; Kelts et al. 1979; Kirschner et al. 1981). Nonetheless, patients with persistently active disease do not enter puberty despite provision of adequate supplements, suggesting that other factors, additional to undernutrition, might be influencing pubertal delay (Brain and Savage 1994). Despite this, what it is clear in the clinical setting is that the surgical removal of the active disease causes first signs of puberty often within 1 year of intestinal resection (Brain and Savage 1994). Food intake also increases after resection of the diseased bowel and it is possible, once again, that the onset of puberty is, in part, also related to improved nutrient uptake. However, these observations also suggest that inflammatory mediators secreted by the inflamed gut may have a direct adverse influence, independent of undernutrition, on the onset and progression of puberty.

Studies performed with rodents with TNBS-induced colitis showed similar results of delayed puberty, T-cell activation and cytokine profile of those present by human Crohn's disease patients (Duchmann et al. 1996; Rachmilewitz et al. 1989; Yamada et al. 1992). In one of these studies, it was assessed the contribution of reduced food intake and inflammation to pubertal delay. The studies controlled daily food intake of healthy rats to match the colitic group and precisely monitor the effect of undernutrition in the progression of puberty. Undernutrition in healthy females resulted in a delay in the onset of puberty and progression of puberty compared with controls. Even so, the onset of puberty was further delayed in the

colitic group, which again gives rise to the hypothesis that inflammatory mediators are potentiating the effects of undernutrition and enhancing the delay in the onset and progression through puberty.

The endocrine mechanisms behind puberty delay are not well defined, which makes it harder to understand the real influence of both nutritional and inflammatory mediators associated with IBDs. This becomes even more challenging since in humans there is no simple model to assess undernutrition and confounding variables influence studies in underweight patients.

In both animals and humans, food deprivation and a reduction in body size or weight are associated with reduced activity of hypothalamic neurons, which reduce their production of GnRH, and, consequently, reduced production of pituitary gonadotropins, mediating, in this manner, puberty delay. However, in human patients, as mentioned previously, there is no simple model for undernutrition and there are many variables that influence underweight patients, including psychological ones. For example, patients with anorexia nervosa present low basal levels of both GnRH and plasma concentrations of gonadotropins for up to a full year after weight normalization. Suggesting that hypogonadism may also be influenced by factors other than nutritional altercations (Devlin et al. 1989; Ohzeki et al. 1989). In animal models, the gonadotropins response to undernutrition has usually only been assessed after periods of extreme calorie deprivation (Ahlma et al. 1996; Farthing and Swarbrick 1982; Slob, Vreeburg, and Van Der Werff Ten Bosch 1979), which is not what is observed in a high percentage of chronic inflammatory patients, with lesser degrees of undernutrition. In other studies, using the TNBS-induced colitis

model, it was observed that the onset and progression of puberty in both colitic and pair-fed groups was delayed. However, plasma concentration of gonadotropins was similar between experiment and healthy free-feeding controls (Azooz et al. 2001). Nonetheless, it was observed that in male rats with TNBS-colitis, plasma concentrations of testosterone were significantly reduced, as well as the concentrations of estradiol in the females. Notwithstanding, this reduction was observed both in colitic and pair-fed groups, suggesting that it is the lack of androgen production that may be contributing to the observed delay. Undernutrition seems to be the main determinant for reduced testosterone concentrations as levels were similar between colitis and pair-fed groups. Nevertheless, the extended delay observed for the colitic group seemed to indicate a possible resistance to circulating testosterone, however partial since exogenous administration of testosterone accelerates puberty in male rats with colitis.

Aside from the steroidal hormone role, it seems to become clearer from the already mentioned studies, that cytokines seem to have a role in pubertal delay in patients with IBDs, nonetheless not much is known about which specific cytokine is mediating this effect.

In vitro studies are starting to shed some light on this case. TNF-α decreased androgen receptor protein and mRNA levels in prostate cancer cell lines and also inhibited the ability of dihydro-testosterone to induce cell proliferation and activate the prostate-specific antigen gene promoter (Mizokami et al., 2000). IL-6, on the other hand, upregulated androgen receptor expression and activated androgen receptor-mediated gene expression in this cell line (Chen, Hua Wang, and Farrar 2000; Lin et al. 2001).

Introduction

The current treatment for IBD patients involved reduction of intestinal inflammation and administration of calorie supplements to correct undernutrition. In patients with Crohn's disease, exclusive enteral feeding with elemental or polymeric feeds for eight weeks combines nutritional treatment with specific anti-inflammatory effects, and thus is ideal for patients with growth failure and pubertal delay.

Aside from IBDs, puberty is frequently delayed in young patients with cystic fibrosis and rheumatoid arthritis. In these cases, similar to IBD, puberty can be delayed despite a normal nutritional intake (Athreya et al. 1993; Corey et al. 1988; Fraser et al. 1988; Johannesson, Gottlieb, and Hjelte 1997; Kindstedt-Arfwidson and Strandvik 1988; Landon and Rosenfeld 1984; Neinstein et al. 1983). In young patients with arthritis or cystic fibrosis, the endocrine profile is similar to that in patients with IBD.

Other high inflammatory diseases have been studied and observed to have a major influence in pubertal development. One of these diseases is highly prevalent among children and has become a major health concern in recent decades. We are referring to childhood obesity. Childhood obesity, a result of relative overnutrition, is associated with a number of medical complications, among some is the increased risk for atherosclerotic vascular disease, increased insulin resistance, hyperglycemia, hypertension and dyslipidemia (Cali and Caprio 2008). Recent data suggests that the excess adiposity during childhood maybe be influencing pubertal development as well. In particular, this excess in adipose tissue during childhood may be advancing puberty in girls and delaying in boys. How obesity may perturb various hormonal aspects of pubertal developmental remains unclear, although cytokine association has not been ruled out. Obesity is often associated with high

inflammation profiles and marked changes in cytokines and adipokines. For instance, the proinflammatory cytokine IL-6 is found to be elevated during obesity, and this cytokine can stimulate adrenal steroidogenesis, including androgen synthesis (Mastorakos, Chrousos, and Weber 1993; Papanicolaou et al. 1998; Päth et al. 1997). Moreover, polymorphisms in the IL-6 gene promoter and IL-6 receptor may be associated with hyperandrogenemia, characterized as the high levels of circulating androgens in females, that might cause, among other symptoms, increased body and facial hair and infrequent or absent menstruation (Escobar-Morreale et al. 2003; Escobar-Morreale, Luque-Ramírez, and San Millán 2005; Villuendas et al. 2002; Walch et al. 2004). Additionally, IL-6 can augment the transactivation of the androgen receptor (Yang et al. 2003), which might play a role in polycystic ovary syndrome development, a syndrome caused by elevated androgens in females, that in obesity experienced during pubertal transitions is highly observed (Franks et al. 2006). However, in the case of female obesity patients, all these altercations act in the sense of advancing puberty and not delaying it, as we saw previously for the IBDs patients. In obesity male patients' pubertal development has been described as being delayed rather than advanced (Kaplowitz, 1998; Lee et al., 2010; Wang, 2002). Nevertheless, the reasons for this phenomenon are still unclear. Obesity in men can be associated with a form of hypogonadotropic hypogonadism (HH), a condition where the male testes do not produce sufficient or any sex hormones due to a problem with the pituitary gland or hypothalamus (Hammoud et al. 2006). Hypothalamic disorders result from a deficiency in GnRH release, while pituitary gland disorders are due to a deficiency in the release of gonadotropins, which influence LH and FSH production, from the anterior pituitary. Specific data collected from Fu and

collaborators show that the morning LH and testosterone levels are undetectable in most subjects, and FSH morning concentrations were also very low. However, these hormonal levels were not so different from age-matched prepubertal controls. What was clear was that obese prepubertal boys showed a greater testicular volume, as well higher levels of inhibin B (a hormone produced by the Sertoli cells which exerts a negative feedback on FSH secretion), DHEA (a hormone produced by the body's adrenal glands, just above the kidneys, that servers as a precursor for sex steroids production), and DHEA-S (a hormone also produced in the adrenal cortex but hormonally inert that can be converted back into DHEA) concentrations (Fu et al. 2006). Nevertheless, associations between puberty delay and interleukin activity in male obese patients has not been established or proposed yet.

In some tumor patients, mainly suffering from tumors of the central nervous system (CNS), puberty delays have also been observed. In these cases, they seem to be mostly due to an interference of GnRH synthesis or secretion. Germinomas are the most common germ cell tumors to cause delayed puberty, although they are very rare in the clinical setting (Shibamoto 2009). Despite their rarity, they are commonly associated with deficiencies in the production and release of pituitary hormones. These pituitary hormone deficiencies are often manifested, also, by diabetes insipidus patients. Although puberty delay and alteration of pituitary hormones is common in these tumoral cases, the reason why they generate such altercations is thought to be treatment-associated. Treatment for CNS tumors depends mostly on cranial irradiation, which might result in gradual development of hypothalamic-pituitary failure (Haas et al. 1983). Growth hormone (GH) deficiency is the most common component of

the radiation-induced hormone disorder, but gonadotropin deficiency also occurs when the radiation dose is high. Development of radiation-induced hypothalamic-pituitary failure may take from 1 year to several years to overcome (Wallace 2011), even so the estimated prevalence of gonadotropin deficiency in childhood cancer survivors is 10.8%.

Although the reason for puberty delay and pituitary hormone alteration is thought to be radiation treatments, it was never explored, for these patients, the impact of inflammation or cytokine release in the onset of puberty.

Nevertheless, what seems clear to us is that highly inflammation prone diseases or disorders have a tendency to affect puberty onset. It is also true that the mechanisms used to do so in each different situation might not be related with inflammation or cytokine release. However, in some cases, such as IBDs, it seems clear that inflammation processes have a role in puberty delay, and in other cases, such as CNS tumors, it seems clear that more studies need to be performed to assess the contribution of this systemic response in puberty delay. Therefore, more projects should be thought in order to gather more information about the potential role of cytokine release and inflammation induction in the production of steroidal hormones.

Off course, it seems also clear that these studies should be performed primarily considering robust animal models, since human patient observations are usually highly complex and sometimes subjected to uncontrollable elements or stimuli, making it difficult to manage certain variables and gather specific information. However,

research in this particular field should be holistic and integrative, in order for it to be possible to reach a higher number of conclusions about the possible impact of inflammation processes and cytokine release in the onset of puberty during tumor development, inflammatory disorders and other diseases prone to cause delays in this particular transition

OBJECTIVES

Systemic effects, such as the inflammation responses, alteration of metabolic requirements or secretion of long-range signaling molecules, have been continuously observed in epithelial tumor models. However, performing studies to assess tumorigenesis impact, not only with a local approach, but also considering their systemic roles, comes with its challenges. These challenges are mostly associated with the fact that, when working with an entire organism, we increase the possibility of encountering redundancy or highly complex signaling networks. Consequently, this increases the difficulty of dissecting the individual role of secreted signals when in a complex background, where moving one element might push the entire system towards a different response.

Nonetheless, *Drosophila* presents itself as an excellent model organism for these systemic effect studies, since it provides less redundancy, a sophisticated genetic tool set and a short life cycle.

In this thesis, I will use the epithelial tumor model of CIN to assess the systemic impacts in the production of the steroidal hormone ecdysone. The lab has generated a model where cells with highly aneuploid karyotypes delaminate from the tissue and, upon additional blockage of the apoptotic response, activate the pro-tumorigenic JNK signaling pathway. JNK promotes the expression of mitogenic molecules responsible for the overgrowth of the epithelium, as well as it promotes the release of other long-range signaling molecules capable of generating a systemic impact in the peripheral tissues.

Objectives

The main objectives of this thesis are:

1) To characterize the systemic impact of CIN epithelial tumors in developmental timing (metamorphosis)
2) To identify the long-range signaling molecules and target tissues involved in the CIN-induced developmental delay
3) To dissect the molecular mechanisms and pathways mediating the CIN-induced developmental delay in peripheral tissues

RESULTS

1. Characterization of CIN induced systemic delay

1.1 Generation of CIN derived epithelial tumors

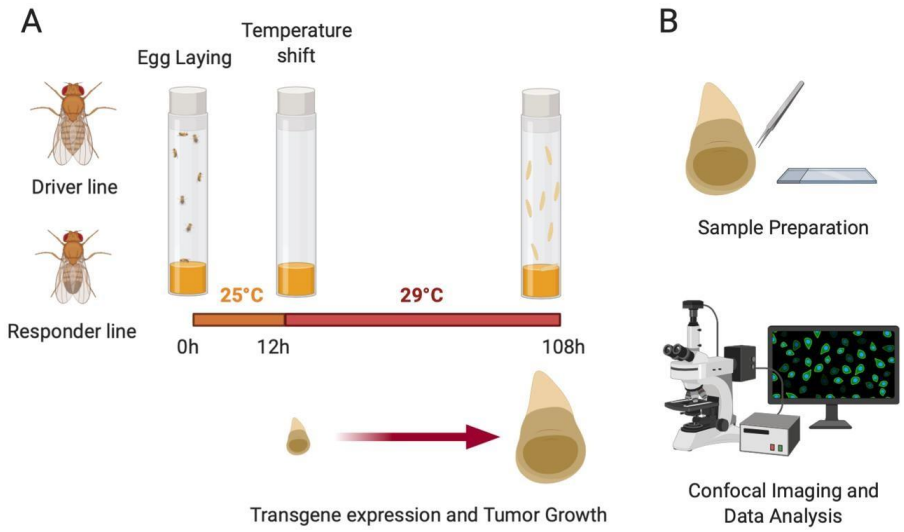

Figure 16. Protocol to induce CIN in the *Drosophila* wing primordium

A – Flies carrying constructs for the tissue-specific generation of CIN were crossed and kept for 12 hours egg laying period at 25º C. Tubes carrying the embryos and recently hatched larvae were then transferred to the induction baths at 29 º C for stronger induction of transgene expression. After 108 hours after egg laying tubes are removed from the induction baths and kept at room temperature for dissection.

B – After dissection, immunostaining and sample preparation, imaginal discs are observed in the confocal for image processing and data is analyzed through measurements or image treatment.

Results

As previously mentioned, in this thesis we used the *Drosophila* wing epithelium to model CIN-derived epithelial tumors. By using the Gal4/UAS system, aneuploid cells were generated by blocking (via interference RNA) the SAC gene *bub3*, in the dorsal compartment of the wing disc (by using *apterous* (*ap*) gal4 driver line). Additionally, the baculovirus protein p35 (Hay et al., 1994) was expressed to prevent the aneuploid cells from undergoing JNK-dependent apoptosis through the activation of effector caspases. The protocol for CIN induction in the wing disc is schematically represented in Figure 16.

Maintaining the highly aneuploid cells in the tissue induces the JNK-dependent transcriptional program that leads to the release of mitogenic molecules, such as Wingless, that promotes tissue overgrowth (Dekanty et al. 2012). Moreover, two populations of cells are formed: 1) the low-aneuploid proliferating epithelium and 2) the highly-aneuploid delaminated population. These two populations of cells were previously described in the lab based on DNA content profile and chromosome labeling. The highly aneuploid cells activate the JNK pathway and can be labelled by one of its downstream targets: MMP1 (Uhlirova and Bohmann 2006) (Figure 17). In addition to MMP1 labeling, they also present loose binding to the other cells, cell cycle arrest and activation of the SASP, characterized by the high secretion of signaling molecules (Dekanty et al. 2012; Muzzopappa, Murcia, and Milán 2017).

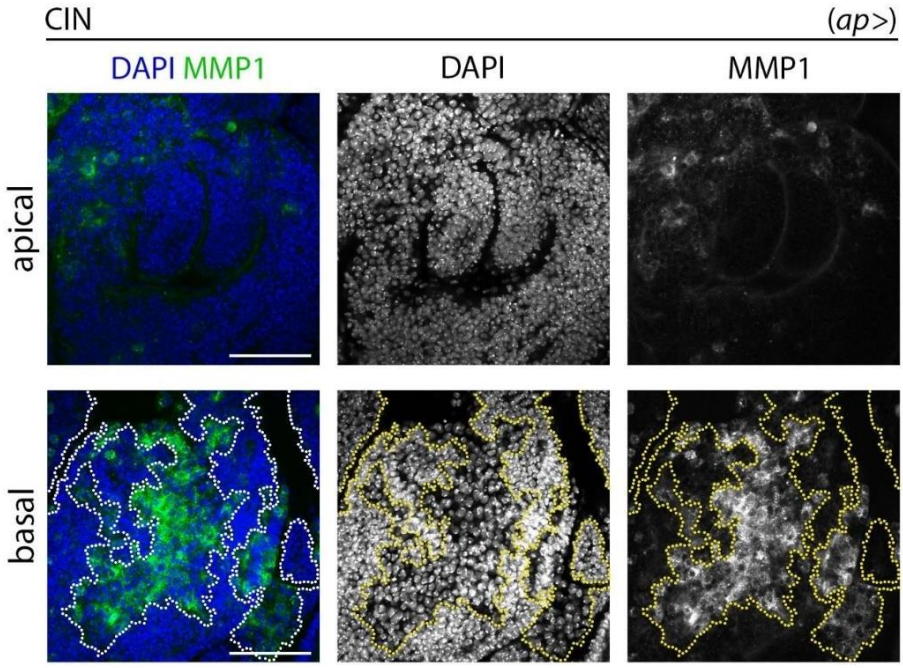

Figure 17. CIN induced epithelial tumors present two cell populations: a growing epithelium and a delaminated population highly secretory

Wing imaginal discs depleted of bub3 spindle assembly checkpoint gene and blocked apoptosis in the dorsal compartment under the control of the *ap>gal4* driver. The apical region is marked by the epithelium tissue morphology which reveals an increased overgrowth visible by the folding in the tissue and absence of MMP1 (stained in green). The basal region is marked by the increased expression in MMP1 and the loose bounds.

1.2. CIN induced epithelial tumors generate developmental delay

When the CIN model was described by the lab back in 2012 (Dekanty et al. 2012), it was clear that these larvae generated these tumor-like structures and this caused them to delay their development for a couple of days, making it possible to dissect

tumors at day 8 after egg laying. These larvae also presented an increase in volume, most probably caused by these extra days spent in the food. Since my goal is to describe and elucidate the systemic effects of CIN derived epithelial tumors, I decided to describe the amount of extra time that these larvae spend in the food as well as their behavior during this time.

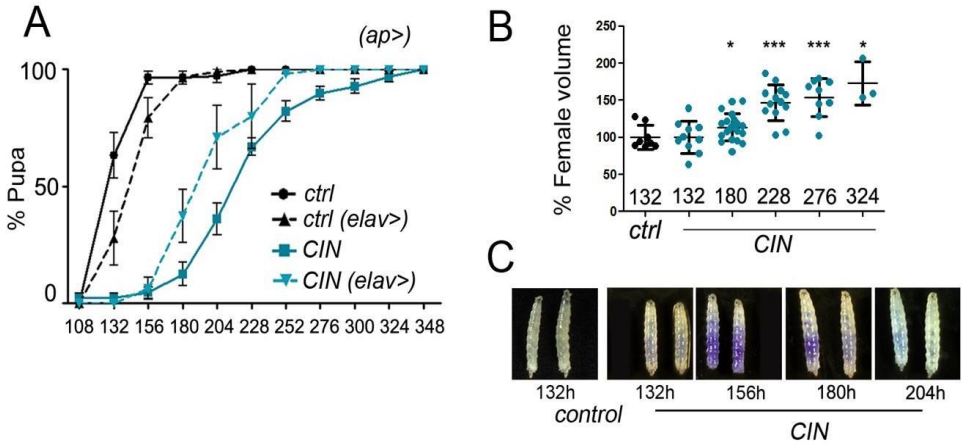

Figure 18. CIN larvae present a delay in pupariation accompanied by increased in feeding and larvae volume

A – CIN larvae with and without elav-Gal80 present an increase in developmental timing, spending and average of 96 extra hours (for CIN tissues with elav-Gal80) or 120 extra hours (for CIN tissues without elav-Gal80) in the food when compared to their controls. Time of comparison is established to be the value of 50% of total pupas formed.

B – CIN larvae were measured during the time of wondering in the food for the following time points: 132, 180, 228, 276 and 324 hours after egg laying. Larvae volume measurements were compared taking into account their increase in size relative to the control larvae measured at 132 hours, or day 6, which average was set to 100%. At the same time point as the control, CIN present a similar larval volume that exponentially increases as time goes by, most probably because of increased feeding behavior.

C – Cessation of feeding usually accompanies entry into puparium. To assess if wondering larva continued feeding an assay using food supplied with bromophenol was performed. Larvae feeding present blue guts while larvae not feeding present yellow guts. CIN larvae presented blue guts for the time points representing wondering larvae in the food, while control larvae presented yellow guts consist with cessation of feeding.

In order to do so, the first thing was to slightly modify the protocol of tumor induction, described previously, to account for only 4 hours of egg laying, in order to synchronize the hatching of the eggs without having to manipulate the larvae. After egg laying, tubes were placed at 29ºC for 4 days to induce tumor formation, and subsequently tubes were removed and kept at room temperature to count the formation of pupal structures during the next 15 consecutive days.

Surprisingly, when comparing CIN-induced tumors to its wild-type counterparts it is possible to observe that these larvae spend approximately three and up to five extra days in the food (Figure 18 A).

The CIN developmental timing assay was also performed taking into account an additional genetic tool: the GAL80 repressor. The GAL80 gene is also a gene from yeast that functions as a negative regulator of the GAL4 transcriptional activator (Suster et al. 2004). A dimer of this repressor is able to bind to a GAL4 dimer in a way that, although the UAS sequence can still bind to the GAL4, it cannot be actively transcribed. This particular GAL80 is defined to prevent the expression of *ap*-GAL4 in the brain (elav-Gal80), as it is clearly shown in the panels of Figure 19 comparing in CIN tumors MyrT expression with control ones. Pupariation assays carrying the elav-Gal80 repressor (Figure 18 A) show a slight decrease in the extra time spent in the food, reducing it from five to approximately three days of developmental delay. However, repressing the activity in the brain was not sufficient to fully rescue the delay, which indicates that the tumor produces signals capable of affecting steroidal hormone production.

Moreover, this developmental delay is accompanied by continuous feeding (Figure 18 C). Feeding assays were performed using

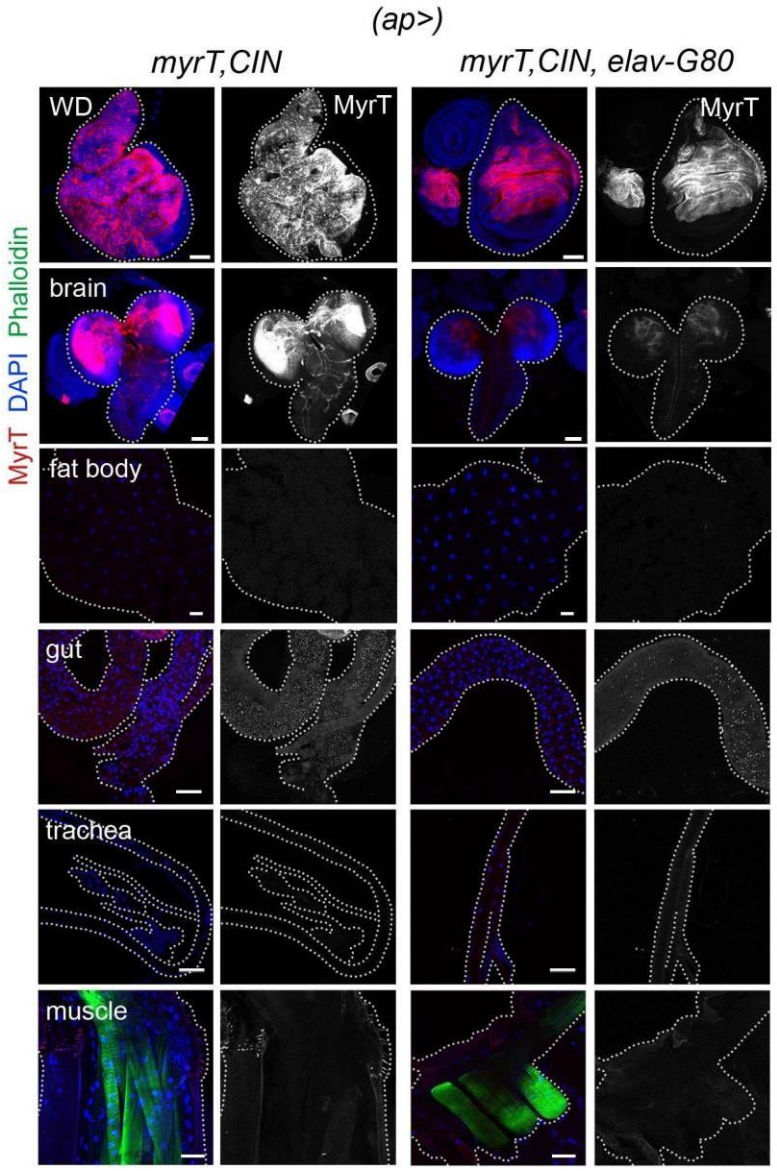

Figure 19. *ap-gal4* driver shows unspecific expressions in different larvae tissues

CIN larvae dissected in L3 under the control of the ap-gal4 driver show unspecific expression in the brain and gut cells. CIN larvae containing elav-Gal80 reduce this unspecific phenotype.

Bromophenol Blue powder which was added to the food until a dark blue color was obtained. Flies were let laying eggs for 4 hours after which the temperature shift took place. However, once they reached L3 stage, larvae were moved from normal yellow food to blue food. Feeding was assessed by imaging larvae every 24 hours. Due to the transparency of the larvae and the fact that they empty their stomachs before they enter into pupariation, scoring for feeding was easily observed by checking the color of the gut. Yellow guts mean no feeding or emptying of the gut prior to the entering in metamorphosis, while blue guts mean that feeding is still taking place. Control larvae show yellow guts at the time of entering into pupariation (approximately 132 hours' time) while CIN larvae present blue colored guts for the time accounting for the developmental delay (from 132 to 204 hours) (Figure 18 C). At the 204 hours' time point some larvae can be found with empty guts preparing for pupa formation. This result makes sense with the Pupariation timing assay since at 204 hours there is a good percentage (higher than 40%) of larvae that have already entered into pupariation. During the extra time spent feeding, CIN larvae also increase in their volume reaching twice their size by day 14 (Figure 18 B). Although this is the case, the number of larvae reaching day 14 without pupating is very low, since more than half of them are able to form a pupa by day 10.

1.3 Feeding Ecdysone in its active form rescues CIN delays

Taking into account that most developmental delays are a consequence of problems developed during ecdysone biosynthesis,

it became pressing to evaluate if the CIN delay was a consequence of missing ecdysone hormone.

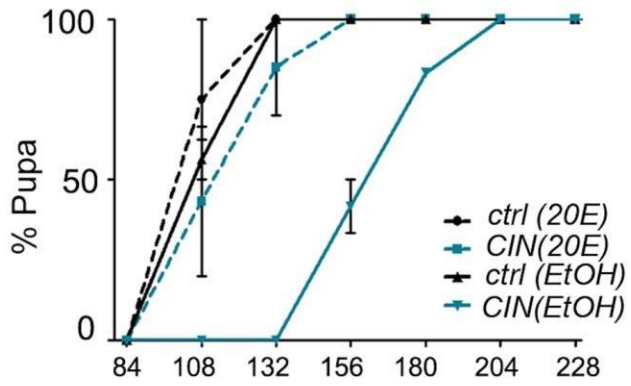

Figure 20. Larvae feed with 20E rescue CIN induced developmental delay

Pupariation assays performed with feeding ecdysone active form to both control and CIN larvae revealed a full rescue of the CIN induced delay. Control groups performed for the feeding experiment were done providing EtOH, the alcohol used to dilute the steroidal hormone. In the control groups no alteration in developmental timing was observed, as expected.

To test this hypothesis, larvae were fed with ecdysone hormone in its activated form: 20-hydroxyecdysone. To do so, larvae were left laying eggs for 4 hours and then placed directly at 29ºC to initiate the temperature shift. After three days, when larvae were starting to make the shift between L2 to L3, larvae were collected from the initial tube and placed into petri dishes with minimal amounts of food in order to be selected at an early L3 stage. This selection was made taking into account phenotypic features such as the morphology of the spiracles. After selection, early L3 larvae were collected and placed in smaller tubes where the activated form of the hormone was

provided twice, every day, to both wild type and CIN samples. For the control groups EtOH was provided instead of the hormone, since this alcohol was the substance used for the steroidal hormone dilutions.

Pupariation graphs were made by counting pupa % for 10 consecutive days, where it was already possible to observe the rescue of CIN larvae supplemented with the hormone (Figure 20).

These results are the confirmation that the delay generated by CIN epithelial tumors, in resemblance to what happens in other tumor models, is a consequence of the absence of ecdysone steroid hormone.

1.4 CIN delay is a consequence of JNK activation but is growth-independent

CIN induction causes high levels of aneuploidy in cells that, if prevented from dying, activate JNK signaling (Clemente-Ruiz et al. 2016; Dekanty et al. 2012; Muzzopappa, Murcia, and Milán 2017). The original role of this stress response pathway is to eliminate cells from the tissue, but since that is prevented by the presence of p35 protein, the continuous activation of JNK leads it to become pro-tumorigenic. This pro-tumorigenic behavior is sensed through the overproliferation stimulated by the release of mitogens, and the increased capacity for invasiveness, dependent on the release of metalloproteases, such as MMP1 (Dekanty et al. 2012) and the modulation of the actin-myosin cytoskeleton (Benhra et al. 2018).

The activation of JNK is also behind one of the main features of the CIN epithelial tumors, which is the highly secretory phenotype of the

senescent-like cells. Senescent cells secrete specific factors that are able to modify the microenvironment and neighboring cells of the tumor. The specific secretome released by senescent cells is called the SASP. The transcriptional profile of highly aneuploid delaminating cells of CIN tumors was analyzed in order to provide information about the secretome of CIN induced tumors. In order to do so, a microarray previously performed in the lab (Clemente-Ruiz et al. 2016) was reanalyzed to assess the most relevant up-regulated genes differentially expressed between delaminating and non-delaminating cells. In the secretome of CIN-induced tumors we can find a collection of long-range signaling molecules from cytokines Upd1-3, growth promoters such as Wnt4 and Wg, metabolic modulators such as ImpL2 and ImpL3 or immune system elements such as PGRP-SA (Table 2).

Table 2. Upregulated signals representing the secretome present in CIN tumors

Symbol	Gene	Reference
wg	wingless	Dekanty et al., 2012
Mmp1	Matrix metalloproteinase 1	Dekanty et al., 2012
upd1	unpaired 1	Clemente-Ruiz et al., 2016
upd2	unpaired 2	Clemente-Ruiz et al., 2016
upd3	unpaired 3	Clemente-Ruiz et al., 2016
egr	eiger	Muzzopappa et al., 2017
spi	spitz	Benhra et al., 2018
vn	vein	Benhra et al.,2018
Dilp8	Drosophila insulin-like peptide 8	Clemente-Ruiz et al., 2016
ImpL2	Ecdysone-inducible gene L2	Clemente-Ruiz et al., 2016
ImpL3	Ecdysone-inducible gene L3	Clemente-Ruiz et al., 2016
NLaz	Neural Lazarillo	Clemente-Ruiz et al., 2016
PGRP-SA	Peptidoglycan-recognition protein SA	Clemente-Ruiz et al., 2016
wnt4	Wnt oncogene analog 4	Clemente-Ruiz et al., 2016
btl	breathless	Clemente-Ruiz et al., 2016

Taking into account the high number of long-range signaling molecules that seem to be direct targets of JNK, the next step was to assess if the CIN-delay was a consequence of JNK triggering.

Pupariation assays were done by blocking JNK activation using *puckered*, a member of the VH-1 family of phosphatases (Martín-Blanco et al. 1998).

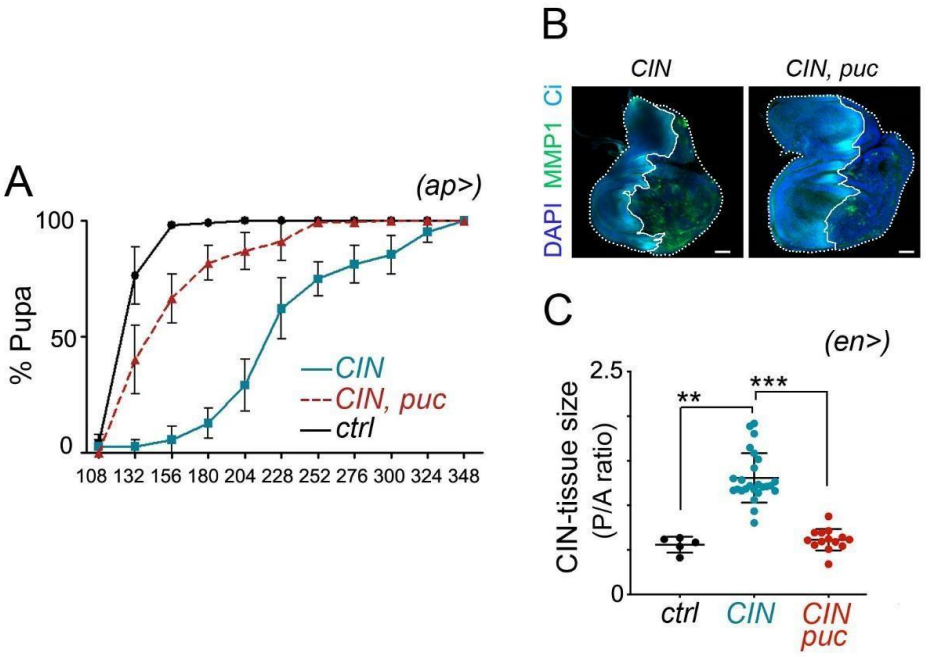

Figure 21. CIN larvae delay is dependent on the activation of JNK signaling pathway

A – Repressing JNK by using UAS-*puc* rescues the developmental timing of CIN larvae almost to control levels with 50% of the pupas being obtained between 132 and 156 hours after egg laying.

B – Larval wing primordium expressing the indicated transgenes in the posterior compartment and stained with DAPI (blue), MMP1 (green) and Ci (cyan).

C – Histogram plotting the P/A ratio values of control, CIN and CIN with blockage of JNK signaling pathway. Blocking JNK clearly rescues tumor size to control levels.

Results

Results show that if JNK is no longer active, the delay observed in CIN induced tumors is fully rescued (Figure 21 A). Even though JNK blockage does not rescue the capacity of forming the delaminated population of cells (Dekanty et al. 2012), repressing this stress response pathway not only fully rescues the delay but it also rescues tumor size (Figure 21 B and C). Tumor size was assessed generating CIN tumors with *engrailed*-Gal4 (driver for the posterior compartment of the wing disc) and expressing *rod*-RNAi and p35.

Measurements were done taking into account the posterior size and the anterior size (labelled by Ci) of the compartments and doing a P/A ratio.

Since rescuing JNK signaling also rescues tumor size, the delay rescue might be a consequence of rescuing tumoral growth.

Therefore, in order to dismiss this hypothesis, we performed Pupariation assays between control, CIN tumors and CIN tumors where the action of the mitogen causing overgrowth was prevented by removing wingless with an interference RNA. This way the tumor with *wg*-RNAi grows less, almost with no difference when compared with control discs, and it is possible to assess if the JNK rescue is due to alterations in tumor size. However, despite rescuing tumor size (Figure 22 B and C), removing *wg* was not sufficient to rescue the developmental delay (Figure 22 A).

These results help us conclude that although CIN delay is mediated by JNK-expressing cells, most probably because of the high capability of secreting long-range signaling molecules, and with tumor growth being a factor - in the sense that the more overgrowth we have the higher the number of JNK-expressing cells -, rescuing tumor size alone is not effective in rescuing CIN induced delay.

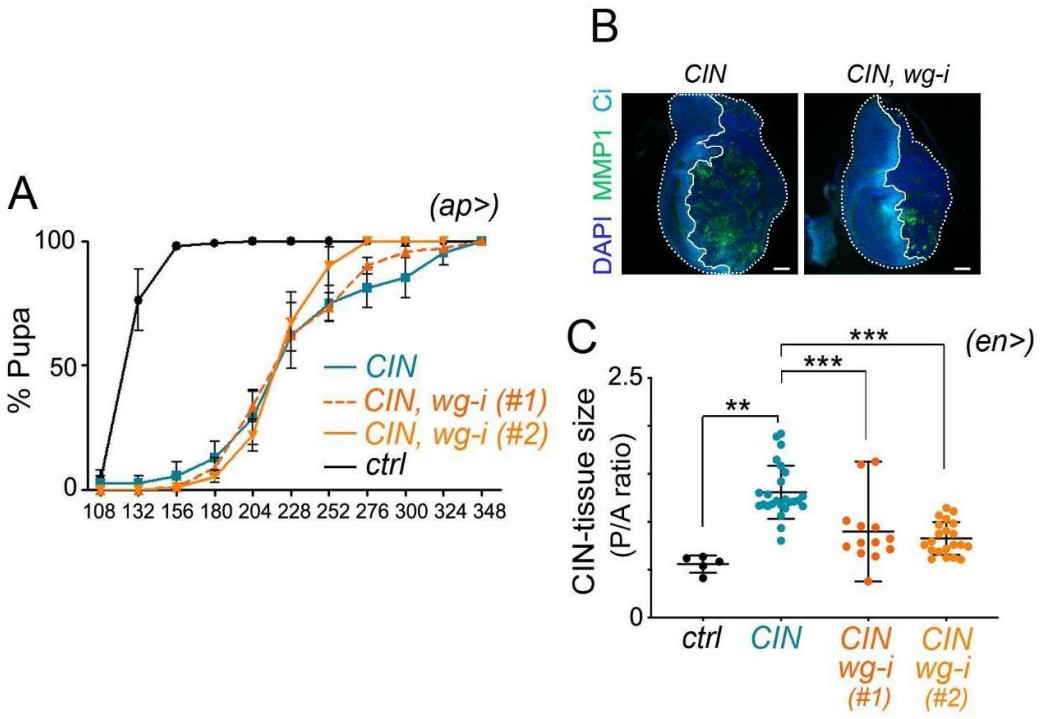

Figure 22. CIN larvae delay is growth independent

A – Larval wing primordium expressing the indicated transgenes in the posterior compartment and stained with DAPI (blue), MMP1 (green) and Ci (cyan).

B – Histogram plotting the P/A ratio values of CIN and CIN with blockage of *wg* mitogen pathway. Blocking the action of mitogen clearly rescues tumor size.

C – Pupariation assay plotting the developmental timing of control, CIN and CIN larvae without *wg* considering hours AED. Absence of this mitogen does not rescue systemic delay, even though the tumor size is much smaller.

1.5 CIN delay is not rescued by the relaxin-like protein Dilp8

Growing organisms sometimes suffer disturbances, such as injuries, which in order to be dealt with require an extension in developmental time. Some of these disturbances might be caused by mechanical injuries, cell death by radiation or chemical compounds, or even tumoral growths. Animals, and in this particular case, *Drosophila,* are able to cope with these disturbances by releasing long-range signaling molecules that help them recover from the injury and regenerate the damaged tissues. They are also able to block other peripheral growth that is taking place to assure correct patterning and correct adult size, as well as block entering into maturation and differentiation stages before full restoration of the damage tissue takes place (Colombani et al., 2012; Garelli et al., 2012; Ray & Lakhotia, 2016).

In the fruit fly, the signaling molecule responsible for blocking ecdysone release, via PTTH-dependent regulation, is the relaxin like protein Dilp8. Dilp8 comes from the damaged disc and binds to its receptor, Lgr3, present in four central nervous system Lgr3-positive neurons, activating, in this way, cyclic-AMP signaling (Gontijo and Garelli 2018). This action delays PTTH production and prevents the surge of the molting hormone ecdysone, postponing the metamorphosis entering point.

Since Dilp8 was already found to be upregulated in our microarrays (Clemente-Ruiz et al. 2016), we decided to further confirm Dilp8 presence in the tumoral tissue by looking at the expression of a *dilp8*-GFP MiMIC line (Figure 23 A). We observed that *dilp8* is expressed

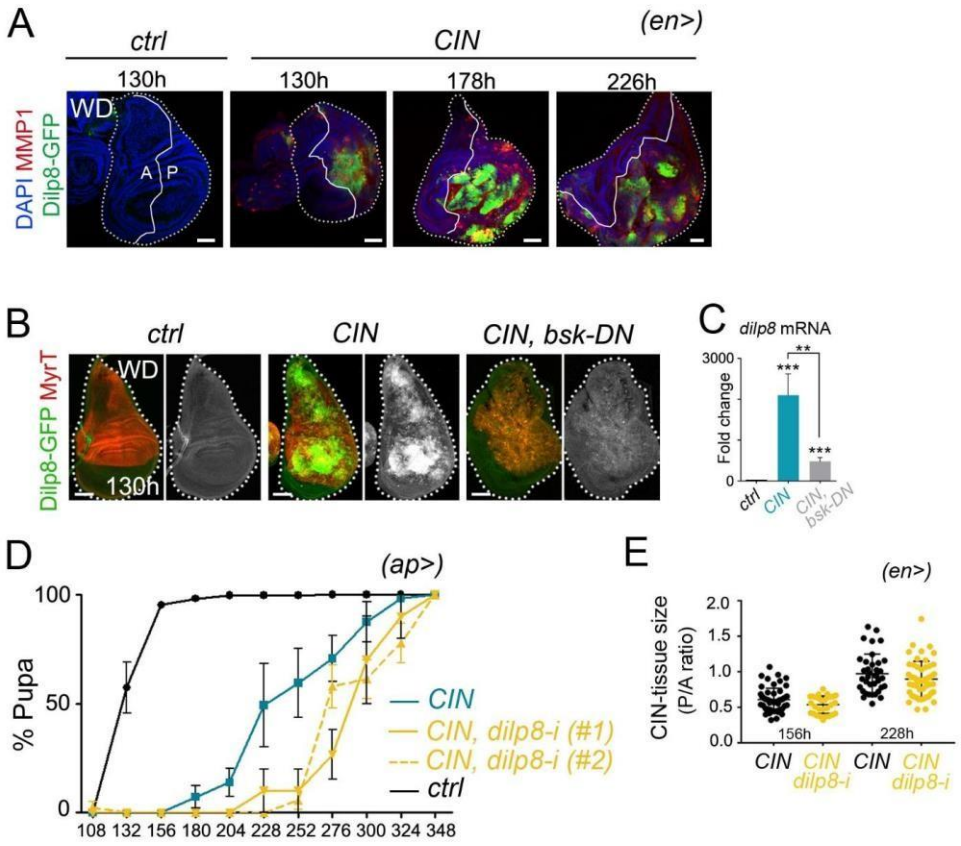

Figure 23. CIN larvae delay is not caused by Dilp8 relaxin-like protein

A – Larval wing primordium expressing the indicated transgenes in the posterior compartment (*en*-gal4) and stained with DAPI (blue), MMP1 (red), Ci (cyan) and *dilp8 mimic-GFP* (green).

B – Larval wing primordium expressing the indicated transgenes in the dorsal compartment (*ap*-gal4) and their fluorescent MyrT area (red) and *dilp8 mimic-GFP* (green).

C – Histogram plotting the qRT-PCR fold change increases of control, CIN and CIN with blockage of JNK. Blocking the JNK stress response pathway reduces significantly dilp8 expression in CIN tumors.

D – Pupariation assay plotting the developmental timing of control, CIN and CIN larvae without *dilp8.* Absence of *dilp8* does not rescue systemic delay, it increases the CIN delay in an extra 48 hours approximately.

E – Histogram plotting the P/A ratio values of CIN and CIN with blockage of *dilp8*. Blocking the action of this relaxin like protein does not alter the P/A ratio of tumors in the time points analyzed.

all throughout tumor development, presenting an increase in expression as days go by.

Moreover, we confirmed that *dilp8* is a direct target of JNK, because blocking JNK using a *Bsk-DN* (*basket* is a target of *hemipterous* (JNKK) phosphorylation for promoting transcription of JNK targets) completely rescues *dilp8*- GFP MiMIC expression levels (Figure 23 B), a result that was also validated via qRT-PCR (Figure 23 C).

Using an interference RNA we removed *dilp8* from the tumors to assess their ability to grow and their developmental delay. Removing *dilp8* in both cases had no rescue effect on P/A ratio or Pupariation timing (Figure 23 D and E). Interestingly, removing this relaxin-like protein from the CIN derived tumors caused the delay to increase to a total of 7 extra days when compared to the control flies. Moreover, although it is true that both the larvae and the tumors grow during those extra days spent in the food, the growth is not significantly different from those presented by the CIN larvae alone.

Since Dilp8 has been recognized as the known molecule responsible for the generation of developmental delays in response to injuries or tumor development, we decided to perform a proof of principle to make sure all lines were working correctly. To do so, we used the Avalanche Model to confirm dilp8's capacity of rescuing tumor-induced developmental delays.

As expected, removing *dilp8* from the Avalanche model (Figure 24 A) produced a rescue of the developmental timing. Within the same line, overexpressing *dilp8* was able to generate a delay, as

previously shown by Colombani and collaborators (J. Colombani, Andersen, and Leopold 2012) (Figure 24 B).

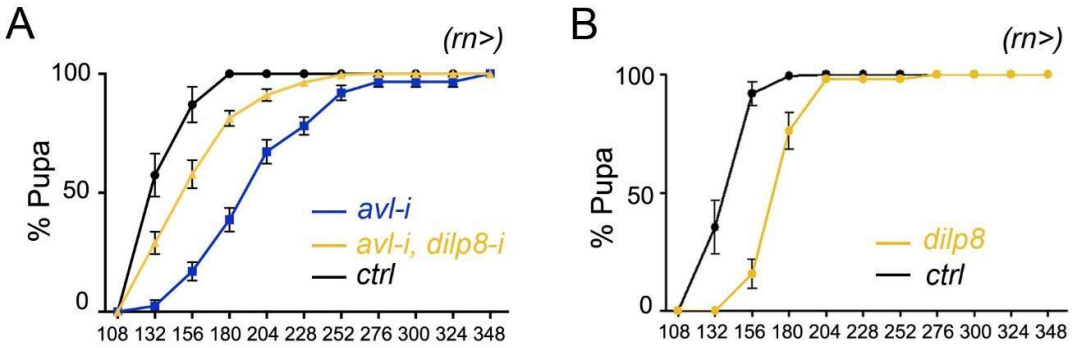

Figure 24. CIN larvae delay is not caused by Dilp8 relaxin-like protein

A – Pupariation assay plotting the developmental timing of control, *avl*-RNAi and *avl*-RNAi combined with *dilp8*-RNAi. In this tumor model depletion of *dilp8* is able to rescue the developmental timing delay almost completely. With 50% of Pupa value occurring between 108 and 132h.

B – Pupariation assay plotting the developmental timing of control and overexpression of *dilp8* in the pouch area of the wing primordium. Overexpression of dilp8 alone is able to generate a systemic delay as previously described by Colombani and collaborators (Colombani et al; 2012)

When previously described as the molecule capable of generating systemic delays, Dilp8 was characterized as capable of binding to Lgr3 and preventing the release of PTTH hormone. The PTTH hormone, as described in the chapter 2, section 2.2 of the Introduction, acts via Torso/Erk pathway to allow for the biosynthesis of ecdysone. Therefore, the next step to assess why *dilp8* was not generating the same effect as previously observed for other tumor models, was to check ERK phosphorylation. If ERK is phosphorylated, then the PTTH responsive regulatory pathway is

active and, most probably, the delay is not a consequence of the misregulation of this branch of ecdysone regulators. When checking for the phosphorylated form of ERK we realized that, although

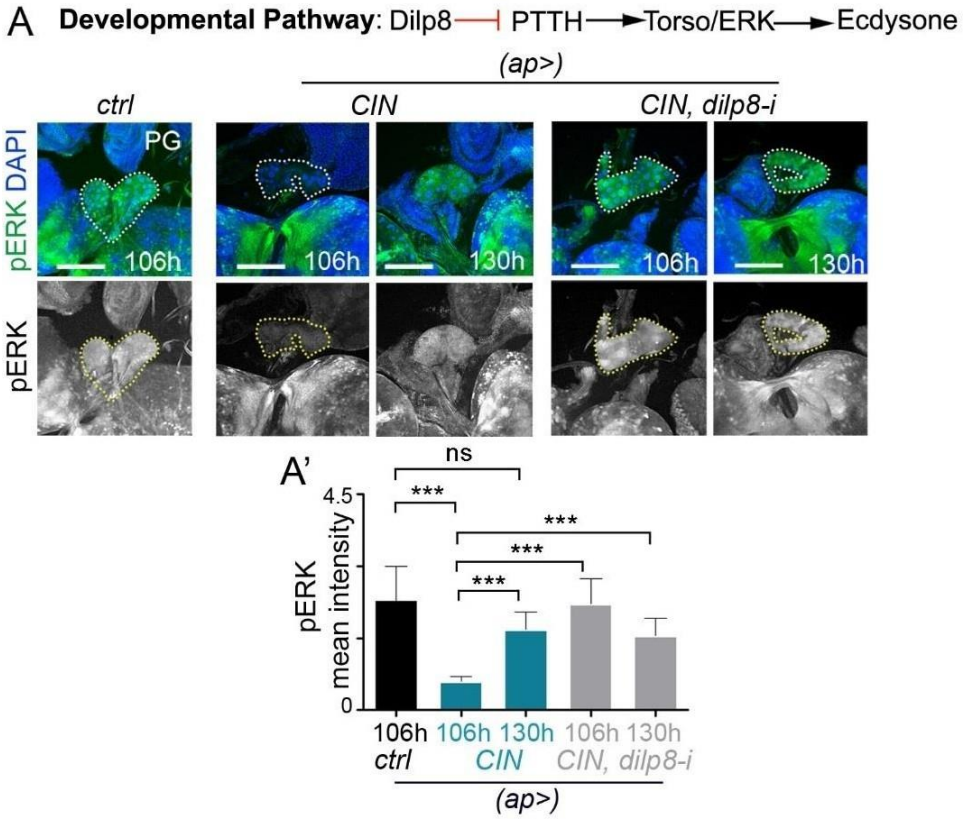

Figure 25. ERK phosphorylation in CIN larvae is not the main responsible for the delay

A – Ring glands from control and CIN tumors with the indicated phenotypes induced in the dorsal part of the wing disc primordium (*ap*-gal4), were dissected from L3 larvae and stained for pERK (green) and DAPI (blue). Results show a delay in the peak of pERK activation that in CIN larvae takes additional 24 hours' time and that it is fully restored to normal timing upon removal of *dilp8*.

A' – Histogram representing mean intensity values of pERK fluorescence of the indicated phenotypes. Blocking dilp8 in the initial tumoral site rescues pERK levels to control values.

delayed, it was present in CIN larvae at 130 hours' time point (Figure 25 A and A').

This result, together with the generation of the delay, seemed to indicate a slight problem within the PTTH dependent regulatory network. Removing dilp8 from the initial tumor site did rescue this slight delay in ERK phosphorylation timing, showing that although it is not the secreted molecule responsible solely for the delay, it does have an influence in this developmental pathway regulatory network.

Taken together, these results show that CIN tumors are able to induce a developmental delay of approximately 96 hours, independently of tumor size but dependent on the activity of the JNK signaling pathway. However, although JNK is able to control *dilp8* expression, Dilp8 relaxin-like protein is not the main responsible for the alterations in developmental timing. As it does not seem to be the PTTH-dependent branch of ecdysone regulation the one mostly affected in the CIN scenario. In a CIN background, the downstream targets of the Torso/ERK pathway are active, indicating that, although suffering from a slight delay, the release of PTTH hormone takes place a great number of hours previously to entering into metamorphosis. Moreover, removing *dilp8* fully rescues this minor timing deviation, without producing a delay rescue. These results indicate that JNK expressing cells are able to release other long-range signaling molecules to act in ecdysone biosynthesis, aside from Dilp8.

2. Monitorization of Ecdysone Regulatory Pathways

2.1 The Insulin-dependent Ecdysone Regulation Pathway is affected in CIN ring glands

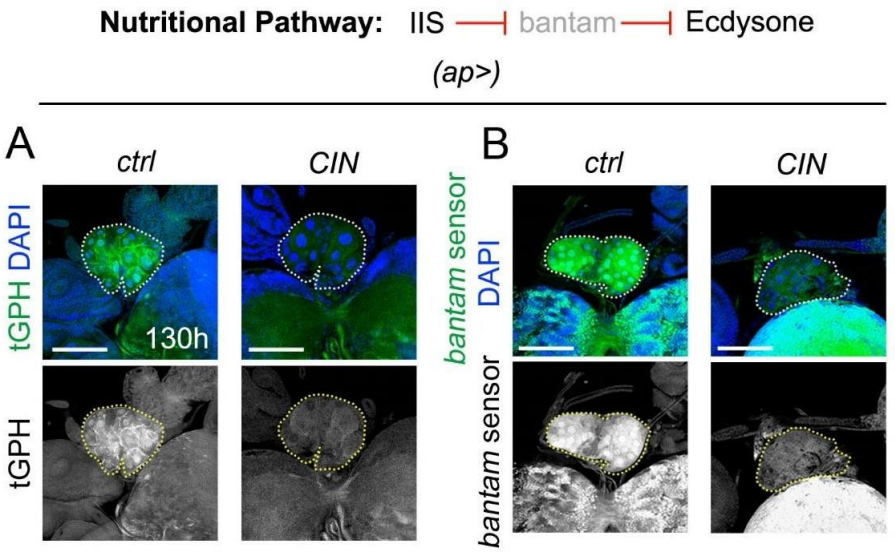

Figure 26. CIN ring glands present insulin resistant phenotype and bantam up-regulation

A – Ring glands of CIN and control larvae dissected at L3 stage and stained with DAPI (blue). Insulin signaling was assess by using the tGPH-sensor (green), a PH-GFP fusion protein used as an indicator of PI3K activity. CIN larvae show low activity of the sensor, meaning that there is low insulin activity in these tissues.

B – Ring glands of CIN and control larvae dissected at L3 stage and stained with DAPI (blue). *bantam* activity was assessed using a bantam sensor expressed under the control of a *tubulin* promoter, carrying at 3'UTR two binding sites for bantam, this way impairing GFP reporter fluorescence when bantam is present. CIN larvae show low acidity of the sensor, indicating that bantam is upregulated in this condition, when compared to control larvae, with high GFP fluorescence.

CIN epithelial tumors present a developmental delay dependent on the activation of JNK stress signaling pathway. Since this delay does not seem to be a consequence of the de-regulation of the PTTH-dependent regulatory network, we decided to check if other regulatory networks for ecdysone production were affected in the ring gland of CIN larvae.

One of the other major regulators of ecdysone production is the insulin pathway. Insulin signaling is able to recognize nutrient cues from peripheral tissues and influence the ring gland accordingly. In the ring gland, IIS acts through the repression of bantam microRNA that is normally repressing ecdysone production (Boulan, Martín, and Milán 2013).

To assess if CIN ring glands presented normal insulin signaling activity we decided to measure two factors: the activity of the signaling pathway via tGPH-sensor (a PH-GFP fusion protein used as an indicator of PI3K activity) (Britton et al. 2002); and the activity of bantam via the bantam-sensor (a sensor that expresses GFP under control of a ubiquitously active tubulin promoter, where the 3'UTR carries two binding sites for bantam, so that when it binds, GFP expression is impaired) (Brennecke et al. 2003).

CIN ring glands presented low insulin activity, shown by an almost absence of the tGPH sensor fluorescence (Figure 26 A), and, consistently, exhibited an increase in bantam expression, visible by the lack of GFP (Figure 26 B). These results indicating that the IIS-dependent regulation of ecdysone is severely impaired.

2.2 NLaz is responsible for the lack of IIS response in CIN ring glands

Since the microarray analysis (Clemente-Ruiz et al. 2016) revealed many secreted molecules that had a metabolic regulation effect were released, we decided to explore this and look out for targets that could function as inhibitors of the insulin pathway.

One that came immediately to our attention was the lipocalin Neural Lazarillo. Neural Lazarillo is a direct target of JNK signaling initially shown to control lipid biogenesis and circulating carbohydrate levels (Hull-Thompson et al., 2009). However, more recent research studies involving *NLaz* showed that this lipocalin is a potential adipokine that antagonizes IIS in order to allow for metabolic regulation. *NLaz* expression in the larval fat body reduces IIS general levels, whereas mutating it shows elevated IIS response capacity (Pasco and Léopold 2012).

This regulation of insulin response behavior made *NLaz* reveal itself as a good candidate to be tested, since it filled the requirements we had so far. In order to validate the microarray results obtained in 2016, we decided to, firstly, perform two different experiments: an *in situ* assay and a qRT-PCR analysis to check for *NLaz* expression levels in the CIN tumors. Results show that *NLaz* is highly expressed in the CIN situation, contrary to what happens in its wild-type counterparts, and that expression drops dramatically once JNK is blocked (Figure 27 A). Within the same line, *in situ* experiments also show differential expression of NLaz in CIN tumors, an expression

that is fully recovered to normal values once JNK is blocked (Figure 27 B). Thus, *NLaz* appears to be a direct target of JNK, highly present in the initial tumor site.

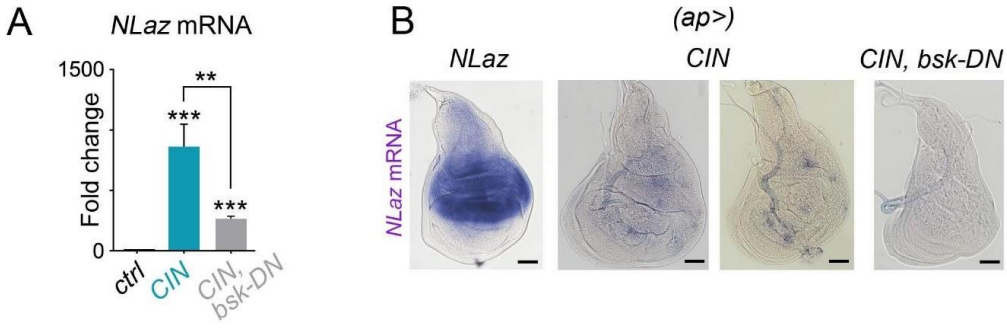

Figure 27. NLaz is highly expressed in CIN tumors

A – Histogram plotting the qRT-PCR fold change increases of control, CIN and CIN with blockage of JNK. Blocking the JNK stress response pathway reduces significantly NLaz expression in CIN tumors.

B – In situ assay done performed in wing disc primordium of NLaz overexpression, CIN and CIN with *NLaz-RNAi*. CIN tumors present differential expression of NLaz when compared with tumors were JNK is blocked.

Due to its capacity of acting as an insulin signaling regulator, we decided to use an interference RNA to deplete *NLaz* from the tumors and check alterations in IIS response patterns.

We realize that the insulin resistance observed in the ring gland of CIN tissues was rescued when NLaz was depleted (Figure 28 A). The same response, as expected, was also visible for the ring glands were JNK signaling was blocked.

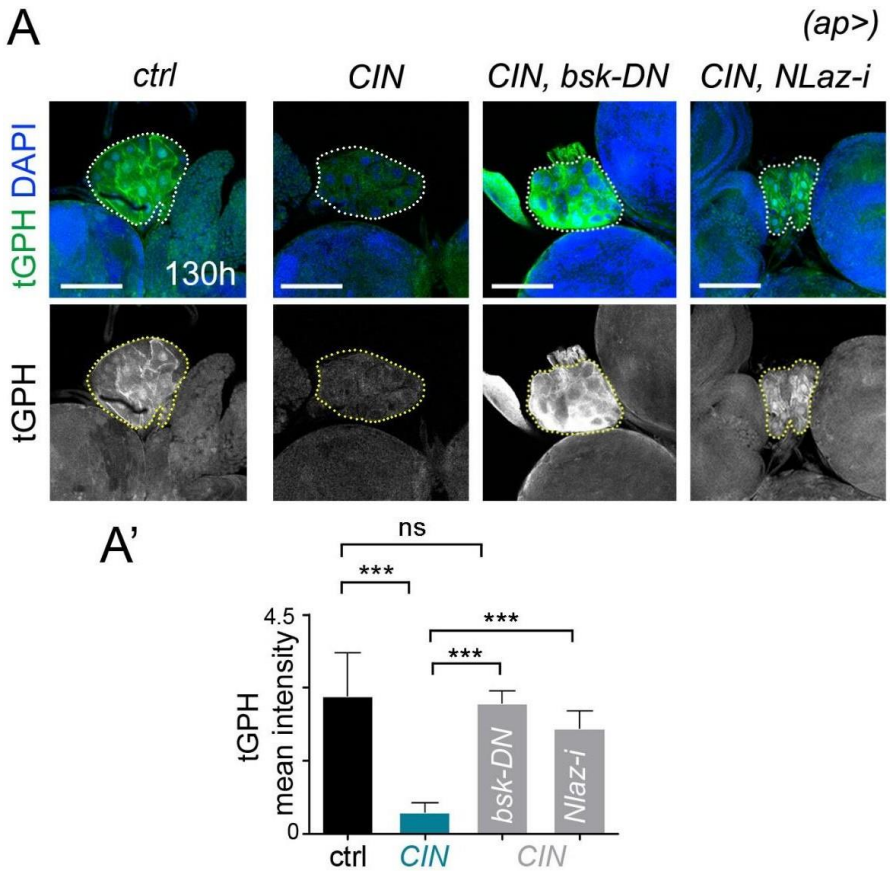

Figure 28. NLaz depletion rescues insulin resistant phenotype of CIN ring glands

A – Ring glands of CIN and control larvae of indicated phenotypes were dissected at L3 stage and stained with DAPI (blue). Insulin signaling was assessed by using the tGPH-sensor (green), a PH-GFP fusion protein used as an indicator of PI3K activity.

A' – Histogram plotting mean tGPH intensity of indicated phenotypes reveals that blocking JNK or removing NLaz from the initial tumor site rescues tGPH levels in the ring gland to control values.

However, depleting *NLaz* from the tumors was not sufficient to rescue bantam upregulation (Figure 29 A).

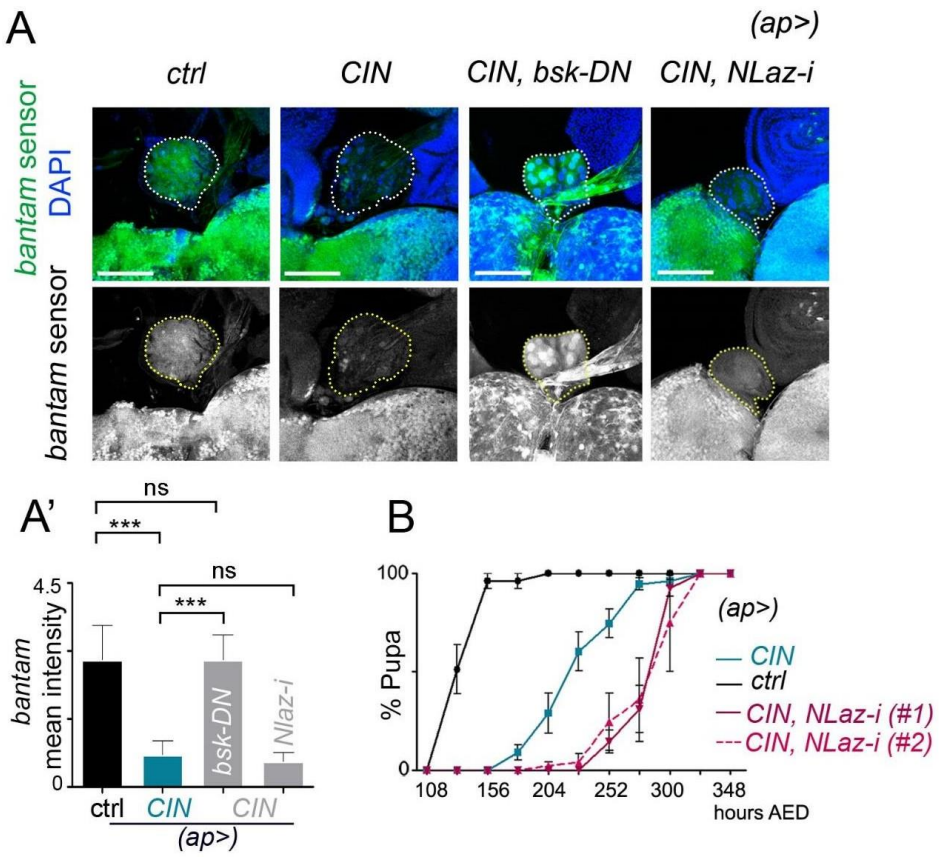

Figure 29. NLaz is not sufficient to rescue the bantam dependent CIN delay

A – Ring glands of CIN and control larvae of indicated phenotypes were dissected at L3 stage and stained with DAPI (blue). bantam activity was assessed by using the bantam-sensor (green).

A' – Histogram plotting mean bantam intensity of indicated phenotypes reveals that removing NLaz from the initial tumor site is not sufficient to rescue bantam levels in the ring gland to control values.

B – Pupariation assay plotting the developmental timing of control, CIN and CIN larvae without *NLaz.* Absence of NLaz does not rescue systemic delay.

As a consequence, the increased developmental timing observed in CIN pupariation assays was also not recovered (Figure 29 B) and, similarly to what happened before with *dilp8*, removing *NLaz* contributed to the generation of an increased delay, now obtaining 50% of total pupa between 276 and 300 hours.

Since depleting *NLaz* did give rise to an increase in insulin response we wondered if, in a normal situation, overexpressing *NLaz* was sufficient to induce insulin resistance in healthy ring glands. To test this hypothesis, we performed an overexpression of UAS-*NLaz* in the ring gland of control larvae, by using the *P0206*-gal4 driver (Figure 30 A). We observed that not only was the overexpression of this lipocalin sufficient to generate a delay, but that it was equally capable of inducing insulin resistance in this tissue (Figure 30 B).

Moreover, the overexpression alone was also able to cause an increase in bantam levels, indicating that in a non-tumoral context *NLaz* is capable of influencing the IIS-dependent regulatory network of ecdysone production following the double repression mechanisms proposed by the lab in 2013 (Boulan, Martín, and Milán 2013).

Overexpressing the UAS-*NLaz* using the wing pouch area driver *rotund*-gal4 was also performed, and able to recreate the results observed previously for the overexpression in the ring gland in terms of developmental timing (Figure 30 C).

Due to the strong correlation between the behavior of NLaz and Dilp8, we hypothesized that these two molecules might be working together to prevent the transition to the next metamorphosis phase.

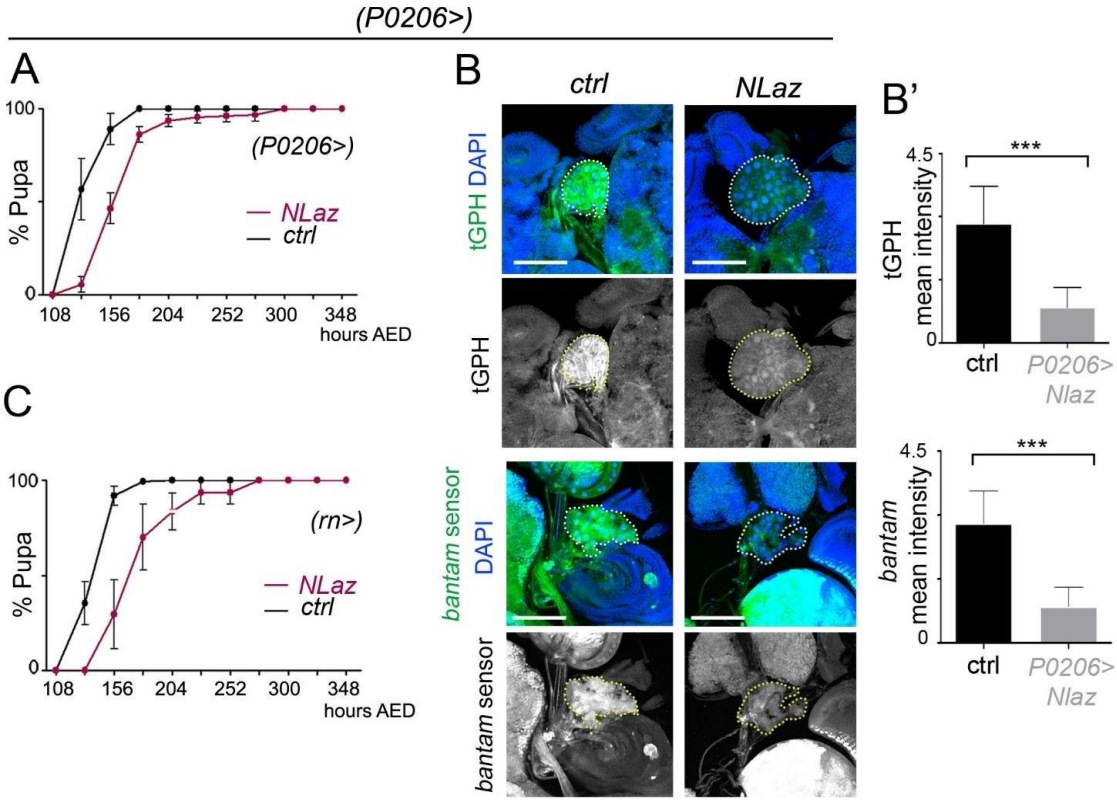

Figure 30. Overexpressing *NLaz* causes insulin resistance and bantam upregulation in the ring gland and a systemic delay in developmental timing

A – Pupariation assays done with control and overexpression of *NLaz-RNAi* in the ring glands of developing larvae caused a delay in development of approximately 24 hours, when comparing 50% of total pupas formed.

B – Ring glands of CIN and control larvae dissected at L3 stage and stained with DAPI (blue). Insulin signaling was assessed by using the tGPH-sensor (green), a PH-GFP fusion protein used as an indicator of PI3K activity. *bantam* activity was assessed using a bantam sensor expressed under the control of a *tubulin* promoter, carrying at 3'UTR two binding sites for bantam, this way impairing GFP reporter fluorescence when bantam is present. Overexpressing *NLaz* in the ring gland causes increase in bantam expression and a decrease in insulin sensibility.

B' – Histograms plotting mean intensity of tGPH and bantam fluorescence of indicated phenotypes. Results reveal that overexpressing NLaz severely affects IIS regulation in the ring gland tissue.

C – Pupariation assay done with control and overexpression of *NLaz-RNAi* in the pouch of developing larvae. Overexpression of *NLaz* is sufficient to cause a delay of approximately 24 hours when compared to control phenotypes.

To verify this hypothesis, we depleted both NLaz and Dilp8 from the initial production site, the CIN tumor. Due to their influence in the two main branches of the ecdysone production network, we thought it might be possible that they would produce a strong enough signal to generate a partial rescue. However, we were not expecting a full rescue since bantam would still be upregulated, given the fact that NLaz is not sufficient to produce a downregulation of this crucial player.

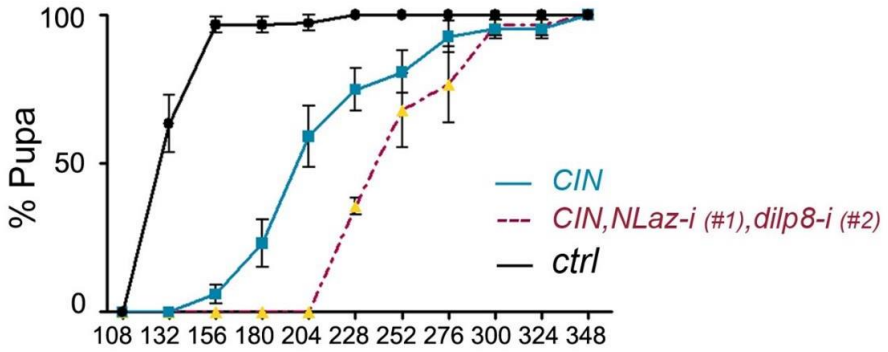

Figure 31. Co-depletion of NLaz and Dilp8 is not sufficient to rescue CIN delay

Pupariation assay plotting the developmental timing (in hours AED) of control, CIN and CIN larvae without co-depletion of both *NLaz* and *dilp8.* Absence of both secreted molecules does not rescue systemic delay present in the tumoral situation.

Results show that eliminating both secreted signals does not produce a rescue of the CIN developmental delay (Figure 31). Meaning that the combination of both secreting molecules is not sufficient to surpass bantam repression.

These results allow us to conclude that although neither *NLaz, dilp8* nor their combination is sufficient to rescue the delay of CIN larvae, although they clearly influence insulin sensibility and the timing of ERK phosphorylation, respectively.

Nevertheless, the observations concerning NLaz provide new information about this lipocalin, revealing it to be capable of acting on the IIS-dependent branch as well as efficient in generating a systemic impact on developmental timing. This delay seems to be a cause of reduced capacity to respond to insulin cues which results in the upregulation of the *bantam* microRNA, which in turn represses ecdysone biosynthesis.

2.3. Travelling cytokines are able to activate JAK/STAT signaling in CIN ring glands generating a *bantam*-dependent delay

The lipocalin NLaz seems to be able to regulate IIS response in the ring gland of CIN induced tumor carrying larvae. However, when depleting *NLaz* from the initial tumor site, *bantam* upregulation is not rescued. Therefore, some other long-range signal is regulating this microRNA independently from the IIS pathway.

Going back to the microarray, we were looking for a long-range signaling molecule or group of molecules, most likely secreted under the control of JNK signaling, with a known systemic impact. This impact could be either by the ability to activate major regulatory networks or the ability to control the response of peripheral tissues to a variety of stresses.

Taking into account these options, the candidates that caught our eye were the Upds (*upd1-3*). These cytokines are most similar to interleukins, in mammals, and they perform an immense number of different activities depending on the tissue or developmental timing experienced by the fruit fly. Upd1, for instance, also known as *outstretched*, is the most potent ligand for activating the JAK/STAT signaling pathway (Rajan and Perrimon 2012), being initially described as responsible for the segmentation defects present in *Drosophila*'s embryos, recapitulating the phenotype of loss of *hop* and *stat92E* (Harrison, Price, and Bell 1998). However, from that point onwards *upd1* has also been described as responsible for growth control in the eye disc (Vollmer et al. 2017); for controlling feeding behaviors, attraction to food cues and weight gain (Beshel, Dubnau, and Zhong 2017); for promoting longevity and pro-aging in a tissue-dependent manner (Moskalev et al. 2019) and for maintaining basal turnover of the midgut epithelium by controlling intestinal stem cell (ISC) maintenance (Osman et al. 2012). Upd3, on the other hand, is the cytokine mostly associated with its role in immunity and inflammation processes, known to be able to be produced by the hemocytes upon bacterial infection, sceptic injury or mechanical damages (Chakrabarti et al., 2016; Lee et al., 2017; Sotillos et al., 2008). This cytokine was also found to be required for enterocytes to induce ISC proliferation (Wisidagama and Thummel 2019); to be responsible for the differentiation of enteroblasts and midgut proliferation (Li et al., 2014); to activate *totA* expression in the fat body in response to septic injury (Agaisse et al. 2003); to cause epithelial renewal upon damage (Buchon et al. 2009) and to promote the encapsulation of parasitoid eggs upon wasp infection, by promoting increased JAK/STAT expression in the skeletal muscle (Yang et al., 2015).

In our particular CIN case, both Upd1 and Upd3 cytokines were found upregulated in the tumoral tissue, and due to their high capacity for mobilization, they arise as possible good candidates for the generation of the CIN larvae delay.

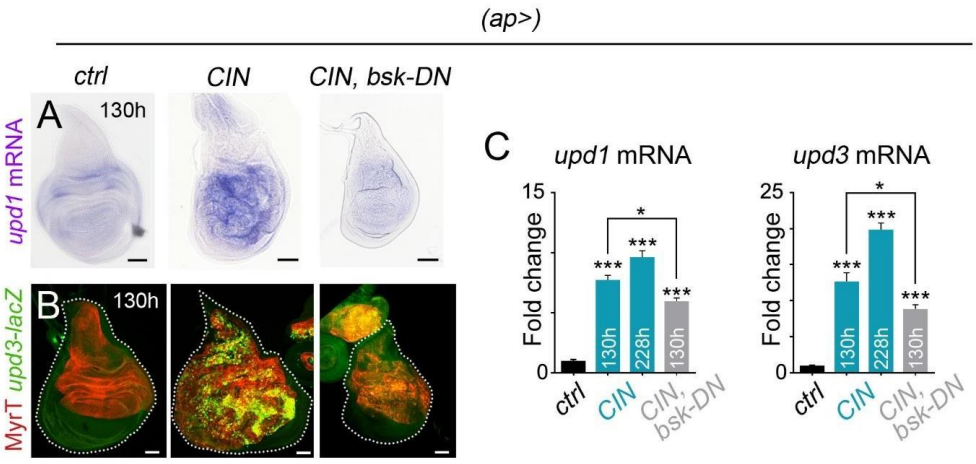

Figure 32. CIN tissues show high levels of both *upd3* and *upd1* expression

A-B – Wing imaginal discs expressing the indicated transgenes (A, B, ap-gal4) and stained for upd1 mRNA (purple, A), upd3-lacZ (green, B) and MyrT (red, B). Both cytokines are shown to be highly expressed in CIN tumors, under the control of JNK signal.

C – Histogram plotting the qRT-PCR fold change increases of control, CIN and CIN with blockage of JNK. *upd1* and *upd3* cytokines are found to be highly expressed throughout CIN tumor development, levels that decrease once JNK signaling is blocked.

Therefore, the first experiment that came to mind, for their activity characterization, was to check for their expression levels in the tumors. Previous reports from the lab had already shown the high activity of these cytokines in CIN (Clemente-Ruiz et al. 2016), namely, microarrays showed that upd1 and upd3 were the highest

produced cytokines with fold change increases of 13,7 and 5,9, respectively, considering differential expression between delaminated and epithelial population.

We confirmed these reports through the usage of both reporter lines, *in situ* hybridization and qRT-PCR assays (Figure 32).

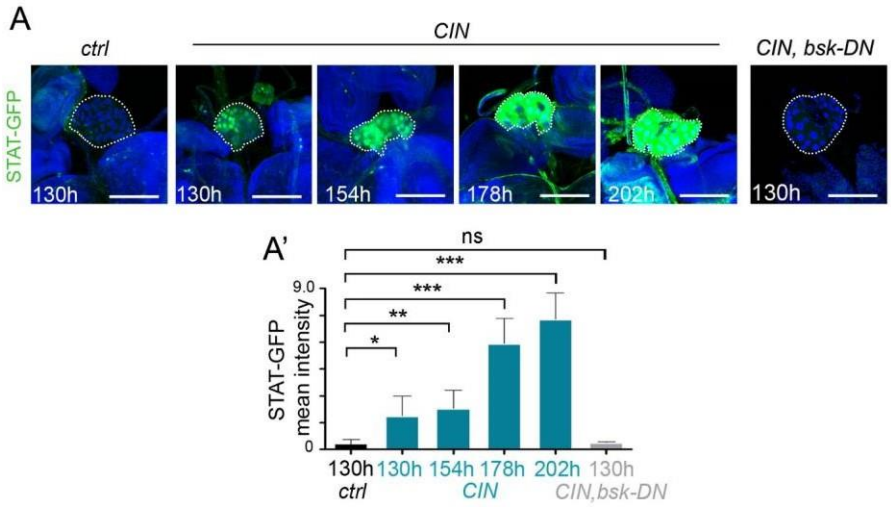

Figure 33. JAK/STAT is highly active in CIN ring glands

A – Ring glands of CIN and control larvae dissected at L3 stage and stained with DAPI (blue). JAK/STAT signaling pathway activation was analyzed with a STAT-GFP reporter line. CIN ring glands present high activity of the JAK/STAT signaling pathway at 4 different time points, whereas no activity is visible in the ring glands of control larvae.

A' – Histogram plotting mean STAT-GFP intensity of indicated phenotypes reveals that JAK/STAT activation in CIN larvae increases exponentially throughout the delayed days.

This cytokines are found to be highly expressed in the tumoral tissues (Figure 32 A and B) which is visible, not only, from the reporter usage upd3-lacZ, but also from the *in situ* hybridization

experiments. Furthermore Upd1 was found to be significantly overexpressed both at 130 and 228 hours' time, suffering a fold change increase from 7,7 to 9,6 when compared to the control larvae. Interestingly, in the overall wing disc primordium, upd3 was found to be even more expressed when compared with upd1, presenting a 12,7 and a 19,9 fold change increase for the same time points considered (Figure 32 C).

These overall increases in cytokine expression are, as expected, rescued once JNK is blocked, since Upds are known to be expressed under the control of JNK signaling (Katsuyama et al. 2015).

Taking into account that these cytokines are highly secretable and capable of reaching peripheral tissues, the next step was to check for JAK/STAT activation was perhaps present in the center for metamorphosis control, the ring gland (Figure 33).

Results show that CIN larvae have high levels of JAK/STAT activation when compared to control ones (Figure 33 A). Furthermore, dissections performed overtime (130, 154, 178 and 202 hours) revealed that this expression is not only continuous throughout the delay but that it exponentially increases in intensity during that time (Figure 33 A').

These results were extremely exciting since no JAK/STAT activation was ever reported for the ring gland tissue neither in tumoral situations nor during development progression, so far to the best of our knowledge. Therefore, it became pressing to assess if this activation was a consequence of upd expression, and if so which of the upd molecules was most involved in such activation.

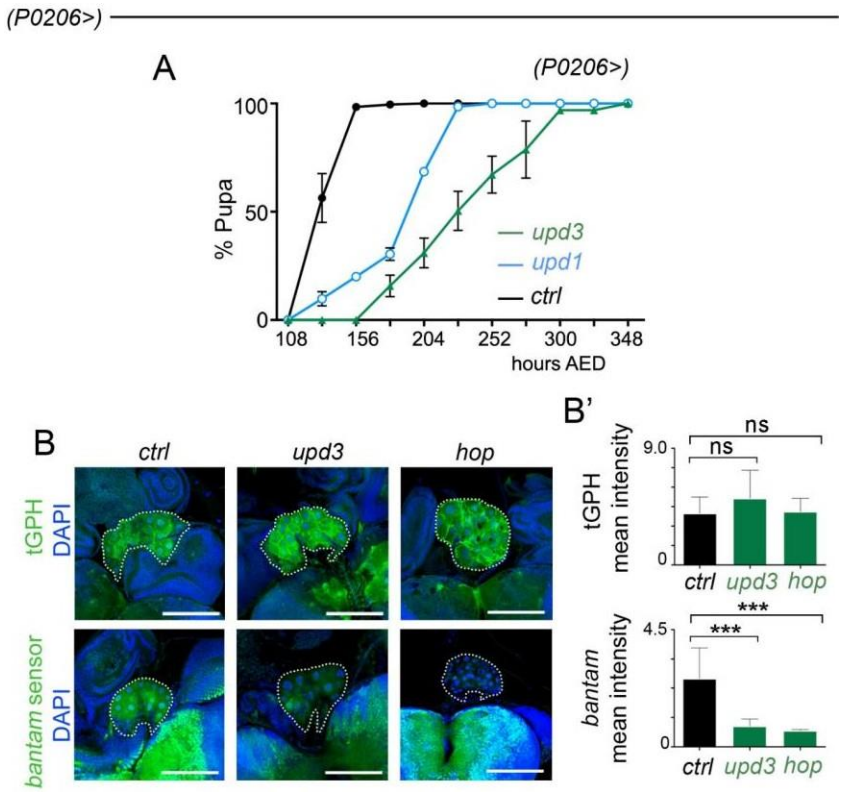

Figure 34. Cytokine overexpression in the ring gland is sufficient to generate a delay

A – Pupariation assays done in control, UAS-*upd3* and UAS-*upd1* in the ring gland of healthy larvae under the control of the *P0206*-gal4 driver. Results reveal strong delay induction in the case of UAS-*upd3*, recapitulated in a slightly softer manner by the overexpression of *UAS-upd1*.

B – Ring glands dissected from L3 larvae were stained with DAPI (blue) and checked for the expression of two reporter lines: *bantam* and tGPH-GFP reporters. Results show that overexpressing JAK/STAT through UAS-*upd3* or UAS-*hop* causes upregulation of the microRNA *bantam*, but does so in an IIS independent manner, since tGPH-sensor levels remain unaltered in all three cases analyzed.

B' – Histograms plotting mean intensity of tGPH and bantam fluorescence of indicated phenotypes. Results show that overexpressing JAK/STAT strongly affects bantam expression in an IIS independent manner.

To prove this we decided to perform several sets of experiments, the first being the overexpression of both cytokines directly in the ring glands of healthy larvae and analyzing pupariation timing. Overexpression of Upd3 is able to recapitulate a CIN like delay, obtaining 50% of total pupal number between 204-208 hours, whilst overexpressing Upd1 generates a slightly smaller delay although still significant, obtaining 50% of pupas between 108-204 hours (Figure 34 A).

Although promising results, the efficiency in the generation of a developmental delay was not sufficient to correlate JAK/STAT with bantam activity in CIN. As previously shown, bantam upregulation seems to be the crucial player in ecdysone production blockage, therefore, we also decided to monitor tGPH and bantam activity once JAK/STAT was active in the ring gland (Figure 34 B and B'). Images report that JAK/STAT activity, either by overexpressing *hop* (the *Drosophila*'s JAK kinase) or *upd3*, is able to influence bantam levels in an insulin-independent manner, since bantam suffers a clear increase in its expression whilst no alterations on tGPH expression were visible. Overexpression of *hop* in otherwise healthy ring glands using the same *P0206*-gal4 driver was also able to generate a systemic delay (Figure 35 B).

The same delay results were replicated when overexpressing *upd3* and *hop*, independently, with an alternative gal4 driver, *phantom* (Figure 35 A). This way confirming the previously reported results.

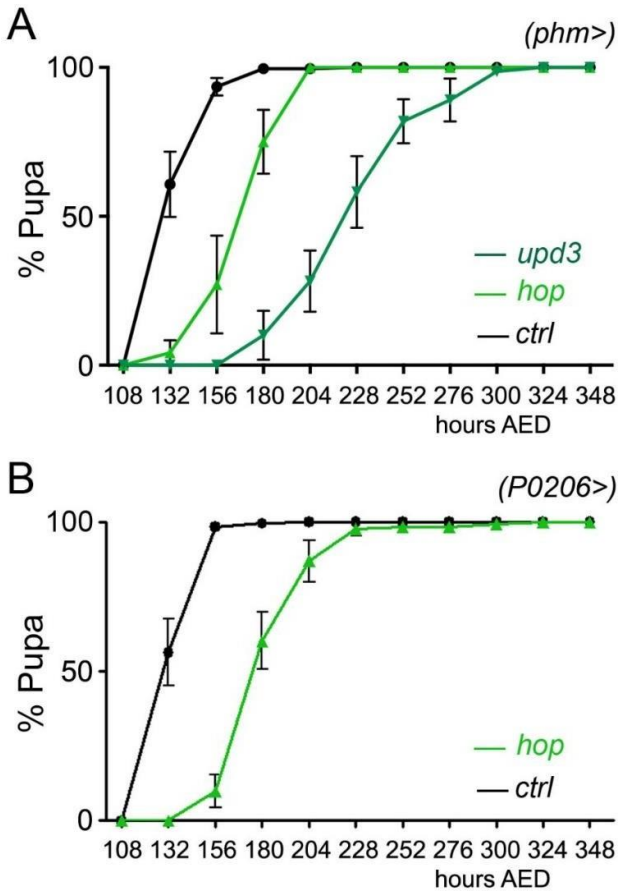

Figure 35. JAK/STAT overexpression in the ring gland is sufficient to generate a delay

A – Pupariation assays done in control, UAS-*upd3* and UAS-*hop* in the ring gland of healthy larvae under the control of the *phm*-gal4 driver. Results reveal strong delay induction in the case of UAS-*upd3*, recapitulated in a softer way by the overexpression of *UAS-hop*.

B – Pupariation assays done in control and UAS-*hop* in the ring gland of healthy larvae under the control of the *P0206*-gal4 driver. Overexpression of *Drosophila's* JAK generates a delay consistent with the one generated under the control of the *phm*-gal4 driver.

Since the CIN delay is thought to be a consequence of the SASP and a major systemic response, we also decided to assess the ability for both cytokines to migrate from the wing disc to the brain area. In order to do so, we overexpressed *upd1* and *upd3* separately in wing discs of healthy larvae and scored for the developmental delay they were able to generate (Figure 36 A).

Surprisingly, only Upd3 was able to reproduce the postponement of pupal transition, mimicking the same pupariation curve as observed for CIN tumors. Upd1, however, was not able to generate a delay *per se*, indicating that this cytokine has trouble performing long-range migrations and that it mostly probably acts as a local signal.

Delay rescue was produced when combining Upd3 with a dominant negative form of the JAK/STAT receptor Dome, a form that prevents its function by trapping the ligands and impeding JAK/STAT activation.

To tackle the question regarding if this was a consequence of JAK/STAT activation in the ring gland, we also performed a reporter line observation for all the elements of the IIS regulatory network (Figure 36 B and B').

Overexpression of Upd3 in the wing disc is able to activate JAK/STAT in the ring gland, as well as allow for the overexpression of bantam microRNA. However, PI3K activity was not affected, consistent with what was previously observed for the direct overexpression of JAK/STAT in the ring gland tissue. Therefore, it seems that the delay observed when overexpressing *upd3* is a consequence of the activation of JAK/STAT in the ring gland that, in turn, regulates bantam overexpression, in an IIS independent manner.

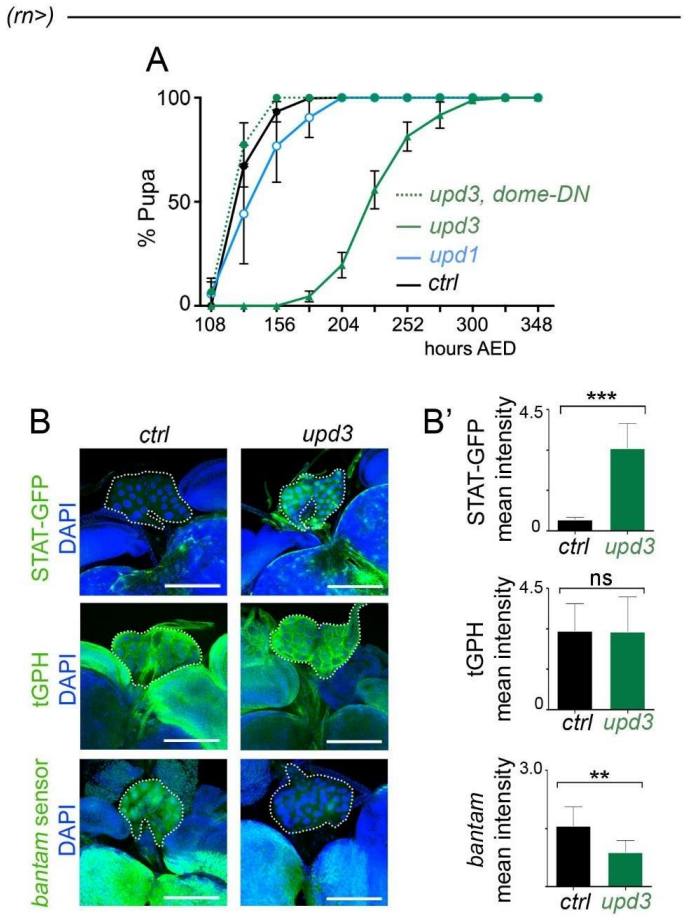

Figure 36. Upd3 overexpression in the wing disc generates a JAK/STAT dependent delay

A – Pupariation assays done in control, UAS-*upd3* and UAS-*upd1* in the wing discs of healthy larvae under the control of the *rn*-gal4 driver. Results reveal strong delay induction in the case of UAS-*upd3*, rescued by using a dominant negative version of the receptor. Upd1 cytokine is not able to recapitulate the CIN delay.

B – Ring glands dissected from L3 larvae were stained with DAPI (blue) and checked for the expression of three reporter lines: *bantam,* STAT-GFP and tGPH-GFP reporters. Results show that overexpressing UAS-*upd3* causes upregulation of the microRNA *bantam* and of the JAK/STAT signaling pathway.

B' – Histograms plotting mean intensity of STAT, tGPH and bantam sensors in indicated phenotypes. Results show that overexpressing Upd3 affects bantam expression in an IIS independent manner.

Considering this results, the only experiment missing seemed to be depleting these cytokines directly from the initial tumor site, to confirm if our hypothesis was applicable for the CIN model.

In order to do so, interference RNA lines for both *upd3* and *upd1* were tested. Pupariation assays show that depleting these cytokines directly from the tumor site did not produce any rescue (Figure 37).

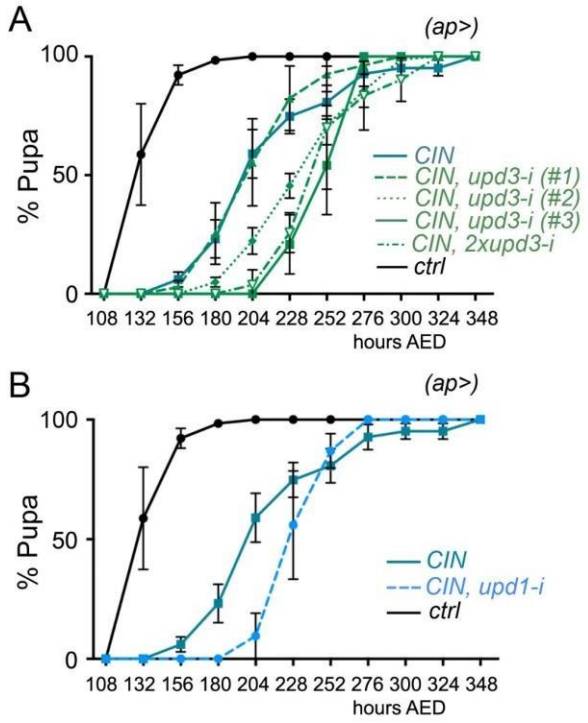

Figure 37. Single depletion of Upd3 and Upd1 cytokines from CIN tumors does not rescue systemic delay

A – Pupariation assays done in control, *upd3* RNAi and CIN under the control of the *ap*-gal4 driver. Results reveal that single depletion and co-depletion of upd3 RNAis is not sufficient to generate a CIN delay rescue.

B – Pupariation assays done in control, *upd1* RNAi and CIN under the control of the *ap*-gal4 driver. Results reveal that single depletion of *upd1* is not sufficient to generate a CIN delay rescue.

However, a rescue was possible when co-depleting both *upd3* and *upd1* from the initial tumor site (Figure 38).

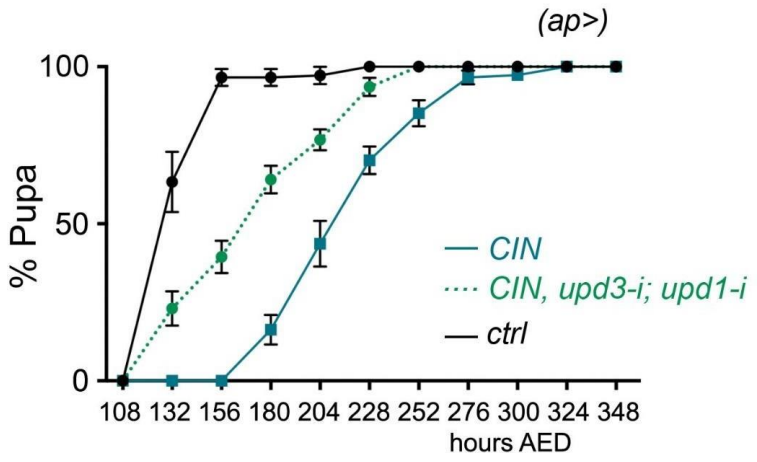

Figure 38. Co-depletion of Upd3 and Upd1 from CIN tumors rescues CIN systemic delay

Pupariation assays done in indicated phenotypes under the control of the *ap*-gal4 driver. Results reveal that co-depletion of upd3 and upd1 RNAis efficiently generates a CIN delay rescue.

Indicating two possible behaviors: either the CIN delay is a consequence of the capacity of the ligands to travel to the brain site or the CIN delay is a direct consequence of JAK/STAT activation directly in the tumoral tissue, an activation that releases several other signals into the hemolymph, some of which are going to act on the regulatory branches of ecdysone biosynthesis (the upd ligands, dilp8 and NLaz, for instance). Such behaviors are not exclusive, and so it is possible that both play a role in generating the CIN systemic delay. However, we believed that it was possible to assess each behavior contribution by: on the one hand, depleting JAK/STAT

activation in the wing disc of CIN larvae (playing with two independent interference RNAs for both dome and hop); and on the other hand, by impeding ligand secretion and traveling (by using two independent dome dominant negative lines (Dome$^{\Delta CYT}$, truncated forms of Dome lacking the intracellular domain and known to act as ligand traps)).

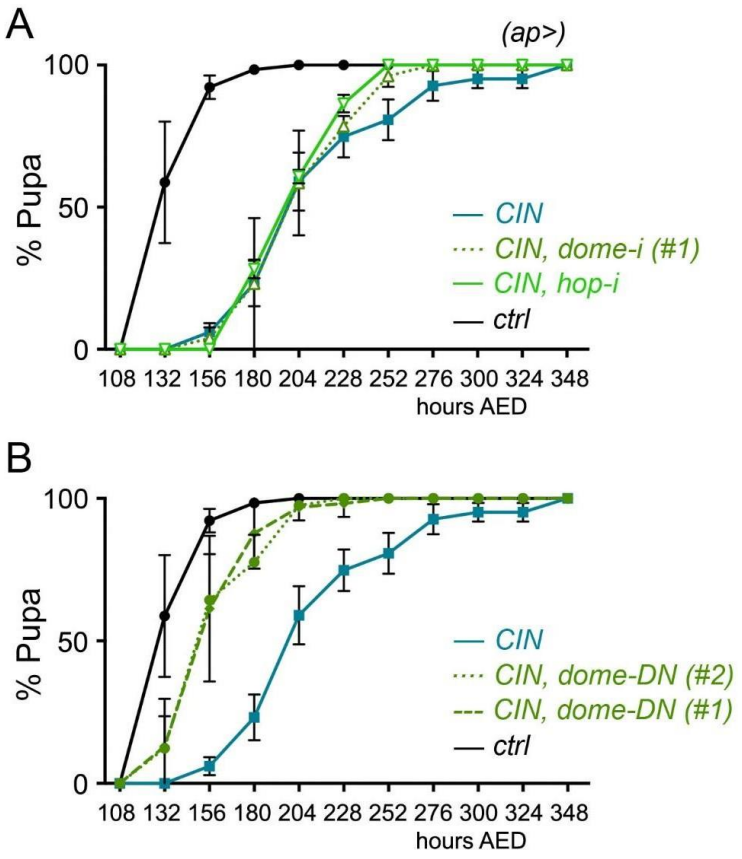

Figure 39. Trapping cytokines is efficient in rescuing CIN delay

A – Pupariation assays done in control, *dome* RNAi, *hop* RNAi and CIN under the control of the *ap*-gal4 driver. Results reveal that single depletion of these crucial JAK/STAT players is insufficient to rescue the CIN delay.

B – Pupariation assays done in control, CIN and CIN plus two independent dome-DN lines under the control of the *ap*-gal4 driver. Results reveal that both dome-DN lines are highly efficient in rescuing CIN developmental timing altercations.

Results

Pupariation assays show that simple reduction of JAK/STAT activity in CIN tissues is not sufficient to produce a rescue, as depletion of JAK/STAT with RNAi lines against dome or hop did not have any effect on the observed developmental delay (Figure 39 A).

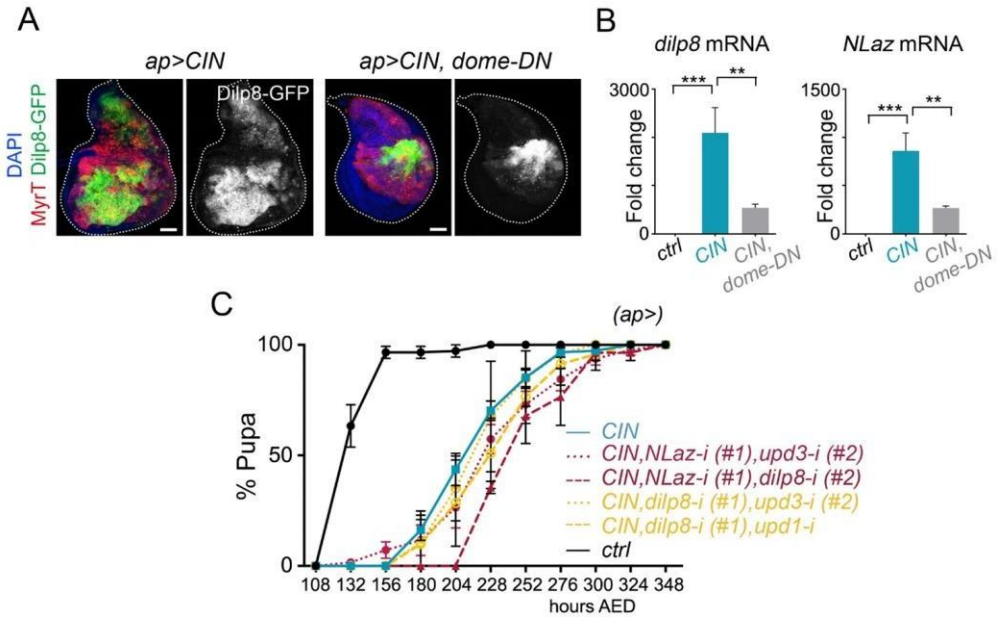

Figure 40. Co-depletion of several systemic players is not sufficient to rescue CIN delay

A – Wing disc primordia dissected from L3 larvae were stained with DAPI (blue) and checked for the expression of Dilp8-GFP reporter (green). Results show that Dilp8 expression is rescued when combining CIN with dome-DN line.

B – Histogram plotting the qRT-PCR fold change increases of control, CIN and CIN with dome-DN. *dilp8* and *NLaz* are found to be highly expressed in CIN tumors, levels that decrease once JAK/STAT signaling is blocked.

C – Pupariation assays done in control, CIN and CIN plus several combinations of indicated RNA-is under the control of the *ap*-gal4 driver. Results reveal the combined depletion of the NLaz and Dilp8 players with the active cytokines are unable to produce a partial rescue of CIN developmental timing.

The return to normal developmental timing is only achieved when Dome-DN lines are used, a result that seems to be mainly related to the capacity of these constructions to trap the Upd ligands (Figure 39 B).

Nonetheless, one might still argue that Dome dominant negative lines would be stronger in their ability to block JAK/STAT activation in the wing disc when compared with the depletion via interference RNAs. To further elucidate on this matter we decided to firstly observe the capacity of Dome-DN lines in reducing the expression of the already described ecdysone regulatory network regulators: Dilp8 and NLaz (Figure 40 A and B); and secondly combine these molecules with the analyzed upd cytokines, in an attempt to obtain at least a partial rescue of the observed developmental alterations (Figure 40 C).

Analysis of both qRT-PCR assays and dilp8-GFP sensor revealed that the usage of Dome-DN indeed significantly reduces both NLaz and Dilp8 levels in CIN tumors (Figure 40 A and B). Nonetheless, the combined depletion of these players with the active cytokines are unable to produce a partial rescue of developmental timing. Such observations, again push us in the direction that CIN delay is most likely due to the activity, both local and systemic, of upd1 and upd3 cytokines *per se*, even though other players might act as minor contributors for ecdysone repression, as previously shown.

Since Dome-DN lines were so efficient in rescuing the developmental timing, we also decide to use them in order to confirm

restoration of tGPH, bantam and JAK/STAT sensor levels to control values, this way corroborating previous pupariation results.

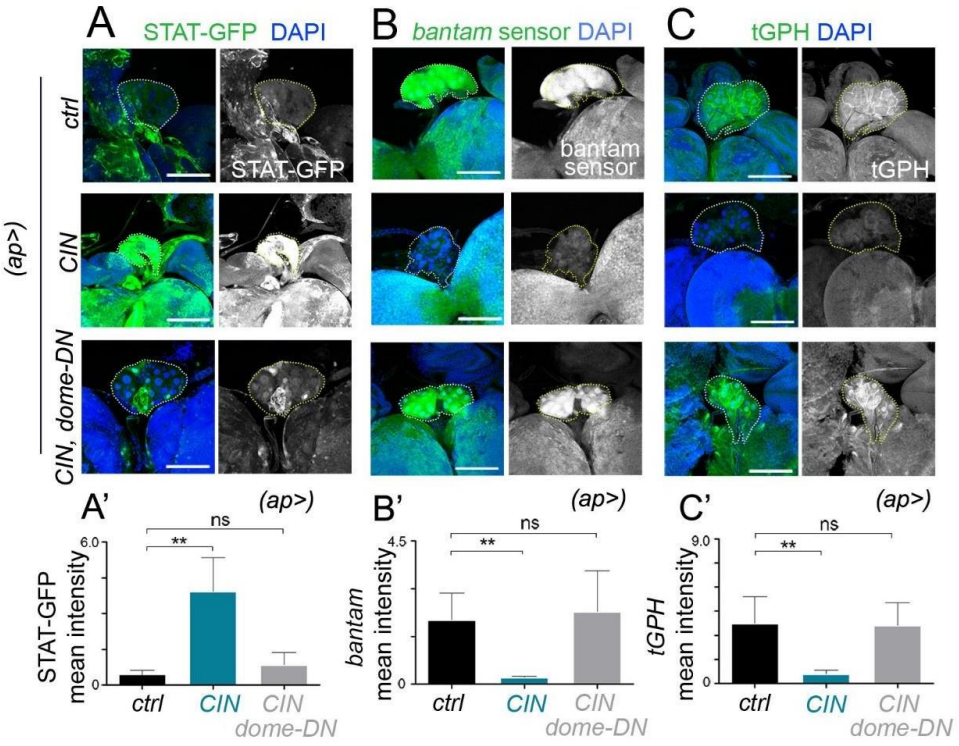

Figure 41. Dome-DN is efficient in rescuing all sensor levels to control values

A-C – Ring glands dissected from L3 larvae were stained with DAPI (blue) and checked for the expression of three reporter lines: *bantam,* STAT-GFP and tGPH-GFP reporters. Results show that *CIN, dome-DN* is able to rescue all reporter sensor levels to control values.

As expected both bantam and JAK/STAT sensor levels are fully restored to control levels (Figure 41 A and B), followed by IIS signaling activity values (Figure 41 C), that are also restored, most likely due to the fact that JAK/STAT seems to act upstream of NLaz production. All sensor fluorescence levels were also quantified to

show that activity reduction, in the case of JAK/STAT and bantam, and increase, in the case of tGPH sensor, were found to be compliant with control values showing no statistically significant difference between one another (Figure 41 A', B' and C').

Taken together these results show that upd1 and upd3 cytokines are deeply involved in the generation of the CIN delay by inducing JAK/STAT activity in the ring gland that, in turn, influences bantam activity in order to block ecdysone production. The involvement of the upd cytokines seem to be both local and systemic, since Upd1 is not able to travel as efficiently as Upd3 but the sole depletion of Upd3 is not sufficient to produce a delay rescue. However, preventing these ligands to travel, either to their local or systemic targets, is what successfully promotes metamorphosis transition. Such observation directs us to think that activity of cytokines in their off-targets has a deep impact in the systemic effects of CIN larvae, although it is undeniable that the primary source for such impacts is the wing disc primordium were the tumor is induced.

3. Characterization of the sources of upd3 cytokine

3.1 JAK/STAT is active in the tumor, gut, muscle and ring gland of CIN larvae

So far we were successful in showing that CIN larvae alterations in developmental timing are a consequence of JAK/STAT activation in the prothoracic gland of tumor-induced animals. Such activation strongly influences bantam activity that acts in order to repress ecdysone biosynthesis, this way preventing larva to pupa transition.

JAK/STAT activation in the CIN background seems to be mainly due to the ability of Upds to travel to their local and systemic targets. This observation is supported by the results obtained with Dome-DN lines, which are fully able to restore sensor and pupariation values to control ones, whilst simple repression of JAK/STAT activity in the initial tumor site is not.

Such results can be explained by two possible scenarios. In the first scenario, Upd1, together with Upd3, might act locally in the wing disc to induce the expression of other long-range systemic signals to regulate, together with Upd3, ecdysone production. Consistent with this notion, overexpression of Dome-DN in CIN tissues reduced the expression levels of Dilp8 and NLaz (Figure 40 A and B), and rescued the impact on the activity levels of the IIS signaling pathway at the PG (Figure 41 C). However, co-depletion of two of the three systemic signals from the tumor site did not rescue, even partially, the developmental delay (Figure 40 C).

The second scenario is deeply related with the ability of Dome-DN to trap the Upd ligands impeding them to reach other tissues. Presumably, if these cytokines are able to migrate to other targets, it raises the possibility that these other tissues might act as contributors to the final cytokine pool, influencing, in this manner, the delay in metamorphosis transition.

To assess this second hypothesis we decided to perform several tissue dissections of control, CIN and CIN co-expressing Dome-DN under the control of the *apterous*-gal4 driver, and checked the activation of the STAT-GFP reporter under these conditions (Figure 42).

Results show that aside from the known sources of JAK/STAT activation, such as the wing disc, the ring gland and adjacent neuronal tissue, this signaling pathway was also highly active in the muscle and the gut of CIN induced tumoral larvae. Despite this peripheral activation of JAK/STAT in the referred tissues, tissues such as the fat body and the trachea did not present any activation of this signaling pathway. This fact was surprising when considering the fat body, since this tissue has been extensively connected with the function and activation of cytokines.

Full rescue of JAK/STAT activation was obtained when co-expressing Dome-DN with CIN for all fluorescent positive tissues. Such results seem to further indicate that Dome-DN is capable of exercising the trapping of the *unpaired* cytokines impeding their action in other peripheral tissues. This is especially clear when observing the reduced JAK/STAT activation in the gut and muscle, so far unknown sources of JAK/STAT activation in the CIN larvae.

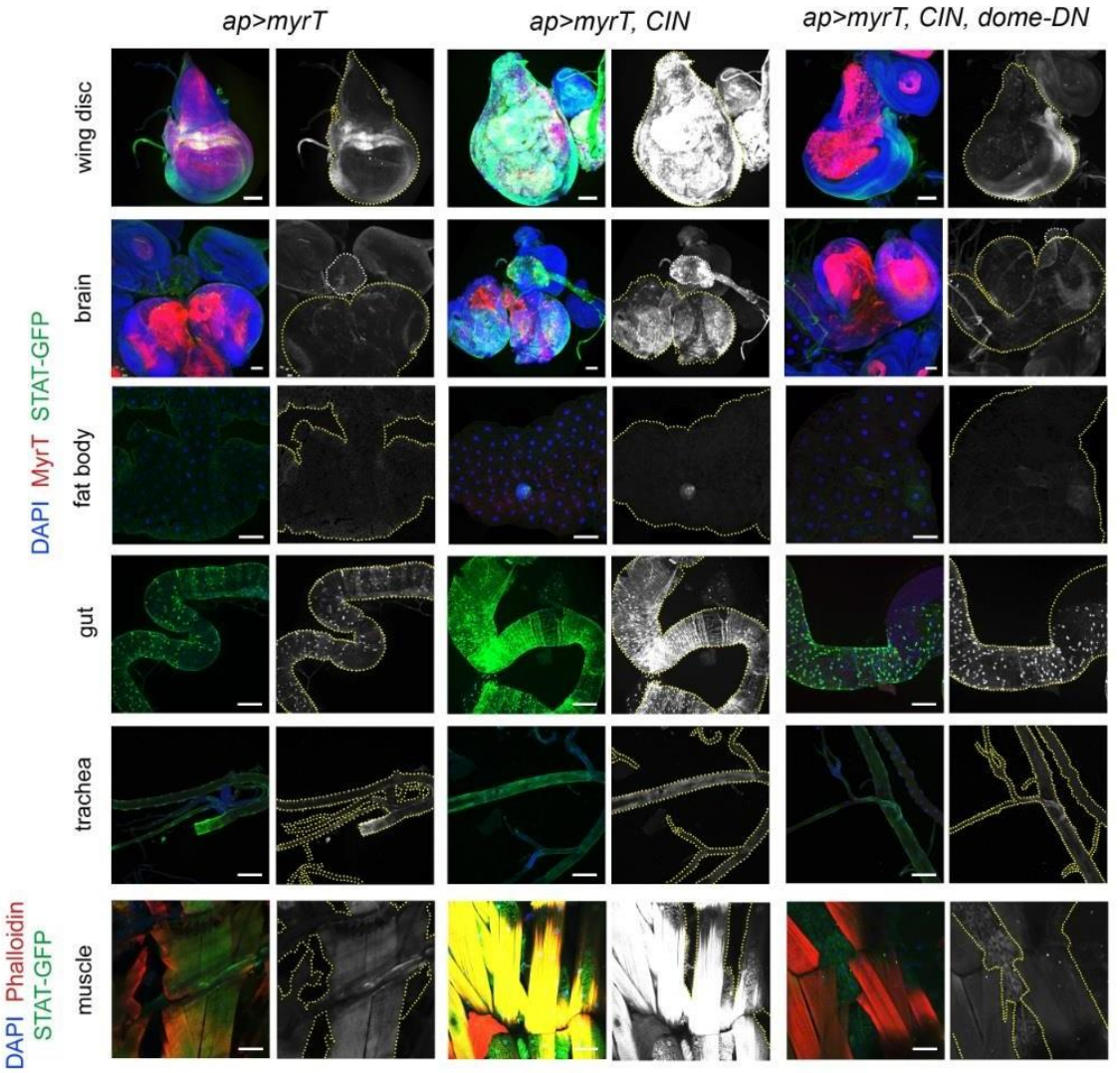

Figure 42. CIN presents JAK/STAT activation in the wing disc, brain, gut and skeletal muscle

Control, CIN and CIN co-expressed with Dome-DN larvae were dissected at L3 stage and stained for DAPI (blue) and phalloidin (red in the muscle tissue). MyrT (red in the brain and wing disc) marks the area of expression of the *apterous*-gal4 driver and STAT-GFP (green) marks the levels of expression of JAK/STAT signaling pathway. Results show activation of JAK/STAT in the tumoral region, neuronal tissues and ring gland, gut and muscle tissues in CIN larvae in comparison with control ones. Restauration to control levels of JAK/STAT activation is obtained when co-expressing Dome-DN with CIN.

Since it seemed clear that other tissues might have a contribution to the pool of cytokines, we decided to dig further and understand the impact of Dome-DN directly in *upd1* and *upd3* expression levels, starting with the wing disc primordium, the known primary source of cytokine production and then moving on to other possible contributors.

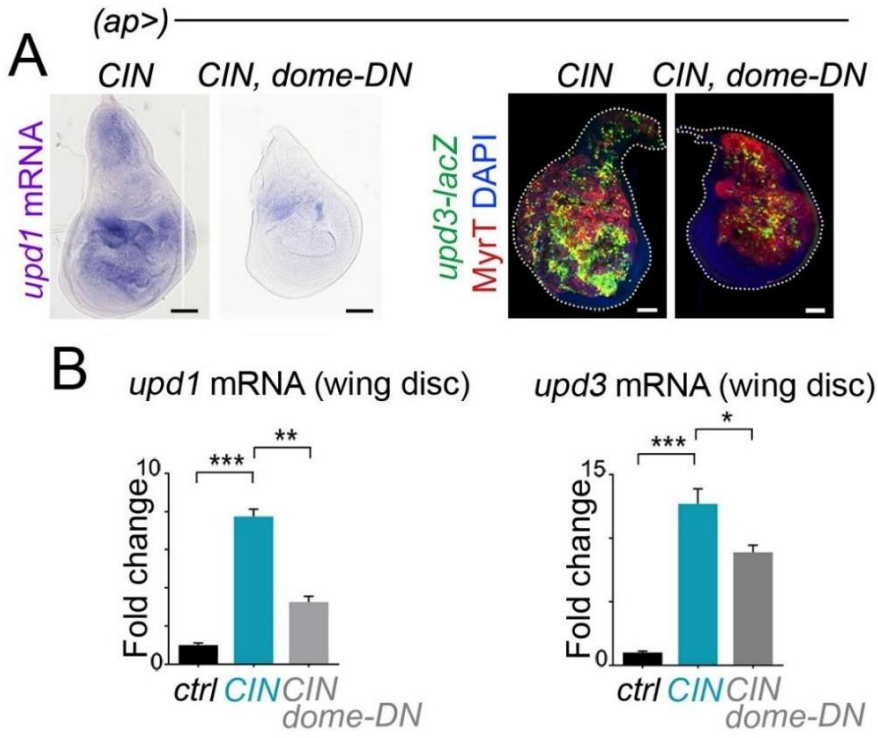

Figure 43. CIN tissues show high levels of both *upd3* and *upd1* expression

A – Wing imaginal discs expressing the indicated transgenes (ap-gal4) and stained for upd1 mRNA (purple), upd3-lacZ (green) and MyrT (red). Both cytokines are shown to be highly expressed in CIN tumors, expression that is rescued by the usage of *dome*-DN.

B – Histogram plotting the qRT-PCR fold change increases of control, CIN and CIN plus Dome-DN. *upd1* and *upd3* cytokines are found to be highly expressed in CIN tumor wing primordia, levels that decrease once JAK/STAT signaling is blocked with *dome*-DN.

Results

Both *in situ* hybridization and upd3-lacZ reporter analysis were performed in CIN and CIN co-expressing Dome-DN. Images show that both cytokine levels are reduced in the presence of Dome-DN (Figure 43 A). An observation that is corroborated by the qRT-PCR assays showing a significant downregulation of both upd1 and upd3 expression levels when the dominant negative line was expressed in the wing disc (Figure 43 B).

Aside from the wing disc, gut and muscle also revealed themselves as positive targets for STAT-GFP expression. Therefore, we decided to further analyze these targets for both ligand expression and capacity of generating STAT-GFP activation directly in the prothoracic gland. Results obtained for these possible contributors are demonstrated in the next sub-chapter.

3.2 JAK/STAT activation in the gut does not seem to contribute for the cytokine pool observed in CIN larvae

Drosophila's gut is divided in three main regions: the foregut, the midgut and the hindgut, separated by their differences in acidification. Cytokine signaling is responsible for coordinating conserved epithelial regeneration and immune responses in the gut. In *Drosophila*, the midgut is strongly influenced by the Upd3 cytokine, which is induced in enterocytes (EC) and enteroblasts (EB) upon infection, triggering intestinal stem cells to divide and repair the tissue.

The gut, or any digestive tract, faces several unique challenges, due to its exposure to the external environment. The transit of nutrients

throughout the gastrointestinal tract is accompanied by the appearance of several biotic and abiotic stress. The digestive tissue is constantly exposed to high density of microbes, which include benign microbiota that help the gut perform its functions, but also pathogens that need to be eliminated by the system (Buchon, Broderick, and Lemaitre 2013).

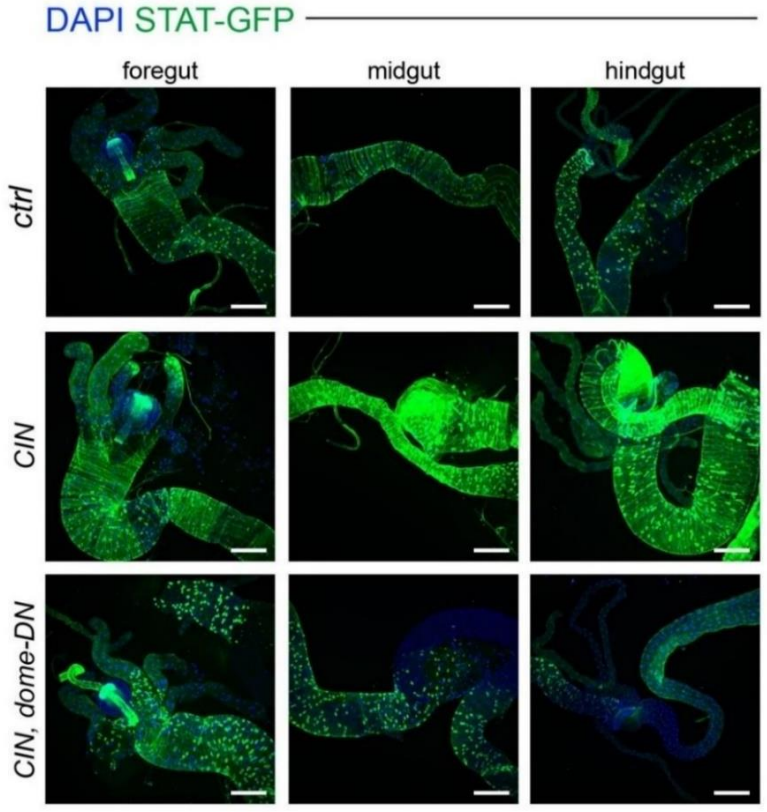

Figure 44. Gut cells are able to activate JAK/STAT in a CIN context

Control, CIN and CIN together with a *Dome-DN* line were dissected at L3 stage (around 130 hours' time point) and stained with DAPI (blue) under the control of *ap-gal4* driver. STAT-GFP (green) reporter marks the area of JAK/STAT activation. Results show that CIN larvae strongly induce the activation of JAK/STAT in their digestive tract, especially in the midgut and hindgut regions. Blocking cytokines secretion by using a *Dome-DN* construct rescues the activation of JAK/STAT in these regions.

Results

Cytokines, which are central to gut homeostasis, are produced by epithelial and immune cells to properly orchestrate immune and repair processes. Upd3 is a major regulator of intestinal epithelial renewal and its expression is induced by several enteric stresses, not only bacterial infections. Gut epithelial cell loss, induced by feeding of bleomycin, for instance, or dextran sulfate sodium, induces *upd3* transcription in the gut to promote intestinal epithelial turnover (Amcheslavsky, Jiang, and Ip 2009).

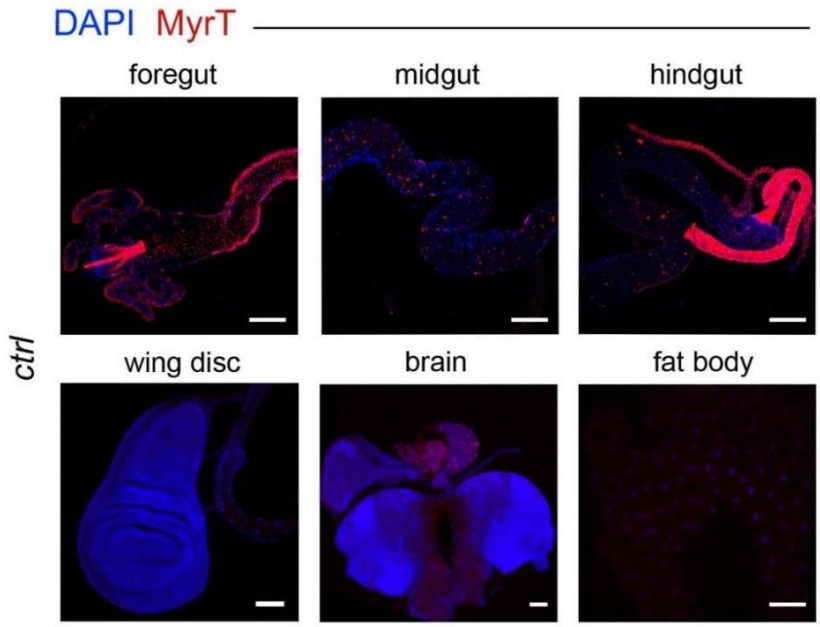

Figure 45. *upd3*-gal4 driver is expressed in the gut and lymph gland of L3 larvae

upd3-gal4 larvae were dissected at L3 stage (around 130 hours' time point) and stained with DAPI (blue). MyrT (red) reporter marks the area under the Gal4 influence. Results show that there is a strong expression all throughout the digestive tract, especially in the hindgut region. Moreover, the lymph gland tissue presented close to the brain region shows mild activation of the reporter. None of the other dissected tissues considered show activation of the Gal4.

In order to have a clear idea of the importance of the upd3 and upd1 cytokines in the gut, and also considering that the gut is a highly complex tissue, we decided first and foremost to characterize JAK/STAT activation considering the three main regions previously mentioned.

Analysis of the STAT-GFP reporter revealed that there is a strong activation of JAK/STAT all throughout the digestive tract of CIN larvae when compared to control ones. However, this activation seems to be stronger in the mid and hindgut regions (Figure 44), than in the foregut, the closest gut region to the brain and ring gland. Blocking cytokine release from the initial tumor site by using *dome-DN*, prevents the activation of JAK/STAT signaling pathway in the gut, especially in the aforementioned regions.

Activating JAK/STAT *per se* is not sufficient to say that the gut is a contributor to the cytokine pool, therefore, and to further understand if the cytokine release from the gut could generate a developmental delay we decided to use a GAL4 driver that would allow expression all throughout the digestive tract. In order to do so we used the *upd3*-gal4 driver. This particular gal4 driver is mainly expressed in the gut of healthy larvae, although it also presents some mild expression in the lymph gland tissue where hemocytes are formed (Figure 45). The main reason for choosing to use this GAL4 and not *escargot*-gal4, the preferential driver for gut studies, was the fact that the latest has a described off-target expression for the wing imaginal discs (Fuse, Hirose, and Hayashi 1996), a tissue that we already

described as being able to generate a delay upon overexpression of JAK/STAT activation.

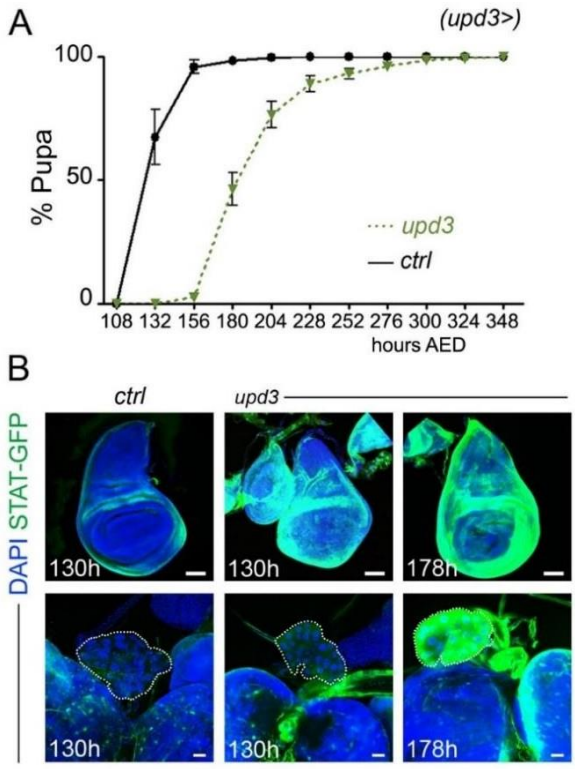

Figure 46. Overexpression of Upd3 in the gut generates a developmental delay

A – Pupariation assays considering control as overexpression of Upd3 cytokine were performed considering the *upd3*-gal4 driver. Results show that overexpressing Upd3 cytokine with *upd3*-gal4 delays developmental timing in approximately three days, since 50% of pupas is attained between 198 and 204 hours, instead of between 108 and 132 hours observed in control larvae.

B – Ring glands and wing imaginal discs of *upd3*-gal4 larvae were dissected at L3 stage and stained with DAPI (blue). STAT-GFP (green) reporter marks the area of JAK/STAT activation. At 130hours there is activation in the wing disc but not in the ring glands of larvae overexpressing *upd3*. At 178 hours' time point it is possible to observe a clear activation of the pathway in both tissues dissected, revealing that the overexpression of the Upd3 cytokine in the gut is able to cause activation of JAK/STAT in the ring gland, as time goes further.

Results considering the *upd3*-gal4 driver for the gut revealed an increase in systemic developmental timing of approximately 72 hours. This result was obtained by comparison of the time point where 50% of pupas were already formed, which revealed itself to be between 108 and 132 hours for control and between 180 and 224 hours for the overexpressing phenotype (Figure 46 A). As previously done for other gal4 drivers, we wanted to assess if this delay in metamorphosis entering was a consequence of JAK/STAT activation. In order to do so, we analyzed the levels of the STAT-GFP reporter line in the wing discs and ring glands of *UAS-upd3* overexpressing larvae.

Much to our surprise, we realized that activation of JAK/STAT signaling pathway in the ring gland is not possible to observe when considering 130 hours of developmental time (Figure 46 B), the same time point previously analyzed for ring gland tissues in a CIN background. However, when considering dissections at a later time, 178 hours, it was possible to observe an activation of the STAT-GFP sensor. This response shows that the overexpression of the Upd3 is able to generate JAK/STAT activation in the ring glands of L3 larvae, although not at the expected time point.

Considering the wing disc, gut overexpression of Upd3 was able to cause JAK/STAT activation in the wing primordium of healthy larvae from the very first time point measured (130 hours), an activation that increases with the passage of time.

Taking into consideration that the *upd3*-gal4 driver has also shown some mild expression in the lymph gland tissues, one might consider

that the effects observed can also be due to a contribution from the immune-related structures. A hypothesis that we will explore later on.

Seeing that overexpression results are able to both generate JAK/STAT activation in peripheral tissues and, consequently, activate a delay in larva to pupa transition, we needed to assess upd1 and upd3 cytokine expression in the guts of CIN tumor-induced animals.

We performed this experiment by qRT-PCR analysis, evaluating differences in fold change increase between control, CIN and CIN co-expressing Dome-DN (Figure 47).

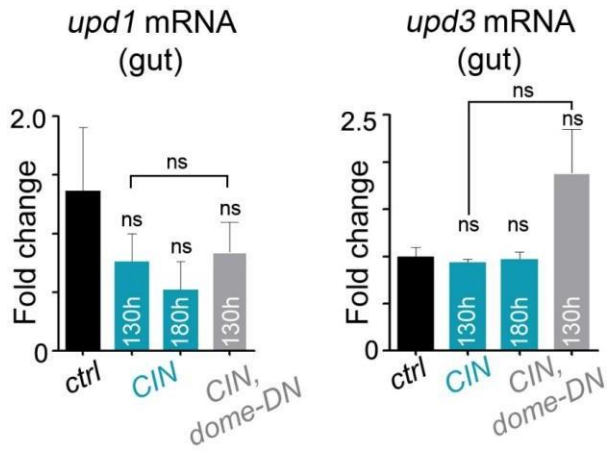

Figure 47. CIN larvae gut tissues do not present any relevant change in cytokine production

Histogram plotting the qRT-PCR fold change increases of control, CIN and CIN with Dome-DN. In the CIN tumoral context, larvae do not seem to present significant upregulation of the targeted cytokines in their gut tissues. Depletion of JAK/STAT signaling pathway in the wing disc whilst trapping the upd ligands also does not produce any significant change in behavior.

Unexpectedly, CIN larvae did not present any difference in cytokine expression when compared with control, not even when a later dissection point was considered. Moreover, expressing Dome-DN in a CIN background was also insufficient to produce a modification in behavior, results showing no significant difference in expression between CIN larvae alone and CIN combined with Dome-DN.

Therefore, despite the overexpression results obtained for healthy larvae it seems that, in the CIN context, the gut is not a major source of cytokine overexpression, even though it activates JAK/STAT signaling pathway in a differential manner when compared to controls. Such activation might, however, contribute to other systemic roles and secretion of other effector molecules that might independently influence the delay. Nonetheless, the gut itself does not seem to contribute to the secretion and release of the upd1 and upd3 cytokines to the circulating hemolymph.

3.3 Hemocytes are not major contributors for the ring gland JAK/STAT signaling activation

Since the *upd3*-gal4 driver presents a slight expression in the lymph gland, and the gut has revealed itself not to be a major contributor for cytokine release, we hypothesized that maybe the delay and consequent activation of the STAT-GFP reporter might be due to an immune system contribution.

Results

Hemocytes are well-known as a source of cytokines, namely, upd3. These effector cells are very important in immunological processes (Hoffmann and Reichhart 2002; Lavine and Strand 2002). They can circulate freely through the body cavities or be found in a sessile state associated with several tissues and organs. They are recruited in response to infection, where they carry out engulfment of the foreign bodies, melanize and secrete antimicrobial peptides (Braun, Hoffmann, and Meister 1998; Rämet et al. 2002; Sorrentino, Carton, and Govind 2002). Furthermore, hemocytes are also responsible for the phagocytosis of apoptotic cells and thus very important in organ remodeling during metamorphosis (Franc et al. 1996). In addition to this, hemocytes are known to express *upd3*, for instance, during a septic injury where hemocytes trigger the expression of this cytokine to mediate the activation of *totA* in the fat body (Agaisse et al. 2003). *totA* is a humoral factor that has a role in resisting the lethal effects of bacterial challenge and stress, that is regulated by JAK/STAT and NF-KB-like Relish pathway in the fat body.

Taking this into account we decided to describe what was happening to the hemocyte population in CIN larvae, to see if it was feasible to hypothesize that hemocytes could be a major contributor for the *upd3* secretion pool and consequent JAK/STAT activation in the PG area.

Staining with NimC1 marker, a phagocytosis receptor observed in the surface of hemocytes (Kurucz et al. 2007), revealed that there is an increase in hemocyte recruitment to the tumor site as time passes by and specially when approaching 202h, the time point closer to 50% of total pupas formed (Figure 48). Thus meaning that, the

continuous proliferation of the wing disc epithelium, combined with the powerful secretion of cytokines released from the delaminated population contributes to an inflammation like state in the CIN tissues.

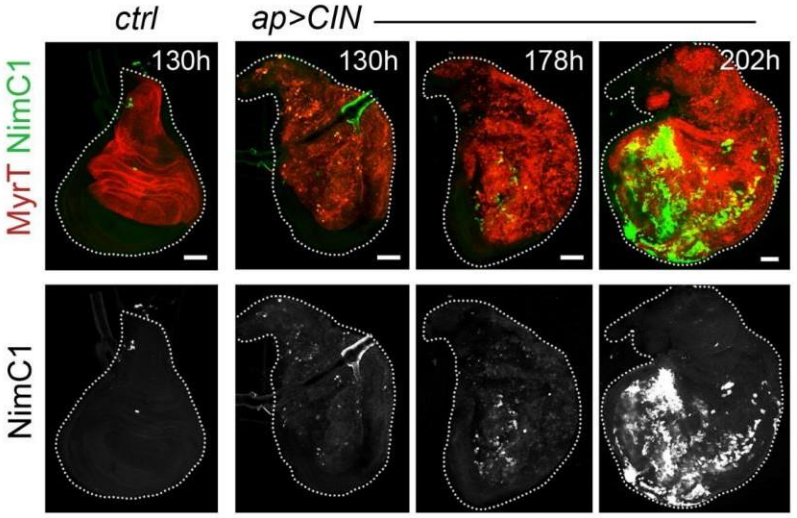

Figure 48. CIN presents increased hemocyte recruitment during the delay

Control and CIN larvae's wing disc primordium were dissected at L3 stage and stained for NimC1 (green). MyrT (red) marks the area of expression of the *apterous*-gal4 driver were the tumor is induced. Results show increasing hemocyte recruitment along the three time points considered, especially visible at 226 hours.

After confirming the increase in hemocyte recruitment we decided to analyze if this phenomenon was affected by the blockage of JNK and JAK/STAT signaling at the initial tumor site, since we already know that these are the main pathways behind CIN delay.

Indeed, blocking JNK, by using a *bsk-DN*, or blocking JAK/STAT, by using a *dome-DN*, produces a rescue in the amount of hemocytes mobilized to the initial tumor site (Figure 49). However, this rescue

is mild taking into account that comparing the same time point for tumor development, control and the rescue experiments, means comparing the time point of CIN development with the least number of immune cells present. Nonetheless, since a mild rescue is produced, hemocytes could still be contributing to the *upd3* pool and delay generation. Therefore, to fully understand the involvement of these immune cells in the generation of a JAK/STAT dependent delay we decided to perform two pupariation assays with two different hemocyte drivers, *hemese*-gal4 and *croquemort*-gal4.

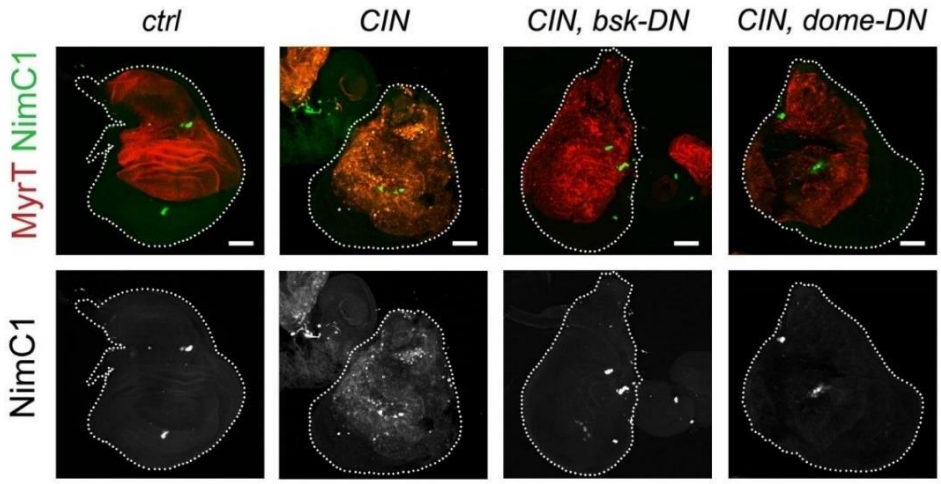

Figure 49. CIN presents increased hemocyte recruitment during the delay

Control, CIN and CIN co-expressing JNK blockage and JAK/STAT blockage via bsk-DN and dome-DN, respectively, larvae's wing disc primordium were dissected at L3 stage and stained for NimC1 (green). MyrT (red) marks the area of expression of the *apterous*-gal4 driver were the tumor is induced. Results show a mild rescue in hemocyte recruitment once the two major stress-response pathways are blocked. However, since the time point of dissection needs to take into account the absence of delay for the control and the rescue phenotypes, the rescue produced is smaller because CIN larvae only reach major immune cell recruitment later in time.

Overexpression of *upd3* in the hemocytes of developing larvae revealed an increase in developmental timing, clearly delaying entering into metamorphosis (Figure 50 A and B). 50% of total pupas were obtained between 204- and 228-hours for both considered drivers. Whilst definitely a good sign, this developmental timing alteration might be due to the inflammation processes themselves and not relatable with what is happening in our CIN tumor situation.

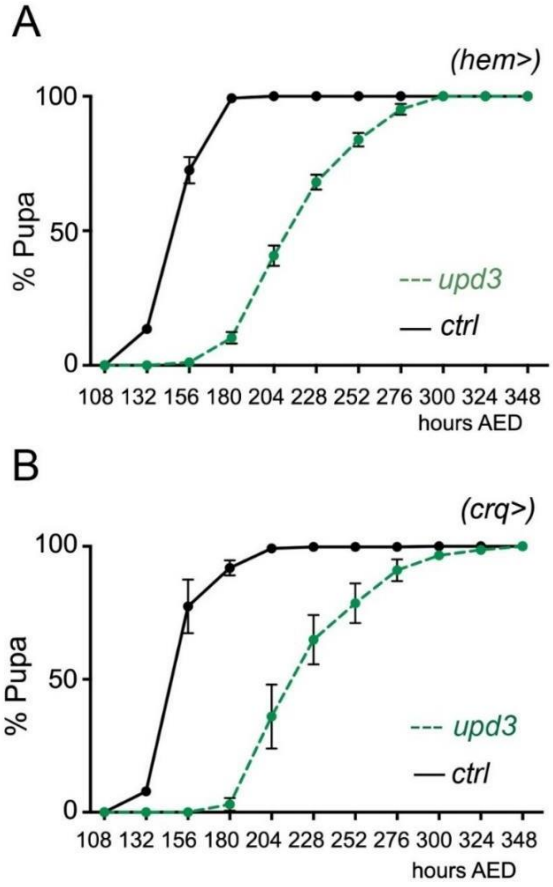

Figure 50. Upd3 direct overexpression in the hemocytes generates a CIN like delay

A-B – Pupariation assays done in control and *upd3* of healthy larvae were performed under the control of the *hem*-gal4 and *crq*-gal4 drivers. Results reveal strong delay induction once *upd3* is overexpressed in both indicated cases.

To assess this, we checked the three main sensors observed so far in previous results: the tGPH-sensor to account for IIS-dependent activation of PI3K; the bantam sensor to account for microRNA repression of ecdysone production and the STAT-GFP sensor, a JAK/STAT activation reporter now a known regulator of bantam expression and ecdysone biosynthesis. However, when these three reporters were observed considering UAS-*upd3* overexpression in the hemocytes, all of them presented the same levels as the control (Figure 51). tGPH-sensor levels were high, revealing that these ring glands do not present any insulin resistance, as well as they do not present upregulated *bantam* levels. Finally, no STAT-GFP was observed, not even when dissecting a later time point to assure maximum hemocyte recruitment conditions.

The sum up of these results led us to believe that, even though hemocyte recruitment increases with tumor growth, these immune cells do not seem to be contributing actively for the activation of JAK/STAT signaling in the ring gland. This does not exclude the fact that the immunological response might be affecting the overall transition between the two metamorphosis phases, but it is true that the *upd3* release from the hemocyte population *per se* is not sufficient to generate an activation of the JAK/STAT signaling pathway in the ring gland. However, hemocytes are known sources of production and release of cytokines, making it impossible to exclude them as contributors to the overall number of circulating interleukins.

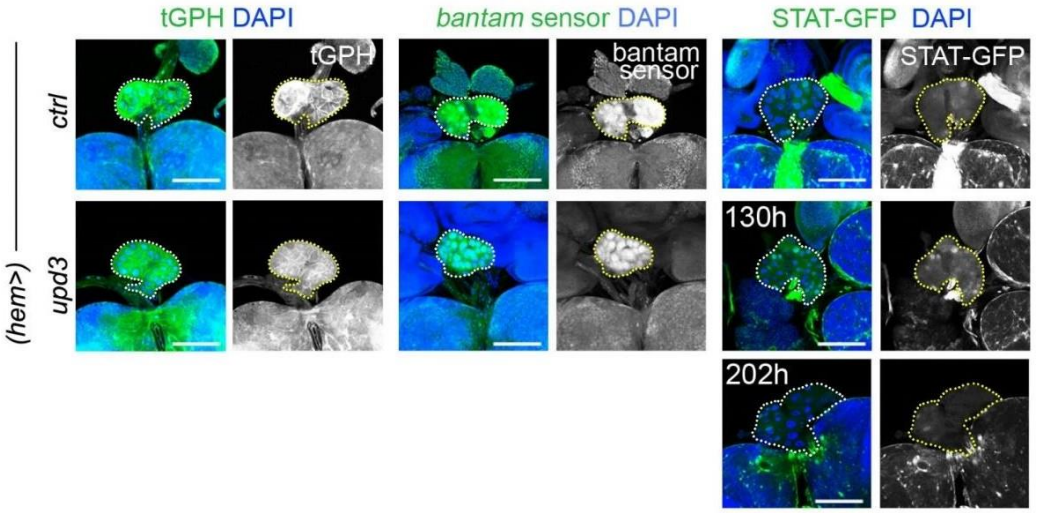

Figure 51. Upd3 overexpression in hemocytes does not activate JAK/STAT in the ring gland tissues

Ring glands dissected from L3 larvae were stained with DAPI (blue) and checked for the expression of three reporter lines: *bantam,* STAT-GFP and tGPH-GFP reporters. Results show that *upd3* overexpression in hemocytes does not show differences in reporter levels when compared with control larvae.

3.4 Myoblasts and the Skeletal Muscle are active contributors for the increase of traveling cytokines

CIN larvae produce a delay that seems to be due mainly to the activation of JAK/STAT signaling in the ring gland. Blocking *upd3* and *upd1* simultaneously in the wing primordia is sufficient to produce a rescue. However, no interference RNA alone was capable of reproducing the same response. Nonetheless, the systemic alteration in larva to pupa transition is not a consequence of the JAK/STAT signaling activation in the initial tumoral area, but a

consequence of the ability for the unpaired ligands to travel. This last behavior leads us to think that there might be more than one source of cytokine release, fed by a positive feedback mechanism that starts in the tumor and spreads to other tissues of the CIN fly. These alternative sources of cytokine production were firstly associated with the tissues that represented differentially STAT-GFP reporter expression when compared with healthy larvae. Such tissues were identified to be the wing disc, the brain and ring gland, the gut and the muscle. The first four alternative sources were already assessed in the previous results, leaving now room to enter into the possible muscle contributions to cytokine production and release.

In the wing disc, aside from the pseudostratified epithelium it is also possible to find adult muscle precursors (AMPs) called the myoblasts. Myogenesis occurs in two phases: the embryonic phase, which makes up the muscles required for the larval stages (Bate et al, 1991); and the post-embryonic phase, which forms the muscles required for adulthood (Fernandes, Bate, and Vijayraghavan 1991; Roy and VijayRaghavan 1998; Sudarsan et al. 2001). The AMPs, lineal derivatives of the mesoderm, are generated in the embryo but proliferate post-embryonically. The AMPs located in the wing imaginal disc contribute to two main steps of muscle formation: the ones expressing Vestigial (Vg) and low levels of the Cut transcription factor generate indirect flight muscles; and the ones expressing high levels of Cut and low levels of Vg give rise to direct flight muscles. Both Vg and Cut act in a mutually repressive manner during muscle formation in order to generate these two distinct muscle types (Vishal et al. 2017). In the wing imaginal disc, during L2 and L3 larval instar stages, the AMPs are present in an undifferentiated state and

undergo a rapid round of proliferation in the notum region, generating approximately 2500 myoblasts within a 120 hours period (Gunage, Reichert, and VijayRaghavan 2014). At the onset of pupa formation most thoracic larval muscle fibers undergo histolysis (degeneration and dissolution of organic tissues resulting from enzymatic activity). However, three dorsal oblique muscles do not undergo this process and, instead, split into six fibers forming the organizer or founder muscles that will serve as dorsal longitudinal muscles templates (Bernard et al. 2003; Roy and VijayRaghavan 1998). The AMPs undergo an additional round of proliferation and myoblast fusion to form six dorsal longitudinal muscles fibers. These muscles increase in volume for the 3 remaining days of pupal development, critical for the formation of indirect flight muscles.

Giving the tight proximity of the larval myoblast population with the wing disc epithelial cells, and, giving that the muscle of CIN larvae were found positive for STAT-GFP reporter signal, we hypothesized that both the myoblast and the skeletal muscle population could be contributors to the secretion of circulating cytokines.

To assess the veracity of this hypothesis, we decided to check the myoblast population first for the activation of *upd3* expression. In order to do so, we used an *upd3*-lacZ enhancer trap construct to report *upd3* expression coupled with Cut staining to identify the myoblast population and differentiate it from the tumoral cells.

Results show that there are some cells positive for both Cut and upd3-lacZ staining (Figure 52 - yellow arrows), and not positive for MyrT, the fluorescent maker that defines the tumoral area. However,

there also seems to be a small population of cells that are positive for upd3-lacZ, but do not present neither MyrT nor Cut staining (Figure 52 - blue arrow).

We hypothesized that these cells might either be hemocytes recruited to the tumoral site or tumoral cells that lost their MyrT identity.

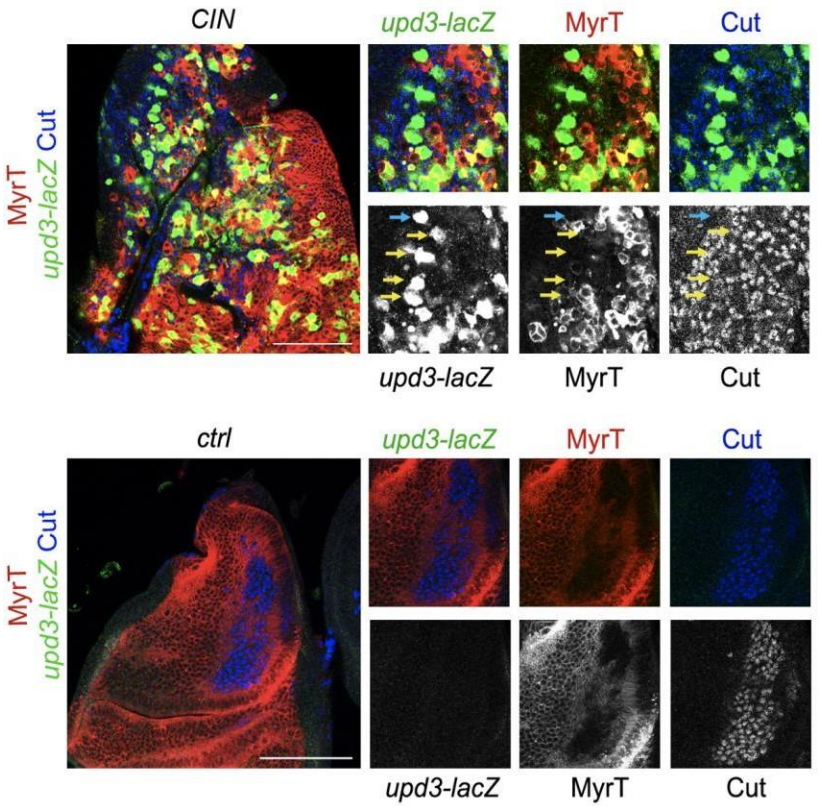

Figure 52. Myoblasts express *upd3* cytokine in the wing disc of CIN larvae

Control and CIN co-expressing *upd3-lacZ* larvae's wing discs were dissected at L3 stage and stained for Cut (blue) and β-gal (green). MyrT (red) marks the area of expression of the *apterous*-gal4 driver were the tumor is induced. Results show that the myoblast population (Cut positive) shows some positive cells for *upd3-lacZ* reporter (green) marked by the yellow arrows. However, some cells appeared not to be positive for either MyrT or Cut, but able to express *upd3* (blue arrows).

Unfortunately, co-staining with NimC1 antibody is not possible in this scenario since the *upd3-lacZ* construct available does not stain these immune cells, making fluorescence overlapping impossible to assess.

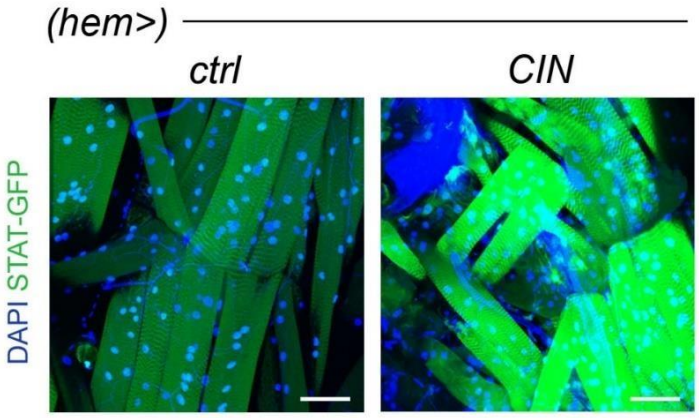

Figure 53. JAK/STAT is strongly activated in the skeletal muscle of CIN larvae

Skeletal muscle fibers were dissected at L3 stage and stained with DAPI (blue). STAT-GFP (green) reporter marks the area of JAK/STAT activation. Images show strong activation of the STAT-GFP reporter for CIN larvae in comparison with control ones.

Even considering that some *upd3-lacZ* positive cells are not a part of the myoblast population, it is clear that cells from this population are able to express upd3. Moreover, skeletal muscle cells of *Drosophila*'s L3 larvae were also shown to be positive for JAK/STAT activation (Figure 53), meaning that there seems to be an involvement of the skeletal muscle and AMPs in the delay response.

To assess this possible contribution, we decided to perform a pupariation assay overexpressing the cytokine UAS-upd3 directly in the myoblast population (Figure 54 A) and in this population together with a skeletal muscle cell driver (Figure 54 B) to see if this would be enough to affect entering into metamorphosis.

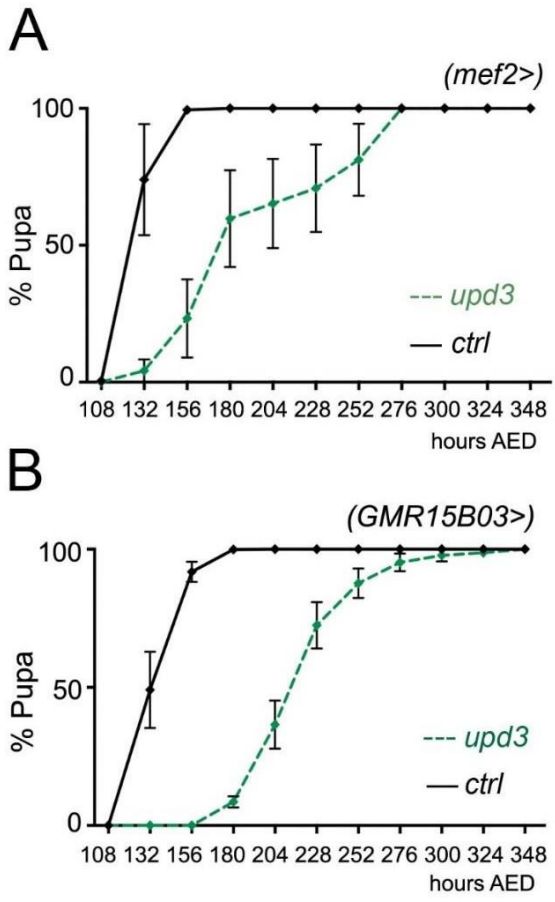

Figure 54. Upd3 expression in muscle cells and their precursors generates developmental delay

A-B – Pupariation assays done in control and *upd3* expressing larvae were performed under the control of the *mef2*-gal4 and *GMR15B03*-gal4 drivers. Results reveal strong delay induction once *upd3* is overexpressed in both indicated cases.

Results show that overexpressing *upd3* in the myoblast population alone, using a *GMR1503*-gal4 driver, is sufficient to generate a developmental timing altercation, delaying the entering into metamorphosis more than 96 hours when compared to a control condition (Figure 54 B). Moreover, the overexpression of this interleukin-like cytokine in the muscle and myoblast population simultaneously, attained by the usage of *mef2*-gal4 driver, also generates a delay in systemic developmental timing (Figure 54 A). In this case, affecting entering in pupariation approximately 72 hours.

Now, giving these results, our aim was to know if the observed delay was a consequence of JAK/STAT activation in the ring gland.

We saw, previously, that CIN delay is a consequence of JAK/STAT activation in the ring gland, which results in the upregulation of bantam, a microRNA described as a repressor of ecdysone biosynthesis. Therefore, in order to know if the muscle and their precursors are contributing to the pool of cytokines responsible for the activation of JAK/STAT in the ring gland, we need to make sure that generating an overexpression of *UAS-upd3* in the referred population of cells is able to cause the activation of the STAT-GFP reporter in the ring gland.

Indeed, images taken using overexpression of *UAS-upd3* with the *mef2*-gal4 driver for the skeletal muscle and myoblast population (Figure 55) show that, aside from the already expected activation of JAK/STAT in the wing disc and muscle fibers, the ring gland of these animals is also positive for the STAT-GFP reporter. This is a strong indication that the *upd3* cytokine is being released from these two populations of cells and is being capable of migrating to the brain

and activate JAK/STAT signaling in the center for metamorphosis control. This activation is clearly visible at 130 hours, the same time point when STAT-GFP is also present in CIN ring glands.

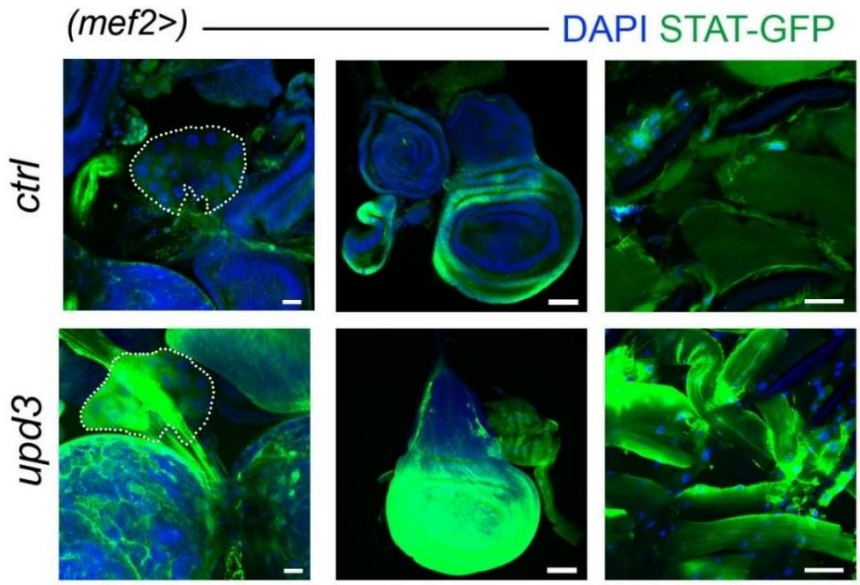

Figure 55. Muscle cells and their myoblast precursors are able to activate JAK/STAT in the ring gland of healthy larvae

Control and *UAS-upd3* overexpressing larvae using a muscle and myoblast population driver (*mef2*-gal4) were dissected at L3 stage (around 130 hours' time point) and stained with DAPI (blue). STAT-GFP (green) reporter marks the area of JAK/STAT activation. Results show that cytokine overexpressing larvae strongly induce the activation of JAK/STAT in skeletal muscle fibers, wing disc and ring glands of healthy developing larvae.

It is also worthy of note that JAK/STAT induction in the wing disc of developing larvae seem to be mostly present in the pouch area and not in the myoblast population area, closer to the notum. This might indicate that myoblasts activate JNK signaling in order to secrete

upds that will then reinforce the activation of JAK/STAT in other tissues or cell populations, such as the developing epithelium.

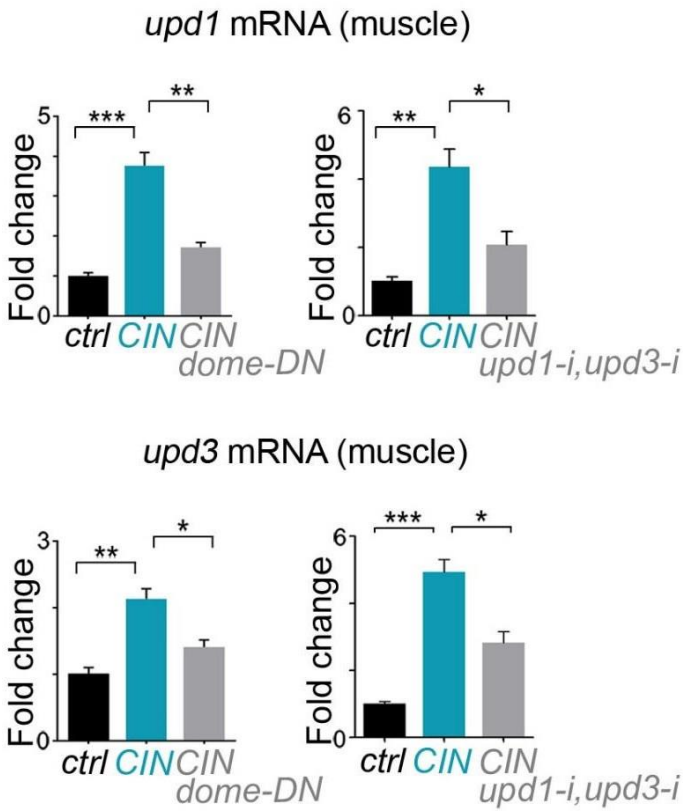

Figure 56. Muscle shows high levels of both *upd3* and *upd1* expression in CIN larvae

Histogram plotting the qRT-PCR fold change increases of control, CIN and CIN with blockage of JAK/STAT. *upd1* and *upd3* cytokines are found to be highly expressed in the muscle of CIN larvae, levels that decrease once JAK/STAT signaling is blocked.

Finally, we decided to check *upd3* and *upd1* expression levels in the muscle tissue, as we did previously for the gut, to confirm that the

contributions for the final cytokine pool were applicable to the CIN context. In order to do so, we performed a qRT-PCR assay for control, CIN and CIN larvae co-expression on the one hand *dome*-DN and on the other *upd1* and *upd3* combined interference RNAs (Figure 56).

Histograms reveal that, in the CIN context, larvae present a strong fold-change increase of cytokine expression when compared with their wild-type counterparts. An increase that is promptly rescued by the trapping or elimination of the upd ligands, this way confirming the muscle as a major contributor to the final circulating cytokine levels.

3.5 JAK/STAT activation in the trachea and fat body does not generate strong developmental delays

The capacity of UAS-upd3 cytokine to generate such strong delays in developmental timing, even in non-tumoral situations, reveals a novel unexplored role of this immune-related interleukin-like molecule. To explore such potential, we decided to perform overexpressions in other tissues aside from the STAT-GFP positive ones in the CIN background. In the CIN larvae, JAK/STAT was clearly activated in the muscle, gut and wing disc, all of which gave delays in healthy situations just by overexpressing UAS-*upd3*. However, neither the trachea nor the fat body presented signs of JAK/STAT in our tumoral context (Figure 57). So, we decided to use these tissues to understand the tissue-dependent or independent ability of *upd3* to delay metamorphosis entering. By using a trachea driver, *btl*-gal4, and a fat body driver, *cg*-gal4, we decided to overexpress the UAS-*upd3* and measure entering into pupariation.

Surprisingly, we obtained a slight delay when the overexpression was performed in tracheal tissues (Figure 58 A), but no delay at all when it was performed in the fat body (Figure 58 B). However, no tissue was able to reproduce a delay as high as the ones obtained for muscle, wing disc and gut.

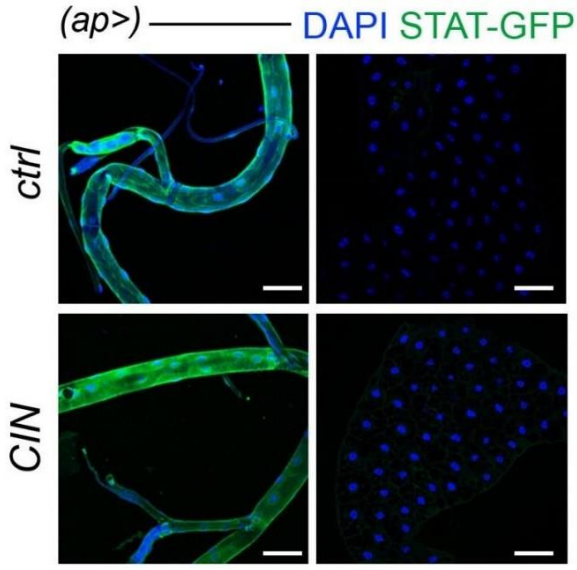

Figure 57. CIN fat body and trachea show no differences in JAK/STAT activation with control

Trachea and fat body of CIN and control larvae were dissected at L3 stage and stained with DAPI (blue). STAT-GFP (green) reporter marks the area of JAK/STAT activation. Control and CIN show the same activation levels of JAK/STAT signaling.

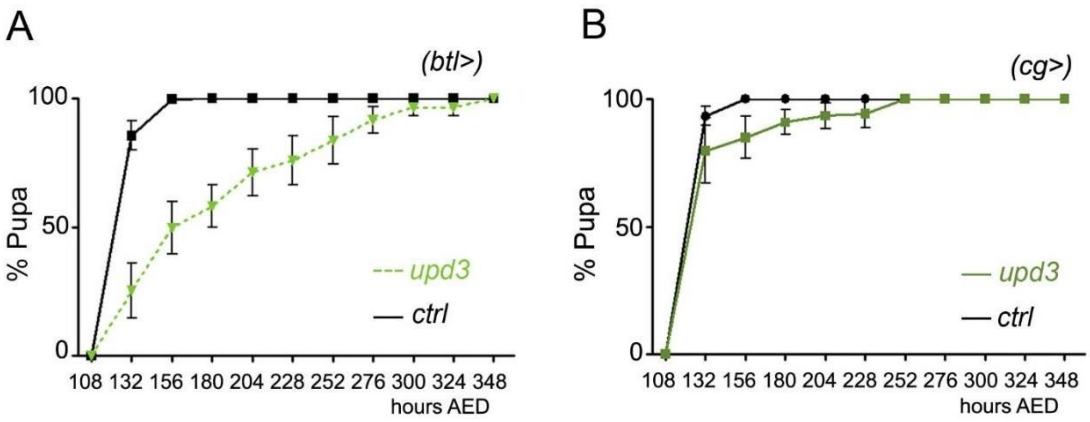

Figure 58. CIN fat body and trachea show no differences in JAK/STAT activation with control

A-B – Pupariation assays of described phenotypes considering the *btl*-gal4 driver (for trachea expression) and the *cg*-gal4 driver (for fat body expression). Results show that overexpressing Upd3 cytokine with *btl*-gal4 slightly delays developmental timing.

4. Blocking JAK/STAT in the ring gland of CIN larvae fully rescues the developmental delay

Taking into account that other cell populations have proven to be important in the contribution to the final cytokine pool in the CIN tumoral context, we started wondering if co-depletion of *upd3* from the initial tumoral site and a contributor peripheral tissue might be able to produce a rescue.

We decided to test three peripheral tissues: the muscle, which is both able to increase cytokine expression and promote JAK/STAT activation in the PG; the gut, which although incapable of increasing upd expression presents differentially STAT-GFP staining in the CIN context; and the hemocytes, known contributors to cytokine release. Taking these targets into account we decided to perform a double gal4 experiment where we co-depleted upd3 in both the initial tumor site and the referred peripheral contributors (Figure 59).

Pupariation results reveal that co-depletion was not efficient in rescuing the CIN derived developmental timing altercations. This might be either because eliminating just two of the potential sources of upd3 is not sufficient to cause a partial rescue; because upd1 contributions are still present and are locally increasing circulating cytokine levels, or even because the usage of a double gal4 mechanism induces CIN conditions in the peripheral host tissue causing unwanted secondary effects that might have unwished systemic repercussions.

In order to tackle the first hypothesis, that we needed to eliminate *upd3* contributions from more than two sources at once, we decided to make usage of the available upd3 mutant line under the control of

the *apterous*-gal4 driver. Therefore, we crossed CIN induced tumor carrying larvae with the mutant line and quantified the timing required for larva to pupa transition, as previously done for the other pupariation experiments.

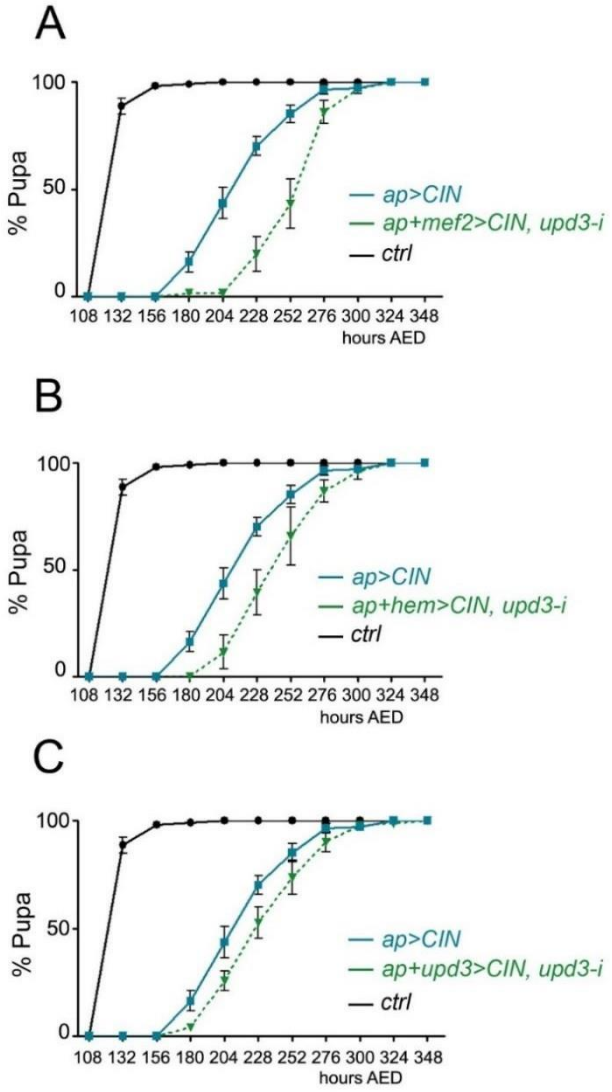

Figure 59. Co-depletion of upd3 in the tumor and peripheral contributors does not rescue the delay

A-C – Pupariation assays done in the indicated phenotypes were performed using a double GAL4 mechanism for co-depletion of the Upd3 cytokine in the wing primordia and muscle (A); hemocytes (B) and gut (C). Results show that no rescue is obtained by Upd3 depletion in two different sources.

Results show that the systemic elimination of *upd3* is sufficient to produce a rescue of the CIN developmental delay (Figure 60 A), through the downregulation of bantam activity in the ring gland (Figure 60 B). This way suggesting that in the CIN delay, the initial tumor site acts producing upd1 and upd3 that are release to act locally and systemically, respectively, in order to activate other potential sources of cytokine release that contribute to the activation of JAK/STAT signaling induction in the ring gland that, in turn, upregulates bantam expression causing the blockage of ecdysone production in the prothoracic gland.

To further proof that JAK/STAT activation in the ring gland is the crucial player responsible for the slowing down of metamorphic transitions, we decided to combine the GAL4/UAS and LexA/LexAop systems to drive CIN in larval wing primordia and deplete stat in the PG. This combination rescued the CIN-induced developmental delay (Figure 61 A) and the activity levels of STAT in the PG (Figure 61 B and B'). bantam activity levels in this same location were also rescued under these circumstances (Figure 61 C). In addition, we also performed a LexA/LexAop-mediated depletion of stat in the PG whilst using a GAL/UAS-driven expression of Upd3 in larval wing primordia of healthy larvae (Figure 61 D). This combination generated a rescue of the delay caused by the overexpression of this cytokine in the wing disc, again reinforcing that metamorphosis transitions are being prevented by the activation of bantam in a JAK/STAT dependent manner, in the PG area.

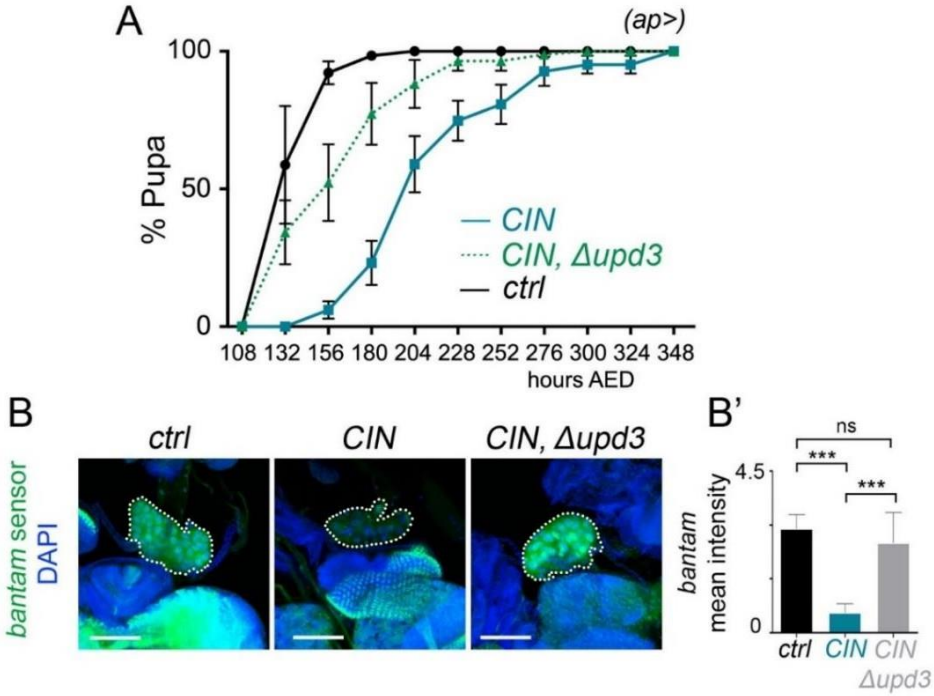

Figure 60. Systemic depletion of upd3 rescues the CIN delay

A – Pupariation assays considering control, CIN and CIN with systemic depletion of Upd3 were performed considering the *ap*-gal4 driver. Comparisons were made by comparing time points relative to 50% of Pupas formed. Results show that systemic depletion of upd3 generates a delay rescue, reinforcing the role of this cytokine in the CIN background.

B-B' – Ring glands of *ap*-gal4 larvae were dissected at L3 stage and stained with DAPI (blue). bantam (green) reporter marks the area of bantam microRNA activation. Mutant upd3 ring glands show rescue of bantam to control levels.

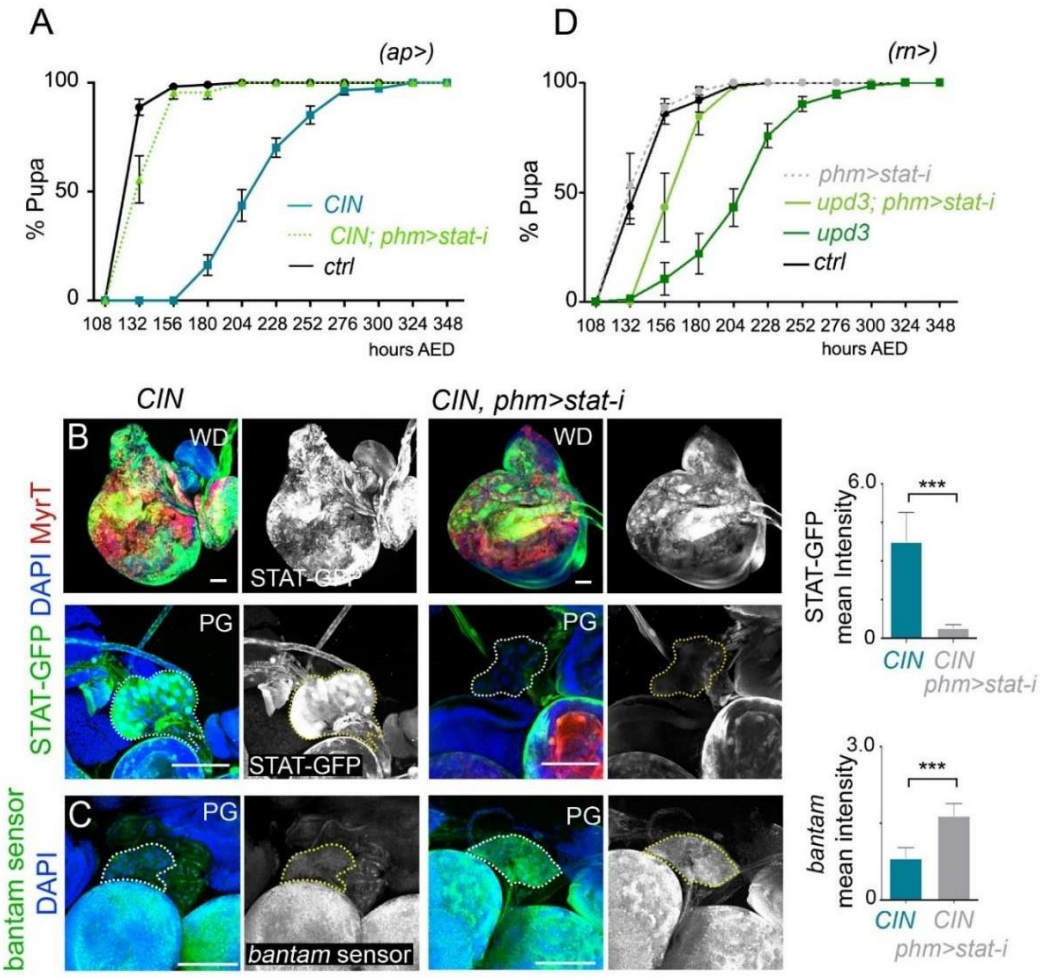

Figure 61. JAK/STAT activation in the ring gland generates a bantam expression dependent delay

A – Pupariation assay considering control depletion of Stat in the PG were performed considering a combination of GAL4/UAS and LexA/LexAop systems. Results show that specific stat depletion in the PG of CIN larvae rescues developmental delay.

B-C – CIN larvae of indicated phenotypes were dissected at L3 stage and stained with DAPI (blue). STAT-GFP and bantam reporters (green) are shown in both the ring gland and wing disc primordia. LexA/LexAop mediated Stat depletion is sufficient to rescue bantam levels.

D – Pupariation assay considering control depletion of Stat in the PG and Upd3 overexpression in the wing primordia. Results show that specific stat depletion in the PG in a condition where Upd3 is being expressed in the wing disc of healthy larvae is sufficient to rescue developmental altercations.

Moreover, we simultaneously depleted Dome receptor in CIN-tumors and PG cells and checked for pupariation progression under these conditions. As we hoped, co-depletion of this receptor in the initial tumor site and the prothoracic gland rescued the CIN-induced developmental delay (Figure 62 A), whereas single depletion of dome in CIN-tumors did not. Attributing, in this way, the returning to normal metamorphosis timing to the absence of the receptor for JAK/STAT in the ring gland tissues. Finally, we also performed simultaneous depletion of bantam activity, with the use of a bantam inhibitor (bantam sponge), in CIN-tumors and PG cells. Pupariation results reveal a rescue in the CIN-induced developmental delay, whereas single depletion of bantam activity in CIN-tumors did not (Figure 62 B). All these results reinforce a central role of the Upd3-JAK/STAT signaling-bantam axis in the CIN-induced developmental delay.

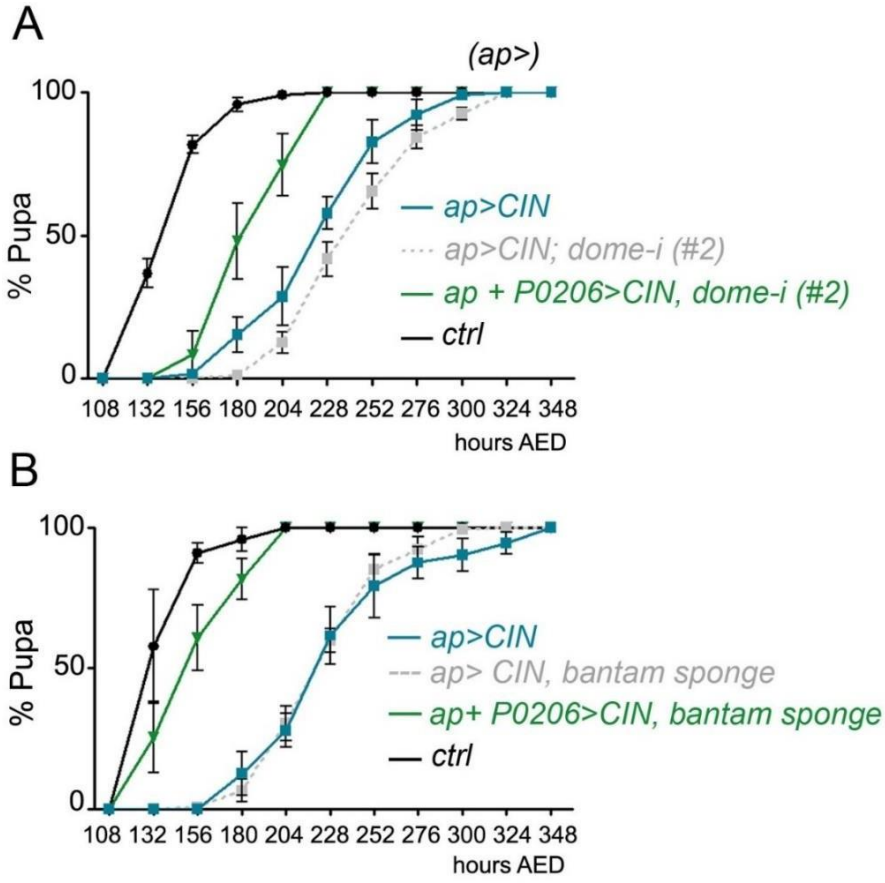

Figure 62. JAK/STAT activation in the ring gland generates a bantam expression dependent delay

A – Pupariation assay considering control depletion of dome in the PG and wing disc were performed considering a combination of two different GAL4/UAS. Results show that specific dome depletion in the PG of CIN larvae rescues developmental delay.

B – Pupariation assay considering bantam sponge expression in the PG and wing disc were performed considering a combination of two different GAL4/UAS. Results show that specific bantam sponge expression in the PG rescues CIN delay.

5. The CIN delay: The intricate interplay between four essential players

During the development of this work, we were successful in showing that Upd3 cytokine and JAK/STAT signaling pathway are the key players in triggering the CIN delay.

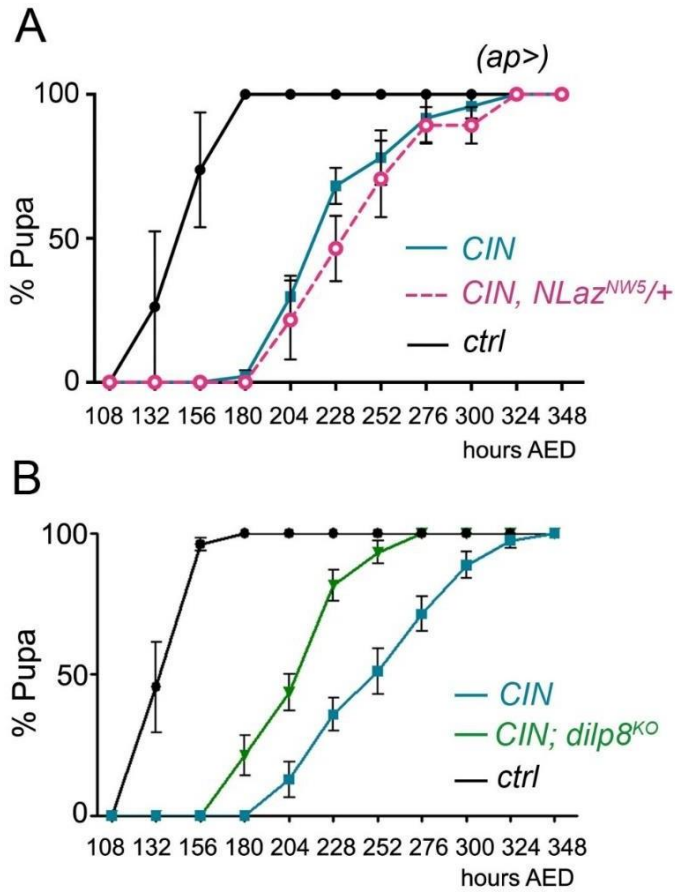

Figure 63. Systemic depletion of Dilp8 generates a 24 hour rescue of CIN delay

A-B – Pupariation assay considering systemic depletion of NLaz (A) and Dilp8 (B) in CIN larvae. Results show in a Dilp8 mutant background, CIN delay is partially rescued, entering into pupa formation 24 hours before expected NLaz however is not capable of generating the same rescue.

However, we also showed that Upd1, NLaz and Dilp8 are important contributors to the process, unveiling, for each of them respectively, local roles in potentiating higher cytokine expression and release; systemic roles in modulating IIS sensitivity in the ring gland; and systemic roles in regulating the PTTH dependent network.

Moreover, we also showed that aside from the initial tumor source, many other peripheral tissues are able to contribute to the delay generation by contributing to the increasing level of circulating cytokines, as the muscle and hemocytes.

Nonetheless, we did not yet observe the possibility for peripheral tissues to be contributors in the release of other important molecules, such as the already identified Dilp8 and NLaz, which have an already demonstrated alternative role in delay generation.

Therefore, to explore this we performed two sets of experiments using mutant lines for NLaz and Dilp8. Systemic elimination of one copy of NLaz was not sufficient to produce a rescue of the CIN delay (Figure 63 A), although it rescued the increased delay observed for the single depletion of NLaz from the initial tumor site (Figure 29 B). However, much to our surprise, in a mutant dilp8 background, a partial rescue of 24 hours was obtained for the CIN delay (Figure 63 B), indicating a possible contribution from other tissues of Dilp8 production and release. A contribution with a small, yet visible, impact in the overall CIN systemic context.

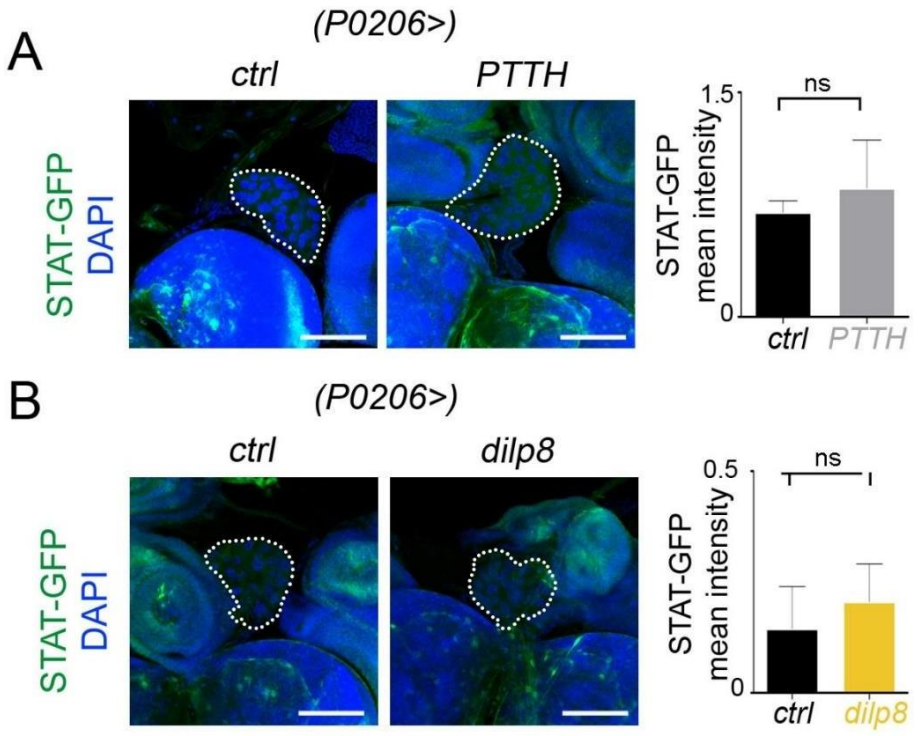

Figure 64. Dilp8 is not responsible for JAK/STAT activation in the PG

A-B – Ring glands of indicated phenotypes were dissected at L3 stage and stained with DAPI (blue). STAT-GFP reporter (green) was used to account for JAK/STAT activation in the ring gland tissues. Results show that neither Dilp8 overexpression nor PTTH were able to generate a strong activation of the STAT-GFP reporter.

Since JAK/STAT has revealed itself as the main pathway responsible for the CIN delay, we also wondered if Dilp8 could be contributing for JAK/STAT activation in this tissue. Therefore, we performed two independent experiments to reach this conclusion:

we overexpressed Dilp8 and PTTH independently in the prothoracic gland and evaluated the fluorescence levels of the STAT-GFP reporter (Figure 64 A and B).

Unfortunately, none of the considered overexpressions gave rise to an alteration in STAT-GFP reporter levels, making us reach the conclusion that Dilp8 contribution in the CIN context is most likely due to an influence on the PTTH regulatory network and not to the JAK/STAT dependent bantam mediated branch of ecdysone biosynthesis.

DISCUSSION

1. CIN epithelial tumors as a model for the study of systemic effects

Chromosomal instability, defined as the loss or gain of whole chromosomes or parts of them, and the resulting high aneuploidy rates, are common features found in most solid tumors. CIN represents the presence of chromosome missegregation during mitosis, and it can be induced through different ways including defects in chromosome cohesion, malfunction or misregulation of the spindle assembly checkpoint (SAC) genes, modifications in centrosome copy number, alterations in kinetochore-microtubule attachment dynamics and cell-cycle misregulation (Thompson, Bakhoum, and Compton 2010).

In our lab, we have been successful in the creation of a CIN tumorigenesis model using the fruit fly *Drosophila melanogaster* epithelial tissues and subjecting them to CIN, through the depletion of bub3 or rod, members of the SAC genes, and the addition of p35, a baculovirus that prevents cell death by acting on effector caspases. This model has been well defined by previous studies of the lab that demonstrated that, as a consequence of CIN induction, these aneuploid cells activate a JNK dependent response, that with time becomes pro-tumorigenic (Clemente-Ruiz et al. 2016; Dekanty et al. 2012; Muzzopappa, Murcia, and Milán 2017). The continuous activation of this major stress response pathway, together with the misplacement of E-cadherin in the high aneuploid cells, promotes the formation of two major cell populations: the growing epithelium and the delaminated population. The delamination process is death-independent and JNK-independent, since blocking JNK activation

prevents cells from dying, but does not prevent their delamination from the main epithelium (Dekanty et al. 2012). However, JNK is the one responsible for the activation of the transcriptional program in the delaminated cells, a program that drives tumorigenesis (Benhra et al. 2018; Clemente-Ruiz et al. 2016; Dekanty et al. 2012; Muzzopappa, Murcia, and Milán 2017). JNK seems to be the main responsible for activating the SASP, a program that allows for the secretion of molecules capable, not only, of affecting the epithelial population, but also of travelling and interacting with other peripheral tissues. JNK exerts a tumor-promoting role by inducing the expression of mitogenic molecules, such as Wg, and boosts tumoral expansion and migration through the expression of metalloproteinases, such as MMP1, which degrades the basement membrane increasing potential invasiveness capacities. JNK also promotes the secretion of other signaling molecules (Table 2) whose functions are not always clear or described in the tumoral situation. This work unravels the role of some of these secreted signaling molecules, namely cytokines and lipocalins, and describes their systemic impact, focusing on the role they play in peripheral tissues, more specifically, focusing on their role in the alterations of developmental timing.

In order for this to be possible, the usage of *Drosophila* and its powerful genetic tools were critical. The fruit fly is an ideal model to study CIN-induced tumorigenesis since induction of CIN can be done in a compartment-specific manner, helping in the study of autonomous and non-autonomous effects in the tissue. Furthermore, it is also an ideal model for the study of systemic effects due to its short life cycle and ease in the moment of tissue dissection and manipulation. Moreover, due to the high conservation

between flies and humans of the intervening pathways and stress responses, from metabolic alterations to inflammation processes, *Drosophila* emerges as an excellent genetic model to the study of complex tissue interactions.

In the context of this thesis, the fruit fly is mainly used to assess alterations in developmental timing during tumorigenesis. Insects, such as *Drosophila*, are subjected to strong evolutionary pressure and high adaptive response to environmental stimuli (Stearns and Stearns 2000). Maintenance of body size and tissue pattern are important traits in the determination of fitness that depend on this tight regulation. That is why organisms have developed adaptive responses to allow for modulation of size in response to different nutrient availability, mechanical injuries or growth disturbances, such as tumorigenesis (Colombani et al., 2012; Garelli et al., 2012; Garelli et al., 2015). Many of these adaptive responses go through the modulation of ecdysone responses, activation or production.

Ecdysone biosynthesis can be modulated via two very important regulatory networks: the PPTH-dependent and the TOR/IIS-dependent regulation pathways. The PTTH pathway signals through Torso receptor, present in the PG. Torso/PTTH ligand-binding causes the activation of the canonical MAPK signaling pathway leading to ERK phosphorylation and consequent activation of ecdysone production. The TOR/IIS-dependent response acts mainly as a nutrient-sensing pathway that coordinates nutritional conditions, systemic metabolism responses and growth. IIS signaling is activated in the PG after the dILPs bind to the InR activating PI3K and Akt, the later responsible for repressing FOXO, an insulin activity inhibitor that suppresses transcriptional activity (Arden 2008; Mora et al. 2004; Oldham and Hafen 2003). Akt signaling is also mediated

by TOR, potentiating this effect (Haar et al. 2007; Kim et al. 2008; Sancak et al. 2007). TOR kinase is also responsible for the phosphorylation of 4EBP, that has a role in increasing translations, and S6K, responsible for increasing ribosome biogenesis (Bruce A. Hay, Huh, and Guo 2004). Aside from these regulatory responses, IIS is also able to modulate microRNA activity. A particular important example of this modulation is the repression of bantam. The expression of bantam in the PG suppresses ecdysone biosynthesis, which reduces basal levels of ecdysone release into the hemolymph. This allows for the increase of IIS in the peripheral tissues, increasing larvae body growth. Once IIS is high and this is sensed within the center for metamorphosis control, bantam is repressed, and ecdysone biosynthesis can occur (Boulan, Martín, and Milán 2013).

Both PTTH-dependent and TOR/IIS-dependent regulatory networks are very important in the regulation of ecdysone biosynthesis; however, most of the alterations in developmental time observed during injuries and tumor development are associated with the PTTH pathway.

Projects developed considering the avalanche syntaxin model and the eyeful model show that during tumorigenesis there is an induction of a delay in developmental timing that is caused by the release of Dilp8 (Colombani et al., 2012; Garelli et al., 2012). Dilp8 acts by inhibiting PTTH production by binding to its receptor Lgr3 in the brain. The Lg3 neurons are therefore activated and can signal the PTTH neurons, preventing the release of PTTH hormone into the ring gland, thus blocking ecdysone production (Colombani et al., 2015; Garelli et al., 2015; Gontijo & Garelli, 2018). Removing *dilp8* expression from the initial tumor site rescues the delay produced.

When the CIN model was firstly described in the lab, it was clear that CIN-induced tumorigenesis caused a developmental delay, since tumors could be dissected two days after control larvae (Dekanty et al. 2012). Indeed, CIN larvae spend 3 to 4 additional days wandering in the food after reaching L3, continuously eating and continuously increasing in size (Figure 18). This response was a consequence of JNK activation, since blocking JNK with *puckered* produced a full rescue of the delay (with 50% of Pupas being obtained between 132- and 156-hours instead of between 204- and 228- hours, the range observed for CIN). However, taking into account that JNK activation is also the one responsible for the generation of epithelial overgrowth, through the release of mitogens like Wg, it was possible that the delay was a consequence of tumor size (Figure 21). The idea behind this hypothesis was based on the knowledge that CIN tissues are formed by two different population of cells. The epithelial population, characterized by being tightly bound and having a normal karyotype; and the delaminated population, that is known to be highly aneuploid, loosely bound and prone to activate the SASP, inducing changes in peripheral and local tissues. One of the main signals secreted by this second population of cells is Wg, which acts on the epithelium to generate a tumoral-like overgrowth. The more the epithelium population grows the more aneuploid cells are formed, due to the CIN input, and the more cells delaminate from the main tissue incorporating the second population. So, one could think about this mechanism as a feedback loop that increases both population sizes as a consequence of continuously active JNK.

That being said, if the delaminated population is growing, this also applies for its capacity to release a high number of secreted signals

capable of generating a stronger systemic effect. However, if the tumoral growth is controlled, without the blockage of JNK, we could discard the hypothesis that the delay is tissue-size dependent.

Therefore, through the usage of *wg* interference RNA, which is also capable of producing a tumor size rescue similar to the one observed for *puc,* we confirmed that developmental timing rescue was independent from growth effects (Figure 22). CIN delay is, thus, JNK dependent but independent from tumor growth.

As previously mentioned, Dilp8 has been described as the responsible signaling molecule for mediating tumor-inducing developmental delays. This molecule was also highly expressed in our CIN tumors, so it arose as a good candidate to be tested for its involvement in the developmental timing alterations. Being secreted under the control of the JNK stress response pathway and expressed all throughout the extra days spent by the CIN larvae in the food, Dilp8 seemed to be accomplishing all the requirements already defined for the secreted molecule responsible for the delay. However, depleting *dilp8* from the initial tumor site, contrary to what was observed for the eyeful and avalanche model, did not produce any rescue of the CIN delay. If anything, it enhanced the delay in approximately three extra days (Figure 23). In spite of increasing the time spent in the food, comparing CIN tumors with CIN tumors with depleted *dilp8* did not show a difference in tumor sizes, not in early time points neither in later ones. However, once CIN larvae pupate, the ones with depleted *dilp8* continued feeding, and continued growing, a consequence of the extended delay, not of the increased growth rate. The extended delay of CIN when depleting this relaxin-like protein was a surprise since it has been well described and used by other tumor models. Despite this, overexpression of Dilp8 in

healthy larvae, by using *rn*-gal4 driver, was able to generate a small delay, confirming that this molecule is indeed capable of generating this systemic alteration (Figure 24).

Dilp8 acts on the release of PTTH hormone, preventing its release, and, consequently, the activation of the Torso/ERK response. Therefore, a good read-out for the PTTH-pathway function is assessing phosphorylation of ERK. The PTTH mRNA levels in healthy larvae starts to increase after 96 hours and peaks approximately at 120 hours after egg laying (Garelli et al., 2012), meaning that the pERK peak should be observed around that time if the developmental timing is not affected. For growth disturbances though, such as the one produced by the *Bx>rpr* regeneration model, the PTTH expression might be altered. In this particular case, for instance, PTTH mRNA levels start to increase after 96 hours but only reach their peak approximately 180 hours after egg laying (Garelli et al., 2012). Meaning that the PTTH and, in consequence, the pERK peaks are pushed back during growth disturbances to delay metamorphosis, and this delay usually coincides with the delay observed in the metamorphosis transition.

In our CIN model we know that we have a very strong developmental delay, which means that the PTTH peak should be strongly pushed back, as previously observed for other growth disturbances. However, the pERK peak in CIN occurs with a small delay of 24 hours, around 130 hours after egg laying, not so far from the original peak, in our case observed in control larvae at 106 hours after egg laying. Considering that the CIN delay is of up to five extra days, we would expect that the pERK peak would occur much later in development. Nonetheless, it is probable that, when CIN larvae start entering into pupariation, another peak is developed.

Discussion

In described tumor and regeneration models, removing *dilp8* expression from the growth disturbance initial site recovers the delay in PTTH expression peak, reestablishing, in this way, the developmental delay produced. In our CIN model, removing *dilp8* from the initial tumor site did produce a rescue in timing observed for CIN of the pERK peak (Figure 25). This led us to believe that although PTTH production is not severely affected in CIN larvae, it is still being regulated by Dilp8 activation coming from the tumor. Moreover, the pERK peak develops quite early in CIN compared to the additional delay these larvae have, showing a peak in the first 24 hours of delay, instead of later, closer to the entering in metamorphosis. Off course, only two time points were monitored, probably indicating the need to check additional time points, closer to the one where 50% of pupa's are already formed. Nonetheless, it appears to be clear that PTTH is being released, and it is not the absence of the activation of the Torso/ERK pathway the main responsible for the delay of CIN. This realization is a turning point for this thesis, since it reveals, at this point, that CIN induced delays experience a clear difference between other analyzed tumor models, scattering the notion that Dilp8 is the sole responsible for this type of delays.

Growth disturbances, however, might not only be a consequence of problems in ecdysone biosynthesis, they might also be related with ecdysone activation, which is performed by the fat body and mediated by the *shade* gene, or with the capacity of the peripheral tissues to respond to ecdysone. A quick way to try and dismiss these alternatives was to check fat body's health, by doing a Nile Red staining to check for lipid droplets amount and structure of the fat body together with an IIS staining, to check fat body capacity of

responding to nutritional cues. We also performed a quick staining for ecdysone receptor core, to see if CIN tissues are producing the receptor in the same amount as the control ones (Figure 65). Concerning the fat body, we can see a healthy tissue, with high ability to respond to IIS activity and a high number of lipid droplets, an indication of increased energy storage. Indeed, continuing in this direction, CIN seems to have a higher activation of PI3K than control larvae a fact that, combined with the high amount of storage vesicles, seems to be due to the larvae continuous feeding during the extended delay.

As for the ecdysone receptor levels, it is true that CIN seems to have, when dissected considering the same time point, slightly less amount of ecdysone receptor when compared with the control. This might be due to the fact that the pupariation peak is later in time, meaning that the increase in ecdysone receptor number will only occur later on.

However, feeding the larvae with an activated form of the hormone clearly produces a rescue of the CIN delay (Figure 20), confirming that the receptors for the hormone are fully functional in our model. The hormone feeding assays also confirm that the delay is caused by an impair ecdysone availability, as it is the case for many other observed models. However, it is not strictly Dilp8-dependent, opening a new window for the intervention of other signaling molecules in the regulatory networks of ecdysone production reveling the high complex regulation these animals are subjected to in order to maintain growth and patterning.

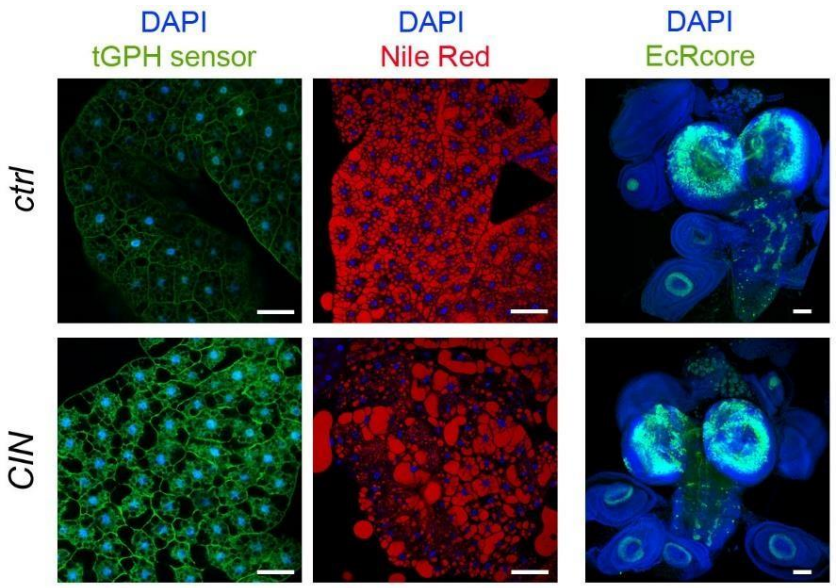

Figure 65. CIN presents a healthy fat body together with normal amounts of EcR

Control and CIN larvae dissected at L3 stage were stained for Nile Red (red) and EcRcore antibody (green). tGPH sensor was used for fat body staining (green). Results show that CIN fat body shows signs of strong catabolism and response to IIS signaling. Brain staining for ecdysone receptor does not show major changes between CIN and control animals.

2. CIN delay is a consequence of impairs in IIS-dependent regulation

Aside from PTTH regulation, TOR/IIS regulatory networks help animals integrate nutritional and environmental cues with their metabolism responses, growth rates and patterning mechanisms. Therefore, it is not surprising that ecdysone biosynthesis is also regulated by these pathways. Insulin, especially, has a very important role in activation of ecdysone production, because it can

regulate such production in distant ways, from repressing FOXO action to acting on microRNAs in order to modulate their activity. One of the microRNAs under IIS influence is *bantam*. The double repression mechanism for which IIS represses bantam, that in turn represses ecdysone biosynthesis, was also described by our lab back in 2013 (Boulan, Martín, and Milán 2013). However, the work had a developmental perspective, so these sensors were never tested for the tumorigenesis condition. Nonetheless, when we realized that PTTH was being released and sensed by the PG, consequently activating the phosphorylation of the downstream target ERK, checking IIS response and bantam expression became a priority. As a result, we observed that CIN ring glands presented no sign of PI3K activation, a sign of insulin resistance, and high expression of bantam microRNA, which appeared to be a direct consequence of the lack of IIS response (Figure 26). Consequently, it became clear that this was the regulatory network affected in CIN tumors, the one responsible for the delay, and probably the reason why Dilp8 depletion was not effective in our model.

After rejection of Dilp8 as the main molecule capable of delay induction, we were looking for other secreted signaling molecules that were expressed under the control of JNK, so far the only one able to produce a rescue, capable of long-range activity, since it had to influence the brain and associated ring gland, and that was being extensively produced by the senescent like population. Moreover, it should have an IIS relation or a bantam relation, since this regulatory network is the one affected in CIN. Complying with these requirements, we found Neuro Lazarillo. NLaz is a lipocalin already described as an antagonizer of IIS and a regulator of metabolic responses, especially in situations of high sugar diets (Pasco &

Léopold, 2012). And assessing NLaz role in our CIN model proved to be a good decision since depleting the tumoral tissue of this lipocalin expression led to a full rescue of the ring gland's IIS resistance (Figure 28). Revealing, therefore, a new role of NLaz as an active intervenient in the IIS regulatory network and able to generate a delay through the impairment of this pathway in healthy conditions (Figure 30).

Being as it may, by only blocking NLaz it was not possible to obtain a rescue of the CIN delay, since the bantam repression over ecdysone production was not lifted just by depleting this signaling molecule (Figure 29). Indeed, in resemblance to Dilp8, depleting NLaz caused an increase of the delay in developmental timing, a surprising response, taking into account these molecules seem to be produced by other tumor, regeneration or metabolic disorder models to delay entering into pupariation. This might indicate that the tumor has a very sensitive equilibrium regarding its secretome, and whenever a molecule from this secretome is removed, the system becomes highly unbalanced and activates a response mechanism that blocks entering into metamorphosis in an even stronger manner. It is also worth disclosure that the pupas arising from these extra delays of Dilp8 and NLaz depletion have much darker color and softer cuticles, indicating bigger problems in generation of these structures. Since overexpression of NLaz and Dilp8 individually gave rise to a similar delay, and their depletion in a CIN tumoral situation gave rise to a similar response, we also considered removing both NLaz and Dilp8 at the same time from the initial tumor site (Figure 31). The response was not a rescue of the CIN delay, but it did produce a rescue of the extra days spent in the food, and a slightly ameliorated phenotype for the pupas formed, meaning, stronger

cuticles and slightly lighter color similar to CIN ones. The rescue of the extra days spent in the food gives us a clearer idea that the tumoral system is quite delicate, and that every alteration produced in the secretome might rapidly unbalance the system towards a higher delay and a more complex systemic impact.

3. CIN delay is a consequence of JAK/STAT activation in the PG

When the lipocalin NLaz rescued the insulin sensibility of the ring gland and, consequently, of the PG it became clear that there was something else regulating *bantam* expression in CIN larvae.

However, we were still looking for a signaling molecule that was able to comply with the aforementioned criteria: JNK dependent expression, long-range action, secretable and probably related with the regulatory networks involving IIS or bantam expression. It was at this point that we hypothesized the involvement of the *unpaired* molecules. All three were highly present in the tumoral tissue, and they had been described throughout the years in several systemic responses for responding to nutritional cues, such as *upd1* (Beshel, Dubnau, and Zhong 2017; Rajan and Perrimon 2012), or highly involved in inflammation responses, a role mostly associated with *upd3* (Chakrabarti et al., 2016; Lee et al., 2017; Sotillos et al., 2008). Moreover, they were clearly a target of JNK signaling (Figure 32) and highly secretable and able to travel long distances and affect the function and activity of peripheral tissues. Finally, in one way or another, they also presented relations with nutritional cues or nutrition-sensing organs, such as the fat body. Appropriately, they

became a clear option to test as responsible for the systemic impact on developmental delay. And since they were all ligands capable of activating the same receptor, Dome, JAK/STAT signaling pathway was also a prospective candidate for the other regulatory network capable of bantam regulation.

Indeed, activation of JAK/STAT was clear in the CIN ring glands, with extremely high levels of expression when compared with control (Figure 33), a phenomenon not yet described for any other situation, to the best of our knowledge. This activation of JAK/STAT was able, on its own, to generate a delay in non-tumoral conditions through the upregulation of *bantam* in an IIS independent manner (Figure 34).

Although it seemed clear that JAK/STAT activation was the one responsible for the delay, depleting the cytokines individually from the tumor region did not produce any rescue (Figure 37). However, a rescue was obtainable when combining both *upd1* and *upd3* depletion, as well as when using a dominant negative of the receptor Dome (Figure 39). Dominant negatives are mutations that alter the gene product in such a way that the resulting product acts antagonistically to the wild-type allele. This means that the product of such mutations is still able to interact with the same elements as the wild-type product, but some aspect of their function is blocked. In this case, the dominant negative is referent to an intracellular domain of the receptor Dome. Dome is activated by the three *upd* cytokines, which, after the ligand-binding to the receptor, activates hop (the JAK present in *Drosophila)* and then consequently promotes the dimerization of STAT, that enters into the nucleus promoting gene target transcription. In the Dome-DN line, the cytokines can bind to the receptor, but the downstream pathway is not activated. However, we do not recapitulate the rescue results

using an interference RNA for *hop*, neither do we rescue using a Dome-RNAi (Figure 39), meaning that the Dome-DN does more than just blocking JAK/STAT activation.

Indeed, we hypothesize that Dome-DN not only blocks JAK/STAT activation but also traps the ligands, blocking any alternative response these cytokines might be generating outside the initial tumor location. Aside from Dome-DN, rescues are only obtained for mutant upd3 (Figure 60), indicating that there is a systemic response coming from peripheral tissues and influencing the delay. Furthermore, it also indicates that this systemic response is mostly connected with *upd3*, leaving *upd1* with a more local role.

The Dome-DN line rescues not only delay, but also all the sensors related with IIS regulatory network of ecdysone biosynthesis (Figure 41). This means that blocking ligand travelling rescues not only JAK/STAT activation in the ring gland, but also the upregulation of *bantam* and even the ability of the tissue to respond to IIS. Equally, it leads us to believe that the expression of *NLaz* in the tumor is under the control of JAK/STAT, because blocking the activity of this pathway is able to rescue PI3K activity in the ring gland. Considering that JAK/STAT is not able to modulate IIS activity in the ring gland upon its overexpression (Figure 34), we explored this connection with NLaz by qRT-PCR and *in situ* staining that clearly show the downregulation of this target upon JAK/STAT blockage.

Aside from the Dome-DN, rescues of the three used sensors are also obtained when using Bsk-DN, something expected since both these lines are able to produce a delay rescue.

To confirm the capacity of the upd3 cytokine to generate a delay in our CIN tumoral situation we also decided to perform the

overexpression of these interleukin-like molecules in healthy wing discs (Figure 36). This experiment confirms the ability of upd3 cytokine to generate a delay on its own, a solid proof that the initial delay-dependent response comes from the initial tumor site. This delay is fully rescued when the overexpression is combined with Dome-DN, again reinforcing that expressing *upd3* cytokine in the wing disc alone is sufficient to generate a delay, whilst trapping it from the initial secretion site is able to rescue. This delay is, as mentioned previously, completely independent from IIS activity, since all ring glands, control and overexpressions, are positive for tGPH sensor with similar levels, supporting the notion that PI3K is highly active in all considered situations. Therefore, in our CIN derived tumor model, JAK/STAT activation is the one responsible for delay generation, a response that acts through the modulation of the activity of the microRNA bantam. This microRNA is active in our tumor situation in order to block ecdysone biosynthesis in an IIS independent manner. The impact of JAK/STAT in delay generation has never been described so far, and the involvement of secreted cytokines in this response is also new in the tumoral situation which, so far in the published models is fully dependent of the action of Dilp8 and its impact in the PTTH regulatory network.

4. CIN delay is caused by different sources of *upd3*

The tumor, induced in the wing disc, is the major source of cytokines and it is the initial trigger for *upds* secretion. Blocking this initial response, using *Dome-DN* lines, completely rescues the delay. However, simple blockage of JAK/STAT signaling in the initial tumor site is unable to reproduce such rescue. This rescue is,

nevertheless, possible if we remove from the tumor both *upd1* and *upd3* ligands simultaneously (Figure 38). Such results give rise to two possible scenarios about the origins of CIN delay. In the first scenario, Upd1, together with Upd3, might act locally in the wing disc to induce the expression of other long-range systemic signals to regulate, together with Upd3, ecdysone production. Consistent with this, overexpression of Dome-DN in CIN tissues reduced the expression levels of Dilp8 and NLaz (Figure 40), and rescued the impact on the activity levels of the IIS mediated regulatory network in the ring gland (Figure 41). However, co-depletion of two of the three systemic signals from the tumor site did not rescue, even partially, the developmental delay (Figure 40). This latest results, pivot us in the direction of the second scenario, where a feed-forward loop mediated by the local activities of Upd1 and Upd3 might expand their expression domains to nearby tissues. Supporting this, we saw that overexpression of Dome-DN in CIN tissues also reduced the expression levels of Upd1 and Upd3 (Figure 43). As well as we noticed that Upd ligands and JAK/STAT signaling were ectopically induced in myoblasts and in larval muscles (Figure 52 and 53), an ectopic induction that was blocked by targeted overexpression of Dome-DN or targeted co-depletion of upd1 and upd3 to CIN-tumors (Figure 56).

Activation of JAK/STAT in the skeletal muscle fibers of *Drosophila* has been previously described in an immune context. More specifically, JAK/STAT was shown to be induced upon parasitoid infection context in the muscle, through the secretion of Upd2 and Upd3 from the hemocytes (Yang et al., 2015). In fact, in this situation the suppression of upd2 and upd3 release, through the usage of single and simultaneous combination of mutants for these cytokines,

reduced the cellular immune response, and suppressed JAK/STAT activity in the muscle cells. Such suppression strongly reduced the encapsulation of wasp eggs and the circulating number of lamellocyte effector cells, a specific type of hemocyte capable of larger body encapsulation. In that study, JAK/STAT activation in somatic muscles together with the cytokine secretion from the hemocytes are able to shut down JAK/STAT signaling in the lymph gland, where this pathways activity is known to keep hemocyte precursors undifferentiated. Lifting this repression leads pro-hemocytes to massively differentiate into effector hemocytes (lamellocytes) that allow for more effective encapsulation (Yang et al., 2015)

In our tumor model, the activation of JAK/STAT signaling in the muscle cells is triggered by the tumor, since blocking cytokine release there completely rescues the activation of this signaling pathway in these cells (Figure 42). This activation seems to be related with the necessity of secreting large amounts of cytokines to help activate JAK/STAT in the ring gland, however, we cannot exclude that the activation of this pathway is also connected, somehow, with an immune response, since this has not been clearly analyzed during this thesis. What we can state is that normal routes of immune response usually trigger JAK/STAT activation in the fat body to mediate a *totA* dependent response. Despite this being the general immune response, in our CIN models we do not see JAK/STAT activation in the fat body, therefore, this opens a window into the possibility of muscle-mediated immunity in these types of tumors.

Whatever the response might be, it is also clear that in our model hemocytes don't play a role in developmental delay generation through the activation of JAK/STAT in the PG. Although overexpression of Upd3 in the hemocytes might be able to generate a delay, this is independent from bantam and JAK/STAT activity in the PG (Figure 51). However, hemocytes are known sources of Upd3 release and they are clearly recruited in the CIN scenario, exponentially increasing their count with the extended delay (Figure 48). High counts of hemocytes in later time points could be thought to be extremely detrimental for the entering into metamorphosis, since in other scenarios larvae would die at this point. However, larvae only die once the pupa is formed, which means that their entering into a pupa stage is not prevented by the hemocytes but does not exclude the impact of the high inflammation response in the blocking of the transition into adulthood.

What is clarified, though, is that for the muscle cells and their precursors in the wing disc, overexpressing the Upd3 cytokine is able to trigger a systemic response of both activation of JAK/STAT in the wing disc, muscle and ring gland, generating a delay in developmental timing as a consequence (Figure 54 and 55). A response that was not obtained for the hemocytes overexpression experiments (Figure 50), which were able to generate a delay in pupa formation but incapable of triggering STAT-GFP sensor activation (Figure 51).

Aside from the muscle cells, the gut was also analyzed as a contributor to the cytokine pool. Promising results were obtained with the single overexpression of Upd3 in the gut tissues of healthy

larvae, where overexpression triggered not only a delay but also JAK/STAT activation in the PG (Figure 46). Although this activation took longer than when the overexpression was triggered in the muscle or wing disc tissues. This might indicate that the cytokines take longer travelling through the gut to the PG, when compared to muscle, which covers all the cuticle of the larvae, or the wing disc, which is close to the brain area. If travelling is distance-based, this later tissues would be in an advantage. However, activation of JAK/STAT in the wing disc, in gut induced Upd3 overexpression animals, happens early on, and this tissue is quite far from the gut, so it might not be a distance-related response and more likely a tissue related one. This hypothesis might explain why expression of Upd3 in the trachea and in the fat body of healthy larvae are less able to generate strong delays in pupariation timing (Figure 58). However, in order to fully get into this subject more experiments should be conducted, with different tissues, analyzing thoroughly STAT-GFP activation in the PG area, to further assess the possibility of a tissue-dependent response.

In the CIN context, nonetheless, the gut does not seem to be contributing for the overall circulating cytokine number, since qRT-PCR assays showed no significant difference between expression of these players in CIN and control larvae (Figure 47).

5. JAK/STAT regulates ecdysone production in CIN-derived tumors

Even considering that the tumor has other tissues contributing to the release of *unpaired* cytokines, blocking JAK/STAT directly in the center of metamorphosis regulation, the ring gland, should be

sufficient to block a JAK/STAT dependent delay. And indeed, that is what happens in our case (Figure 61).

By making use of a double expression system, through the combination of tumor generation via the UAS-Gal4 system and the blockage of JAK/STAT in the ring gland through the usage of the LexA/LexAop system (Figure 61), we were able to rescue the developmental delay caused by the tumor. This confirms a new JAK/STAT regulation network for ecdysone production that acts through bantam microRNA. MicroRNAs are a class of small, noncoding RNAs that negatively regulate target gene expression. In recent years it has become increasingly clear that most biological processes involve microRNA regulation or activity. JAK/STAT associated regulation of microRNAs has also been demonstrated previously for *Drosophila* although not related with ecdysone biosynthesis. For instance, JAK/STAT has been reported as a target of miR-279 that through *upd1* is able to manipulate JAK/STAT signaling and disrupt rest: activity rhythms of the circadian clock (Luo and Sehgal 2012). However, there is not much information about microRNA regulation through JAK/STAT in *Drosophila*. In mammals, on the other hand, this is not the case. miR-19a, for instance, has been shown to regulate SOCS1 expression during multiple myeloma through the induction dependent response of anti-viral cytokine interferon α (IFN-α) (Pichiorri et al. 2008). It has also been shown to target SOCS3, a strong regulator of the JAK/STAT pathway enhancing INF-α and lL-6 through STAT3 in inflammatory regulation (Collins et al. 2013). miR-155 has been shown to also target SOCS1, but in macrophages, positively regulating antiviral response in the host by promoting type I IFN signaling, thus suppressing viral replication (Wang et al., 2010); and in breast cancer cells miR-155

suppression of SOCS1 plays an oncogenic role resulting in the constitutive activation of JAK/STAT pathway through the activation of STAT3, stimulating of breast cancer cells proliferation. This activation causes the stimulation of an inflammatory response through the expression of inflammatory cytokines, namely, IFN-gamma and IL6 which contributes to miR-155 expression, suggesting that this microRNA might serve as a bridge between inflammation and cancer (Chakraborty et al. 2020). Other microRNAs have also been associated with SOCS targeting, miR-9 has been shown to target SOCS5 and promoting endothelial cell migration and angiogenesis (Zhuang et al. 2012); miR-98 has been described as targeting SOCS4 in response to biliary infection (Hu et al. 2010); miR-203 has been shown to be produced by the pathogen *Porphyromonas gingivalis* during gingival infection and capable of suppressing SOCS3 to potentiate infection and cause severe periodontal disease (Moffatt and Lamont 2011); miR-146a and miR-146b-5p have been shown to regulate the inflammatory response of the retinal pigment epithelial during a pathogenesis caused by age-degeneration, increasing their expression in response to proinflammatory cytokines (Kutty et al. 2013) and miR-373 produced by the hepatitis C virus has been shown to impair JAK/STAT signaling (Mukherjee, Di Bisceglie, and Ray 2015). These results confirm that the relationship between JAK/STAT and microRNAs is extensive, but also show that most of the already described interactions are in the direction of microRNA regulating JAK/STAT activity and mediating the consequent inflammatory responses, not the other way around, which is what we describe in this project.

Aside from shedding a light onto new regulatory networks that might be affecting ecdysone production, this project also dismissed the

idea that Dilp8 is the only signaling molecule responsible for delay entering into metamorphosis in tumoral models. Although profusely described as the molecule responsible for systemic delays and pupa homeostasis (Colombani et al., 2012; Garelli et al., 2012; Ray & Lakhotia, 2016), with this work we propose other signaling molecules, lipocalin and cytokines, as crucial for ecdysone biosynthesis regulation. Moreover, we open a door about Dilp8-independent tumor regulation of developmental timing, since we show cytokines in the CIN context are the ones mainly mediating ecdysone production. And we also open a door about different strategies tumors might have in order to impact systemic processes and peripheral organs.

With this we do not mean to discard the action of Dilp8. In a dilp8 mutant background we were able to obtain a 24 hour rescue of the CIN delay, reinforcing that this secreted relaxin-like protein is still important in the regulation of metamorphosis. However, such regulation is JAK/STAT independent, since neither by overexpressing it or its downstream target, PTTH, was possible to induce STAT-GFP reporter in the PG of healthy animals. We also do not discard the extreme importance of Dilp8 expression described for the previous models, nor do we want to state that JAK/STAT is the major regulatory network for ecdysone production. What we want to state is that different tumors might have different ways of impacting developmental time, through the regulation of different regulatory networks that might be used single or simultaneously to modulate organ growth, patterning and developmental transitions.

In the case of the Avalanche Model described by Colombani and collaborators, for instance, it is clear the involvement of Dilp8 in the delay rescue. But it is also clear that the pathway mainly affected in

that case seems to be PTTH-dependent network: indeed, a quick check for the tGPH and bantam sensors used throughout this project revealed that the ring gland in this tumor model responds to insulin, showing high activity of PI3K and low bantam expression levels (Figure 66). In this way confirming that the altercations experienced in this tumor model are not the same present in CIN, and also demonstrating clearly that tumors might secrete different factors, according with their particular growth and development situation, in order to cause alterations in developmental delay timing or activity of peripheral organs.

To sum up all of what was mentioned above, during the development of this thesis we were able to describe the systemic mechanism used by CIN larvae to alter their developmental timing. In this tumor model the delay is not dependent on tumoral growth, neither is it dependent solely on the action of Dilp8. In our scenario, however, the delay is deeply connected with JNK signaling. JNK activity allows for the secretion of a number of different signaling molecules that have the potential of impacting peripheral tissues. Lipocalins, such as NLaz, are a good example of this potential. In our tumoral model NLaz acts in order to promote insulin resistance in the ring gland, acting on the IIS-dependent regulatory network of ecdysone production that depends on a double repression mechanism where IIS blocks bantam repression of ecdysone production.

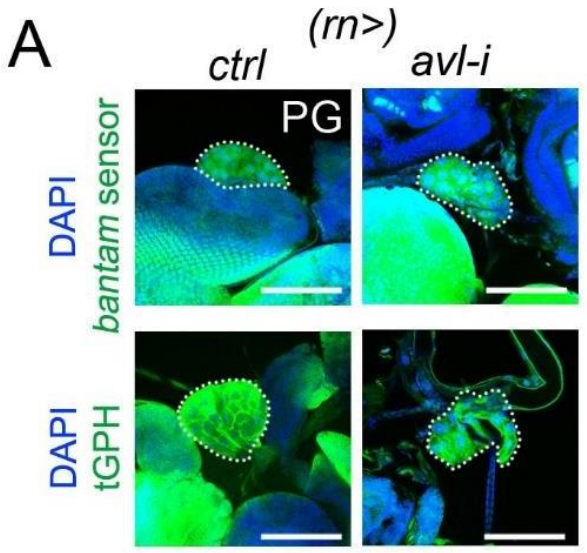

Figure 66. The Avalanche Model presents correct function of the IIS-dependent regulatory network of ecdysone biosynthesis

Avalanche model larvae under the expression of the *rn*-gal4 driver were dissected at L3 stage and stained for DAPI (blue). tGPH and bantam sensors (green) were evaluated to check for the function of the IIS-dependent regulatory network. Results show that the ring gland of these tumoral larvae are positive for insulin activity and show low levels of bantam expression, indicating the correct function of the IIS-dependent regulation on ecdysone biosynthesis.

Even though NLaz has the potential for bantam regulation in a normal scenario, in a CIN context NLaz lifts the repression of IIS sensitivity but it is unable to downregulate bantam expression. This incapacity is mainly due to another regulatory network working on bantam expression which is mediated by JAK/STAT activity. In CIN tumors, cytokines are released, and they travel to many tissues to influence their function and the activity of regulatory signaling pathways. These cytokines promote JAK/STAT activation not only in the ring gland, but also in the gut, muscle and myoblast cells.

Some of these tissues, such as the muscle and its precursors, function as alternative sources of *upd3* release, acting as contributors to the systemic impact of CIN tumors in developmental timing altercations. That is why only by blocking cytokine release from the initial tumor site, or by blocking *upd3* systemically, are we able to obtain a complete rescue of the delay.

The interplay between the different elements secreted by the tumor and their impact in steroidal hormone production in Drosophila's larvae are summarized in the model below (Figure 67).

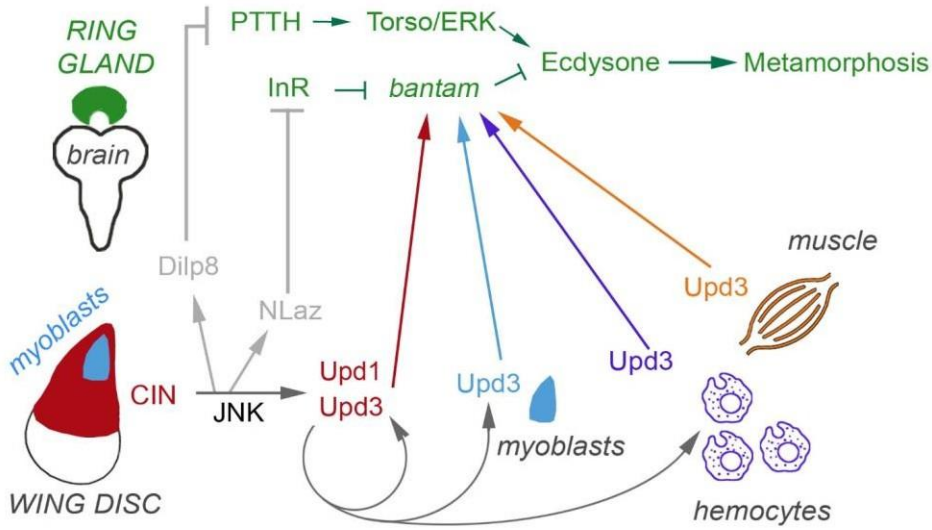

Figure 67. CIN Model of Developmental Timing Systemic Effects

This interplay between systemic impacts and tumoral development brings a new perspective on the regulation of steroidal hormone production during tumorigenesis and opens a new door about the great potential the tumor secretome has in modulating systemic activity.

6. High cytokine levels might be in the base of puberty onset delays in humans

Although in flies the relaxin-like protein Dilp8 has been the molecule most associated with developmental delays in larvae to pupa transitions, in human pathology it is not unforeseen the involvement of cytokines in delaying developmental transitions.

As mentioned before during the Introduction section 3.3 of this thesis, in humans and rodent animal models the release of cytokines and the generation of a strong inflammatory response have been linked to delays in the onset of pupariation. This is particularly true for IBDs, meaning inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis. Patients suffering from these diseases in a prepubertal phase usually present delays in the onset of their puberty transition as well as increased duration of this state (Brain and Savage 1994; Ferguson and Sedgwick 1994). However, these alterations are usually surpassed when removal of the inflamed bowel is done through surgery (Brain and Savage 1994) a strong indication that the inflammation processes in the gut are mediating the childhood to adolescent transition. Despite this observation, patients suffering for IBDs also present strong signs of undernutrition strongly connected to their food-intake behaviors, more than increased losses or needs. Undernutrition has been clearly demonstrated to be a cause of delay in puberty onset. Food deprivation and starving conditions have been observed in both animals and humans as resulting in body and weight reduction, aside from their influence in reducing the activity of the hypothalamic neurons, which in turn reduces the amount of GnRH produced, consequently reducing the production of pituitary gonadotropins,

which affect LH and FSH levels, the precursors for sex steroidal hormone production. Since in human patients' surgical removal also ameliorates food intake, apart from reducing the inflammatory dependent responses, the onset of puberty might also be related with nutrient uptake (Brain and Savage 1994). Aside from surgery, treatment options for these patients usually combine anti-inflammatory drugs with nutritional supplementation (Aiges et al. 1989; Kelts et al. 1979; Kirschner et al. 1981), so it is harder to analyze these variables separately in humans.

Studies in animal models, however, shed some light onto this matter. The TNBS-induced colitis model, the rodent equivalent of Crohn's disease in humans, showed, by controlling food intake in control animals to match the colitis group, that undernutrition does cause a delay on the onset of puberty, but that this delay is smaller than the one presented by animals with TNBS-induced colitis (Duchmann et al. 1996; Rachmilewitz et al. 1989; Yamada et al. 1992). These results strongly support the notion that additional delay is a consequence of the inflammatory mediators that, aside from helping in the generation of inflammation responses can also potentiate the effects of undernutrition, therefore enhancing the delay in the onset and progression through puberty.

Aside from IBDs, delays in puberty onset is also frequent in patients with cystic fibrosis and rheumatoid arthritis, despite the normal nutritional intake observed in these patients (Athreya et al. 1993; Corey et al. 1988; Fraser et al. 1988; Johannesson, Gottlieb, and Hjelte 1997; Kindstedt-Arfwidson and Strandvik 1988; Landon and Rosenfeld 1984; Neinstein et al. 1983). These disorders are

characterized, once again, for having high inflammatory responses, that might be mediating this delay. However, although a correlation between inflammation and high cytokine number and puberty delays have been put forward by the medical community, no concrete study has been put in place to tackle this question. One of the reasons behind this might be that the medical community has been able to resolve these delays in puberty onset either by using a combination of anti-inflammatory drugs and nutritional supplements, surgery or administration of sex steroids.

Although the treatment for these patients has been more or less established for many years, in this thesis we provide proof that other high inflammatory disorders, such as tumorigenesis development, can also be using the same mechanisms to push back developmental transitions.

In tumor patients, mainly the ones with central nervous system associated tumors, puberty delays have also been observed. These delays have been attributed as a consequence of treatment, which nowadays is mainly radiation based. Cranial irradiation is thought to affect the hypothalamic-pituitary axis, preventing the release of either the growth hormone, GH, or the pituitary hormones, LH and FSH (Haas et al. 1983). Development of radiation-induced hypothalamic-pituitary failure may take several years to overcome, and the prevalence of gonadotropin deficiencies in childhood cancer survivors might reach the 10.8% (Wallace 2011).

Although it is true that radiation might influence the puberty onset in these CNS tumors, an inflammation-dependent response was never carefully analyzed for these situations.

Discussion

In this thesis we focus mainly on CIN-derived tumorigenesis, normally associated with carcinoma development, and present in 80% of all the solid tumors diagnosed. We present evidence that these tumors are highly secretory masses able to mediate systemic responses in peripheral organs and hijack the timing of developmental transitions. Within these secreted molecules, cytokines are highly produced and released from the initial tumoral site and they act to delay the larvae to pupa transition, the equivalent of puberty onset in humans, by preventing the production of ecdysone, the equivalent to humans' sex steroidal hormones. Although in our model we show that the immune response mediator cells, the hemocytes, are solely involved as sources of upd3 release, and not in the repression of ecdysone biosynthesis directly, we provide strong evidence that the high systemic levels of cytokines are influencing the developmental transition. Tumors, like the one represented in our model, can also be considered highly inflammatory disorders since the presence of cytokines in these systems is incredibly high, and they can be used to establish the connection between the impact of these secretory molecules in developmental transitions.

That being stated, in this thesis we provide a strong evidence that cytokines might be involved in the control of life cycle progression in highly inflammatory situations, as well as we provide new evidence that JAK/STAT might be a major influencer in the regulatory network for steroidal hormone production.

CONCLUSIONS

Conclusions

Based on the experiments carried out during the elaboration of this thesis, I can extract the following conclusions:

1. CIN induction in the wing primordia of otherwise healthy flies leads to severe alterations in pupariation timing.

2. Providing an active form of ecdysone steroid hormone rescues the CIN delay.

3. The CIN delay is JNK-dependent but independent of tumor size.

4. Dilp8 relaxing-like protein is able to produce a 24 hours rescue of the pERK peak in the PG, acting on the PTTH-dependent regulatory network of ecdysone production.

5. The PG of CIN larvae shows low levels of IIS signaling and high bantam expression levels, a sign of severe affection of the IIS dependent regulatory network for ecdysone production.

6. NLaz, a lipocalin that mediates IIS response, is capable of rescuing these low levels of IIS in CIN ring glands but it is not able to regulate bantam.

7. Co-depletion of both Dilp8 and NLaz is unable to provide a partial rescue of the CIN delay however; both proteins are able to generate delays when overexpressed in wing discs of healthy larvae.

8. JAK/STAT induction in the CIN PG increases exponentially over time and regulates bantam expression independently of IIS response.

Conclusions

9. Upd1 and Upd3 cytokines are highly expressed in CIN wing primordia and their co-depletion or trapping causes a complete rescue of the CIN delay.

10. Overexpression of Upd3 alone in the wing primordia recapitulates CIN delay, but Upd1 is not able to produce the same effect on its own.

11. Single depletion of either Upd3, Upd1 or Dome from the initial tumor site is not able to rescue CIN delay.

12. Hemocytes and Muscle tissues are able to contribute to the systemic Upd3 cytokine pool in a CIN background.

13. Systemic depletion of Upd3 is able to provide a fully bantam-dependent rescue of CIN delay.

14. Specific depletion of JAK/STAT players in the ring gland of CIN larvae perfectly rescues CIN delay, STAT-GFP and bantam reporter levels to control values.

15. CIN delay is dependent on a new regulatory network for ecdysone production that acts through JAK/STAT regulation of bantam expression.

MATERIALS AND METHODS

Experimental Model and Subject Details

Fly Husbandry

Tumor induction: Flies were let laying eggs on standard ecologic fly food for 12 hours at 25°C, and switched to 29°C for 4 days after which they were kept at room temperature and dissected at 130 hours, for all experiments with just one time point dissection, or for additional time points in particular experiments mentioned in the Results section.

Pupariation assays: Flies were allowed to lay eggs on standard ecologic fly food for 4 hours at 25°C and switched to 29°C for 4 days after which they were kept at room temperature. Pupas were counted every day at the same defined hour from 108 to 358 hours, accounting for a period of approximately 5 to 15 days' time.

Feeding assays: Flies were let laying eggs on standard ecologic fly food for 5 hours at 25°C and switched to 29°C for 4 days after which they were switched to standard ecologic blue fly food. Every 24 hours larvae were photographed and measured.

Ecdysone assays: Flies were allowed to lay eggs on standard ecologic fly food for 4 hours at 25°C and switched to 29°C. At 84 hours they were selected for early L3 stage characteristic and switched to food supplemented with ecdysone. Pupas were counted until all larvae made the transition to the next metamorphosis stage.

Materials and Methods

Drosophila Strains

The following strains were provided by the following sources: (1) Bloomington Drosophila Stock Center (BDSC): *ap-GAL4; UAS-myristoylated-Tomato; UAS-gfp^{RNAi}; elav-Gal80; UAS- wg^{RNAi}; UAS-wg^{RNAi}; UAS-dilp8mimic-GFP; UAS-p35; UAS-bskDN; en-GAL4; UAS-STATGFP10x (III); UAS- ∆upd3; UAS- upd1osupd3os; hemese-GAL4; croquemort-GAL4; UAS-upd1^{RNAi}; UAS-upd3^{RNAi}*; *UAS-hop^{RNAi}; UAS-upd2^{RNAi}*; *UAS- ∆upd2;* (2) Vienna Drosophila RNAi Center (VDRC): UAS-bub3^{RNAi}; UAS-dilp8^{RNAi}; UAS-rod^{RNAi}; UAS-NLaz^{RNAi}; UAS-NLaz; UAS-avalanche^{RNAi}; UAS-Dome^{RNAi} and (3) *UAS-puc;* kindly provided by E. Martín Blanco*; UAS-bantam sensor* kindly given by S. Cohen*; UAS-tGPH sensor* gifted from H. Stocker *; UAS-dilp8* granted by P. Leopold*; UAS-upd3lacZ* offered by H. Jiang*; UAS STATGFP10X* reporter line provided by M. Dominguez*, UAS hop* donated by J. Castelli*, UAS upd3* kindly provided by D. Harrison*, P0206-gal4* given by H. Jasper, *UAS-DomeDN* (II) kindly sent by James Hombría, *escargot*-gal4 was kindly facilitated by J. Casanova, the *byn*-gal4 line was kindly sent by Ross Cagan, *upd3*-gal4 kindly granted by B. Lemaitre and the *phm*-lexA line was kindly given by Gary Struhl's lab. Flies were allowed to lay eggs on fly food for 12 h at 25°C for dissection assays and 4h at 25°C for pupariation assays. Larvae were then switched to 29ºC for 4 days before dissection or pupa counts took place.

Immunostaining and Confocal Imaging

Wing imaginal discs, brains, ring glands, trachea and fat body tissues of third instar larvae were dissected in cold PBS, fixed in

4% formaldehyde for 20 min and immunolabeled in BBT (PBS with 0.3% BSA, 0.2% Triton X-100) with the following antibodies: mouse anti-MMP1 (1:20) (14A3D2, Developmental Studies Hybridoma Bank, DSHB); rat anti-Ci (1:10; 2A1, DSHB); rabbit and mouse anti-GFP (1:600) (Molecular Probe, A6455 and A11120, respectively); rabbit anti β-galactosidase (1:600) (Cappel; 0855976); mouse anti-Cut (1:100) (2B10, Developmental Studies Hybridoma Bank, DSHB), rabbit polyclonal anti-p-ERK (1:100) (4370, Cell Signaling Technology) and mouse anti-NIMC1 kindly provided by I. Andó.

For the muscle tissues, the general proceeding was the same as described previously except for an extended time of fixation of 30 minutes after filet dissection after which STATGFP10X reporter was analyzed. Secondary antibodies Cy2 and Cy5 (1:400) were obtained from Jackson ImmunoResearch. Leica TCS SP5 MP confocal microscope co was used to perform high resolution images.

LexO-STAT-RNAi cloning

To generate the LexAop-stat RNAi construct, the following oligos were used.

stat-i Fwd (EcoRI)

CTA GCA GTT CGC AGC TTT ATG TAC GAT AGT TAT ATT CAA GCA TAT CGT AGT ACA AGC TGC GAG CG

stat-i Rev (XbaI)

AAT TCG CTC GCA GCT TTA TGT ACT ACG ATA TGC TTG AAT ATA ACT ATC GTA CAT AAA GCT GCG AAC TG

9.5µl of each oligo (100µM) was used to mix with 1µl of annealing buffer (20X SSC). The mix was heated at 100°C for 10 minutes and let to cool down overnight for an efficient annealing. 1/200 dilution of the annealed mix was used to ligate in the pLOT plasmid (carrying the lexA operator), previously digested with EcoRI and XbaI endonucleases.. The plasmid was transformed in DH5alpha competent cells and 5 colonies were selected for checking. To assess for the correct generation of vector and oligos combined, the absence of the XhoI3461bp digestion site was checked. Plasmids with the correct size and absence of the XhoI site were sequenced and one positive clone was used to generate transgenic flies with standard protocols.

Pupariation Assays

Flies were allowed to lay eggs for 4 h (or a maximum of 5 h for difficult phenotypes, namely the mutants) at 25°C after which they were switched to 29ºC for a total of 4 days. After these 4 days of induction, tubes were taken from the bath and placed at room temperature to allow for counting from 108 to 358 h after egg deposition, accounting for a period of approximately referent from 5 to 15 days' time. Counts were performed always at the same time point and 24h apart. The resulting percentage of pupae was calculated accordingly with the total number of larvae capable of transitioning to the next developmental phase.

Larval Feeding Assays

For the blue food feeding assays flies were allowed to lay eggs for a maximum of 5 hours at 25°C and they switched for 4 days at 29ºC. After this induction period larvae were transferred to plates with food supplemented with Bromophenol Blue (Sigma-Aldrich 114391_5G). After which L3 larvae were isolated from each plate at different time points. The presented data refer to the highly relevant time points of 132h, 156h, 180h and 204h imaged by the Inverted Microscope Olympus IX71 SCMOS.

Ecdysone Feeding Assays

Flies were allowed to lay eggs on standard ecologic fly food for 4 hours at 25°C and switched to 29°C. At 84 hours they were selected for early L3 stage characteristics and switched to food supplemented with an activated form of ecdysone. Stock solution for the steroidal hormone was prepared for a 5mg/ml concentration from 5mg 20-ecdysone powder (Sigma) in 1mL of EtOH. Working solution was adjusted by diluting the initial stock solution in PBS until a final concentration of 0,2 mg/ml. Control solution was done by preparing the same amount of EtOH in PBS. The amount of hormone and control solution were calculated to allow for the dilution to add up to a total volume of 100 microliters. Every dilution was prepared fresh every day, and 50 microliters were added first thing in the morning and latest thing in the afternoon, respecting a 10-hour window. In each vial, food was

moved slightly previously to addition of the hormone or control solution to facilitate diffusion.

Quantitative RT-PCR

For the quantification of mRNA levels, total RNA was extracted from wing discs and muscles of at least 25 and 15 larvae, respectively, using TRIzol reagent (Invitrogen). A total of 1.5 µg of total RNA was treated with DNase and used as a template for cDNA synthesis using Maxima Reverse Transcriptase (Thermo Scientific). Maxima SYBR Green/ROX qRT-PCR Master Mix (Thermo Scientific) was used, and reactions were run in a QuantStudio 6 Pro Real-Time PCR Systems (Applied Biosystems). Samples were normalized to the levels of forkhead domain 68A (Mnf, for wing disc samples), or Actin42A (Act, for muscle samples) transcript levels and fold changes were calculated using the ΔΔCt method. Three separate biological samples were collected for each experiment. The following primer pairs were used: Mnf-Fw: 5'- GAG CAG AAG AGC CCC TAC CT-3', Mnf-Rv: 5'-AAT GAA ACC CTG ACG TGG AC-3' (Ponton et al., 2011); Act-Fw: 5'-GCG TCG GTC AAT TCA ATC TT-3', Act-Rv: 5'-AAG CTG CAA CCT CTT CGT CA-3'(Ponton et al., 2011); Dilp8-Fw: 5'- GCA CCA CCA TCT GAA TCG AC-3', Dilp8-Rv: 5'- CTG AGG CGA TTG AAG TGC TC -3'; NLaz-Fw:5'- GCC AGA AGT AGA ACG GAT ACC A-3', NLaz-Rv:5'- ACT GGT GCA GCT GTA GAC GAC-3' (Hull-Thompson et al., 2009); Upd1-Fw: 5'-AGA CAG CCG TCA ACC AGA C-3', Upd1-Rv: 5'- AGA CAG CCG TCA ACC AGA C-3' (36, wing disc samples); Upd1-Fw: 5'- TGT AAC CCC GTT CGC TGT AT-3', Upd1-Rv: 5'- GCT GAT GTT TCC GTT TCC GT-3' (muscle samples); Upd3-Fw: 5'- ATC CCA CCA

ATC CCC TGA AG-3', Upd3-Rv: 5'-AGA TTG CAG GTG TTC TCC CA-3'

Quantification and Statistical Analysis

Image Processing and Analysis

Fiji [National Institute of Health (NIH) Bethesda, MD] was used to measure the size of the anterior (a), dorsal (d) compartments (based on Ci and MyrT expression respectively), or the whole wing discs (based on DAPI staining), and to monitor reporter intensities (specifically bantam, tGPH, STATGFP and upd3-lacZ reporter lines). Image stacks were obtained using a Leica TCS SP5 MP confocal microscope, 20X dry objective with 1 µm per optical section to cover the entire thickness of each disc. Maximum intensity Z-projection was performed on the stacks prior to reporter analysis. Control wing discs grown in parallel and subjected to the same experimental conditions (temperature and time of transgene induction) were analyzed in parallel. At least 10 wing discs per genotype were scored. In the special case of the myoblast population, 40x glycerol immersion objective was chosen to facilitate the acquisition of high-quality images of both tumor and myoblast cells.

Statistical Analysis

Statistical analysis was generally performed by unpaired equal-variance two-tail t-test. Differences were considered significant when p values were less than 0.001 (***), 0.01 (**), or 0.05 (*). All genotypes included in each histogram were analyzed in parallel. All data points were graphed in Prism 7.0 (Graphpad) statistical software.

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PUBLICATIONS

The Upd3 cytokine couples inflammation to maturation defects

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Summary

Developmental transitions such as puberty or metamorphosis are tightly controlled by steroid hormones and can be delayed by the appearance of growth abnormalities, developmental tumors or inflammatory disorders, such as inflamed bowel disease or cystic fibrosis (Andersen et al., 2013; Ballinger et al., 2003; Brain and Savage, 1994; Tennessen and Thummel, 2011). Here we used a highly inflammatory epithelial model of malignant transformation in *Drosophila* (Dekanty et al., 2012; Muzzopappa et al., 2017) to unravel the role of Upd3 - a cytokine with homology to interleukin 6 - and the JAK/STAT signaling pathway in coupling inflammation to a delay in metamorphosis. We present evidence that Upd3 produced by malignant cell populations signals to the prothoracic gland - an endocrine tissue primarily dedicated to the production of the steroid hormone ecdysone - to activate JAK/STAT and *bantam* miRNA and to delay metamorphosis. Upd cytokines produced by the tumor site contribute to increasing the systemic levels of Upd3 by amplifying its expression levels in a cell autonomous manner and by inducing Upd3 expression in neighboring tissues in a non-autonomous manner, culminating in a major systemic response to prevent larvae from initiating pupa transition. Our results identify a new regulatory network impacting on ecdysone biosynthesis and provide new insights into the potential role of inflammatory cytokines and the JAK/STAT signaling pathway in coupling inflammation to delays in puberty.

Highlights

- Highly inflammatory epithelial tumors cause a strong developmental delay
- Upd3 cytokine produced by malignant cells signals to the prothoracic gland
- JAK/STAT signaling in the ecdysone-producing compartment impacts on the *bantam* miRNA
- JAK/STAT signal amplifies the systemic levels of Upd3

eTOC Blurb

Inflammatory tissues cause developmental delay and animal lethality. *Romäo et al* present evidence that the Upd3 cytokine is produced by inflammatory and adjacent tissues to cause developmental delay by interfering with the production of the hormone ecdysone.

Results

Highly inflammatory tumors produce Dilp8 and Neural Lazarillo to signal to the prothoracic gland

The *Drosophila* wing primordium, which proliferates exponentially during the larval stage to give rise to the adult wing after metamorphosis, has been extensively used to model epithelial tumorigenesis (Pastor-Pareja and Xu, 2013). RNAi-mediated downregulation of the Spindle Assembly Checkpoint (SAC) gene *bub3* in wing primordia leads to high levels of chromosomal instability (CIN, an increased rate in the loss or gain of chromosomes and a hallmark of most solid tumors of epithelial origin) which, upon apoptosis inhibition, induces tumor-like overgrowths with an unlimited growth potential [CIN-tumors, (Dekanty et al., 2012; Muzzopappa et al., 2017)]. This growth potential relies on the activation of the JNK pathway to drive the expression of several secreted proteins including the mitogenic molecule Wingless. Larvae containing CIN-tumors entered metamorphosis three and a half days after control larvae (Figure 1A). The presence of an *elav-gal80* transgene to block Gal4 activity in the brain (Figure S1A) partially reduced the delay but these larvae still entered metamorphosis two and a half days after control ones (Figure 1A). The observed developmental delay was accompanied by an increase in larval volume and non-interrupted feeding behavior (Figure 1B) and was rescued upon feeding with an activated form of the steroid hormone ecdysone (20E, Figure 1C). Blocking JNK also rescued the CIN-induced delay (Figure 1D), and this rescue was not a consequence of reduced tumor growth. Thus, whereas JNK blockage and Wingless depletion reduced tumor growth, the developmental delay was rescued only in the first case (Figure 1D and S1B, C).

Ecdysone biosynthesis in the prothoracic gland (PG) can be positively regulated by two pathways: the developmental pathway, which depends on the release and action of the prothoracic hormone (PTTH) from the brain to activate ERK signaling in the PG (McBrayer et al., 2007), and the nutritional pathway, which depends on insulin (IIS) signaling positively regulating ecdysone production through a double repression mechanism mediated by *bantam* miRNA (Boulan et al., 2013; Colombani et al., 2005). Dilp8, a relaxin-like protein released from abnormally growing primordia to inhibit entry into metamorphosis by preventing the release of the PTTH hormone (Colombani et al., 2015; Garelli et al., 2015; Vallejo et al., 2015), was ectopically induced in CIN-tissues in a JNK-dependent manner (Figure 1E, F and S1D). Consistently, the peak of ERK phosphorylation caused by PTTH release was delayed by 24 h in larvae containing CIN-

tumors, and Dilp8 depletion restored the ERK peak to its control values (Figure 1G). Surprisingly, depletion of Dilp8 by two different RNAi lines did not rescue the CIN-induced developmental delay (Figure 1H). Consistent with previous reports (Colombani et al., 2012; Garelli et al., 2012), Dilp8 depletion partially rescued the developmental delay of aberrantly growing wing discs and Dilp8 caused a developmental delay when overexpressed with the wing specific *rn-gal4* driver in otherwise healthy wing discs (Figure S1E, F). We observed that IIS signaling [monitored by a PH-GFP fusion protein used as an indicator of PI3K activity, (Britton et al., 2002)] was downregulated and *bantam* activity [monitored by a sensor that ubiquitously expresses GFP, carries two perfect *bantam* fixation sites in its 3´UTR and is therefore repressed in the presence of the miRNA, (Brennecke et al., 2003)] was increased in the PG of CIN-larvae, and that these two changes were restored to control levels upon depletion of JNK activity at the tumor site (Figure 1I). Initially described as a target of JNK signaling in *Drosophila* (Hull-Thompson et al., 2009), the lipocalin Neural Lazarillo (NLaz) was upregulated in CIN-tumors by JNK activity (Figure 1F and S1H). Consistent with the role of NLaz in antagonizing IIS signaling in peripheral tissues (Pasco and Léopold, 2012), ectopic expression of this lipocalin in the PG (with the *P0206-gal4* driver) caused a reduction in IIS signaling, an increase in *bantam* activity and a developmental delay (Figure 1I, L). Overexpression of NLaz in wing discs (with *rn-gal4*) caused a similar effect on developmental timing (Figure 1K). Although RNAi-mediated depletion of *NLaz* from the initial tumor site restored IIS signaling levels in the PG, the developmental delay was not rescued and *bantam* activity levels remained high (Figure 1I, J, see also Figure S1I). These results indicate that CIN-tumors impact on the activity of the developmental and nutritional pathways in the PG through Dilp8 and NLaz, and that *bantam* activity levels in the PG are independently regulated by another JNK-target. We did not detect any major change in IIS signaling or *bantam* activity in the PG of larvae containing aberrantly growing wing discs (Figure S1G), suggesting that the impact on the nutritional pathway is specific to highly inflammatory conditions.

Upd3 produced by tumor cells regulates *bantam* activity in the prothoracic gland

We found that the cytokines Upd1 and Upd3 - which bind to the receptor Domeless [Dome, (Brown et al., 2001)] to activate the JAK-STAT pathway – were induced in CIN-tumors (Fig. 2A-C), and that the JAK/STAT pathway was ectopically activated in the PG of larvae carrying CIN-tumors (Fig. 2D). JNK blockage in CIN-

tumors reduced Upd1 and Upd3 expression levels and JAK/STAT signaling in the PG (Fig. 2C, D). These results point to a role of JNK-driven expression of Upds in the CIN-induced delay and suggest that this delay is a result of JAK/STAT signaling in the PG. Consistent with this proposal, targeted expression of Upd1, Upd3 or activation of JAK/STAT in the PG caused a developmental delay (Fig. 2F and S2B). Interestingly, *bantam* was also activated under these circumstances (Figure 2G). In contrast, IIS signaling was not affected, thereby suggesting that the observed impact of the CIN-tumors on IIS signaling (Figure 1I) is a consequence of NLaz activity downstream of JNK. We next addressed the capacity of Upd1 and Upd3 to signal from the wing disc to the PG to regulate developmental timing. Whereas Upd3 caused a developmental delay when overexpressed in otherwise healthy wing discs and this delay was accompanied by the activation of JAK/STAT signaling and *bantam* in the PG (Figure 2F, H), overexpression of Upd1 in wing discs did not have any impact on developmental timing (Figure 2F). These results indicate that Upd3 is the systemic signal produced by tumor cells and that it acts directly on the PG to positively regulate *bantam* activity levels and, consequently, block the production of ecdysone.

CIN-induced developmental delay relies on Upd3 produced by tumor cells and nearby cell populations

Surprisingly, depletion of *upd3* specifically in CIN-tumors did not rescue the observed developmental delay (Figure 3A) and co-depletion of *upd1* and *upd3* was required to achieve successful rescue of developmental timing (Figure 3B and S3A). Overexpression of Dome$^{\Delta CYT}$, a truncated form of Dome lacking the intracellular domain [Dome-DN, (Brown et al., 2001)], also rescued the CIN-induced developmental delay (Figure 3C) and the JAK/STAT and *bantam* activity levels in the PG (Figure 3E, G). This rescue was not the simple result of reducing the activity of the JAK-STAT pathway in CIN tissues, as depletion of JAK/STAT with RNAi lines against *dome* or *hop* did not have any effect on the observed developmental delay (Figure 3D). We support the proposal that the capacity of Dome-DN to rescue the CIN-induced developmental delay relies mainly on its ability to trap Upd ligands. Consistent with this proposal, overexpression of Dome-DN rescued the developmental delay caused by Upd3 overexpression in wing disc cells (Figure 2F). All these results indicate that CIN-tumors produce Upd1 and Upd3 to induce a developmental delay, but only

Upd3 is able to act as a systemic signal to activate JAK/STAT signaling and *bantam* miRNA in the ecdysone-producing compartment.

These results can be explained by two possible scenarios. In the first scenario, Upd1, together with Upd3, might act locally in the wing disc to induce the expression of other long-range systemic signals to regulate, together with Upd3, ecdysone production. Consistent with this notion, overexpression of Dome-DN in CIN tissues reduced the expression levels of Dilp8 and NLaz (Figure S4A, B), and rescued the impact on the activity levels of the IIS signaling pathway at the PG (Figure 3F). However, co-depletion of two of the three systemic signals from the tumor site did not rescue, even partially, the developmental delay (Figure S4C). In a second scenario, a feed-forward loop mediated by the local activities of Upd1 and Upd3 might amplify their expression levels in a cell autonomous manner and spread the expression of Upd3, the major contributor to the developmental delay, in a non-autonomous manner to nearby cell populations. Consistent with the first part of this proposal, Dome-DN overexpression in CIN-tumors reduced the expression levels of Upd1 and Upd3 (Figure 4A, B). In agreement with the notion of the spread of Upd3 expression to nearby cell populations, JAK/STAT signaling and Upd3 expression were ectopically induced in myoblasts (a nearby non-epithelial population of precursors of adult muscles) and in larval muscles, and this ectopic induction was blocked by targeted overexpression of Dome-DN to CIN-tumors (Figure 4C, D, S4D). We also observed the recruitment of a considerable number of hemocytes (a physiological source of Upd3) to the CIN-tumor (Figure S4E). Interestingly, targeted overexpression of Upd3 in myoblasts, larval muscles or hemocytes induced a developmental delay, thereby validating their potential role as a source of Upd3 and their contribution to the CIN-induced delay (Figure S4F-I). Two independent experimental observations support the notion that the CIN-induced developmental delay relies mainly on the activity of Upd3 produced by different tissues acting on the PG. First, a deletion allele of *upd3* (*Δupd3*, (Osman et al., 2012)) rescued the CIN-induced developmental delay and restored the activity of the nutritional pathway to control levels in the PG (Figure 4E, F). Second, combining the GAL4/UAS and LexA/LexAop systems to drive CIN in larval wing primordia and genetically blocking JAK/STAT signaling in the PG rescued the CIN-induced developmental delay and the activity levels of STAT in the PG but not in the wing disc (Figure 4G-I). *bantam* activity levels in the PG were also rescued under these circumstances (Figure 4J). These results

unequivocally demonstrate the central roles of Upd3 and JAK/STAT signaling in the CIN-induced developmental delay.

Discussion

The developmental regulation of puberty in humans and metamorphosis in flies relies on the timely production of steroid hormones (Sisk and Foster, 2004; Tennessen and Thummel, 2011). Here we present evidence that flies carrying highly inflammatory tissues present a very strong delay in developmental maturation, similar to human patients suffering from inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, and we identify fundamental roles of the Upd3 cytokine and the JAK/STAT signaling pathway in triggering this delay. In this work, we used an epithelial model of CIN (Clemente-Ruiz et al., 2016; Dekanty et al., 2012; Muzzopappa et al., 2017), to first show that highly inflammatory epithelial tumors cause a developmental delay that is independent of the size of the tumor. We identify the relaxin Dilp8, the lipocalin Neural Lazarillo (NLaz) and the cytokine Upd3 as three secreted proteins regulated by JNK at the tumor site that have a strong capacity to act systemically and block from a distance ecdysone production in the PG (Figure 4K). Whereas Dilp8 is known to reduce ecdysone production indirectly by impinging negatively on the production of the PTTH neuropeptide (Colombani et al., 2012; Garelli et al., 2012), our data indicate that NLaz and Upd3 signal directly to the PG to activate, through the ISS and JAK/STAT signaling pathways, *bantam* miRNA, a repressor of ecdysone production (Boulan et al., 2013). Despite the expression of Dilp8 and NLaz in CIN tissues and their strong capacity to block ecdysone production in the prothoracic gland when overexpressed in epithelial tissues, our experimental data indicate that the major contributor to the CIN-induced developmental delay is Upd3 and that local and systemic activities of the JAK/STAT pathway play fundamental roles in this delay. Whereas autocrine JAK/STAT signaling in the tumor site, driven by JNK-dependent expression of the systemic cytokine Upd3 and the locally acting cytokine Upd1, contributes to amplifying Upd3 expression levels at the tumor site, paracrine JAK/STAT signaling mediated by Upd1 and Upd3 contributes to induce ectopic sources of Upd3 expression in nearby cell populations. Non-autonomous induction of Upd3 expression in myoblasts and larval muscles, recruitment of hemocytes (a natural source of Upd3 expression) and the most probable amplification of these hemocytes as a consequence of high levels of Upd cytokines in the medium would all contribute to

amplifying the systemic levels of Upd3. These results will certainly provide key insights into a potential role of IL-6, the human homologue of Upd3, and the JAK/STAT pathway in puberty delays caused by inflammation.

Acknowledgments

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Author Contributions

D.R. and M.Mi. conceived and designed the experiments. D.R., L.B. and M.Mu. performed the experiments. All authors analyzed the data. M.Mi., with the help of D.R., wrote the paper.

Declaration of interests

The authors declare no competing interests.

Figures

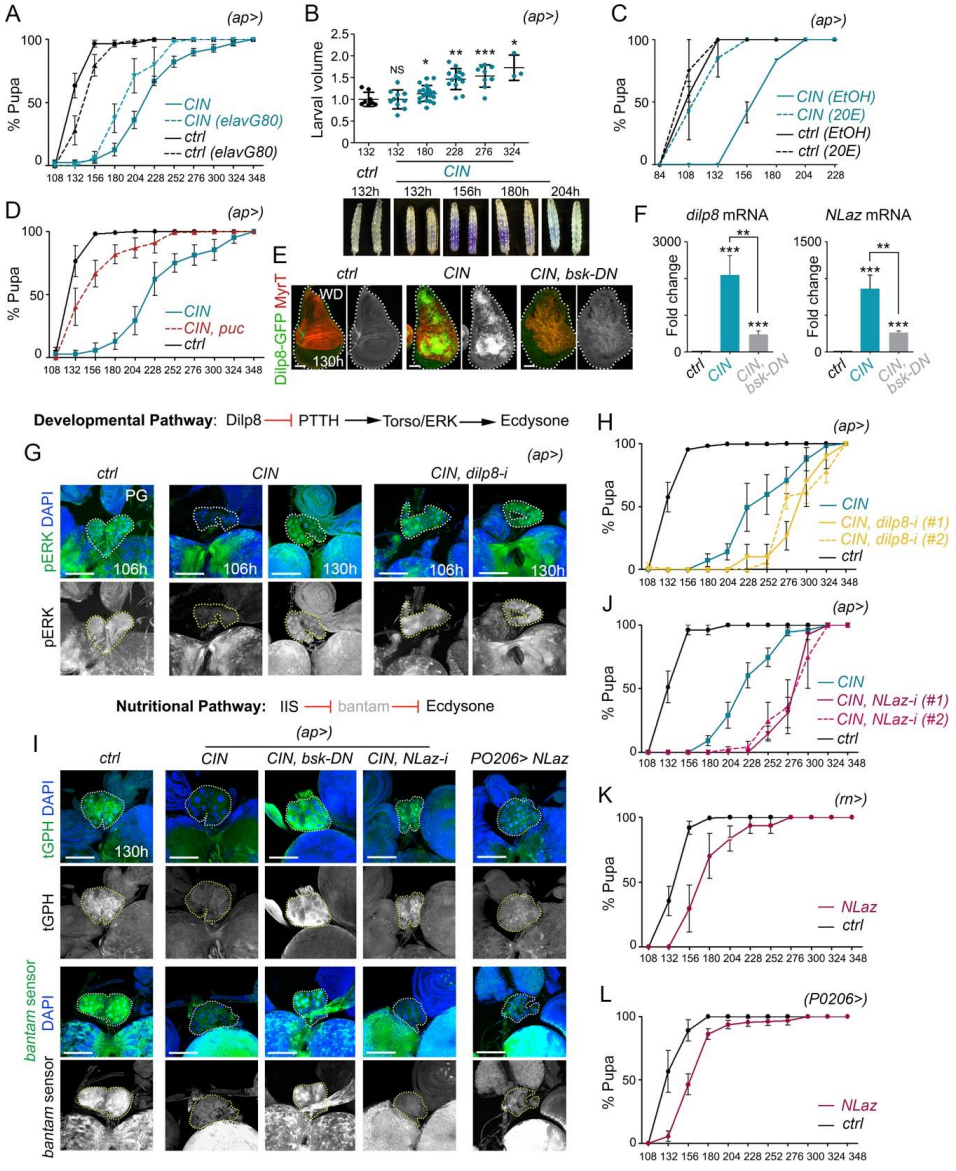
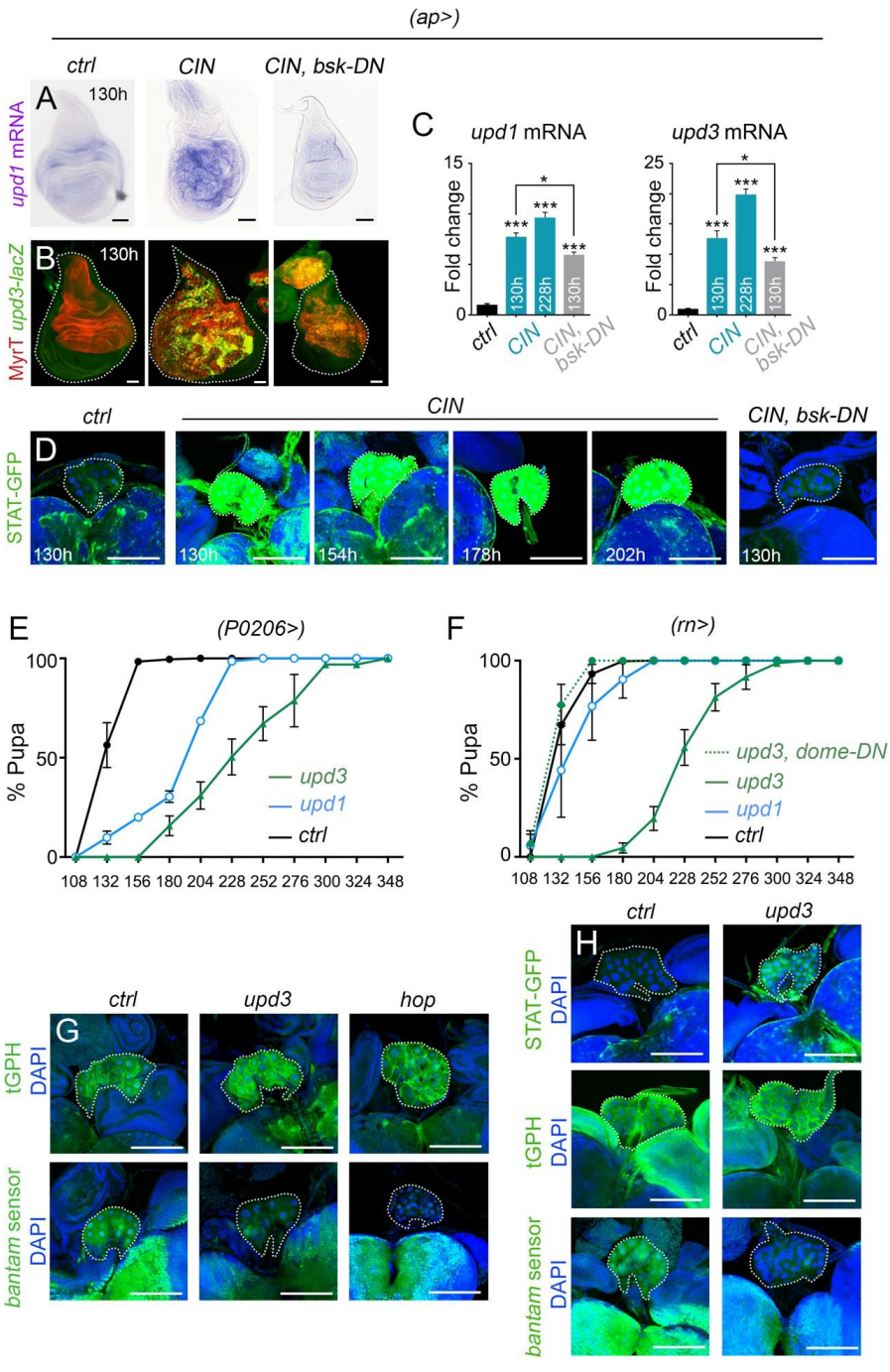

A
(ap>)
% Pupa
CIN
CIN (elavG80)
ctrl
ctrl (elavG80)
B
(ap>)
Larval volume
NS
ctrl
CIN
132h
156h
180h
204h
C
(ap>)
CIN (EtOH)
CIN (20E)
ctrl (EtOH)
ctrl (20E)
D
(ap>)
CIN
CIN, puc
ctrl
E
ctrl
CIN
CIN, bsk-DN
Dilp8-GFP MyrT
WD
130h
F
dilp8 mRNA
NLaz mRNA
Fold change
Developmental Pathway: Dilp8 ⊣ PTTH → Torso/ERK → Ecdysone
G
(ap>)
ctrl
CIN
CIN, dilp8-i
pERK DAPI
pERK
PG
106h
130h
Nutritional Pathway: IIS ⊣ bantam ⊣ Ecdysone
H
(ap>)
CIN
CIN, dilp8-i (#1)
CIN, dilp8-i (#2)
ctrl
I
(ap>)
ctrl
CIN
CIN, bsk-DN
CIN, NLaz-i
PO206> NLaz
tGPH DAPI
tGPH
bantam sensor DAPI
bantam sensor
J
(ap>)
CIN
CIN, NLaz-i (#1)
CIN, NLaz-i (#2)
ctrl
K
(rn>)
NLaz
ctrl
L
(P0206>)
NLaz
ctrl

Figure 1. CIN tissues induce a JNK-dependent developmental delay.

(**A**, **B**) Developmental timing (**A**), volume (**B**, top) and feeding behavior (**B**, bottom) of larvae carrying wing discs subjected to CIN with the *ap-gal4* driver. (**C**, **D**, **H**, **J**) 20E treatment (**C**) and *puc* expression (**D**) but not Dilp8 or NLaz depletion (**H**, **J**) rescued the developmental delay of CIN larvae. (**E**) Wing imaginal discs expressing the indicated transgenes and stained for MyrT (red) and Dilp8-GFP (green). (**F**) *dilp8* and *NLaz* mRNA levels by qRT-PCR in wing discs expressing the indicated transgenes. (**G, I**) Schematic representation of the ecdysone production regulatory networks and ring glands of larvae expressing the indicated transgenes in wing discs (*ap-gal4*) or in the PG (*P0206-gal4*), and stained for pERK (green or white, **G**), tGPH (green or white, **I**), *bantam* sensor (green or white, **I**) and DAPI (blue). (**K**, **J**) Developmental timing of larvae expressing the indicated transgenes in wing discs (**K,** *rn-gal4*) or in the PG (**J,** *P0206-gal4*). Contour of wing discs and ring glands is marked by a dotted line. Scale bars, 50 µm (**E**), 100 µm (**G**, **I**). Data represent mean ± SEM (puparation assays and qRT-PCR) or SD (larval volume). ***p < 0.001, **p < 0.01, *p< 0.05. n=10-272 (puparation assays), n=3-20 (larval volume). See also Figure S1.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ctrl
CIN
CIN, bsk-DN
A
130h
upd1 mRNA
B
130h
MyrT upd3-lacZ
C
upd1 mRNA
upd3 mRNA
Fold change
15
25
0
130h
228h
130h
ctrl
CIN
CIN, bsk-DN
D
STAT-GFP
130h
154h
178h
202h
E
(P0206>)
% Pupa
100
50
0
upd3
upd1
ctrl
108 132 156 180 204 228 252 276 300 324 348
F
(rn>)
upd3, dome-DN
G
upd3
hop
tGPH
DAPI
bantam sensor
H
STAT-GFP

Figure 2. Upd3 produced by tumor cells regulates *bantam* activity in the prothoracic gland.

(**A-B**) Wing imaginal discs expressing the indicated transgenes (**A**, **B,** *ap-gal4*) and stained for *upd1* mRNA (purple, **A**), *upd3-lacZ* (green, **B**) and MyrT (red, **B**). (**C**) *upd1* and *upd3* mRNA levels by qRT-PCR in wing discs expressing the indicated transgenes. ***p < 0.001, *p< 0.05, data represent mean ± SEM. (**D**, **G**, **H**) Ring glands of larvae expressing the indicated transgenes in wing discs (*ap-gal4,* **D***,* or *rn-gal4,* **F**) or in the PG (*P0206-gal4,* **E**), and stained for DAPI (blue), *STAT-GFP* (green, **D**, **H**), and tGPH or *bantam* sensor (green, **G**, **H**). (**E**, **F**) Developmental timing of larvae expressing the indicated transgenes in the PG (**E**, *P0206-gal4*) or in wing discs (**F**, *rn-gal4*). n=38-282, data represent mean ± SEM. Contour of wing discs and ring glands is marked by a dotted line. Scale bars, 50 μm (**A**, **B**), 100 μm (**D**, **G**, **H**). See also Figure S2.

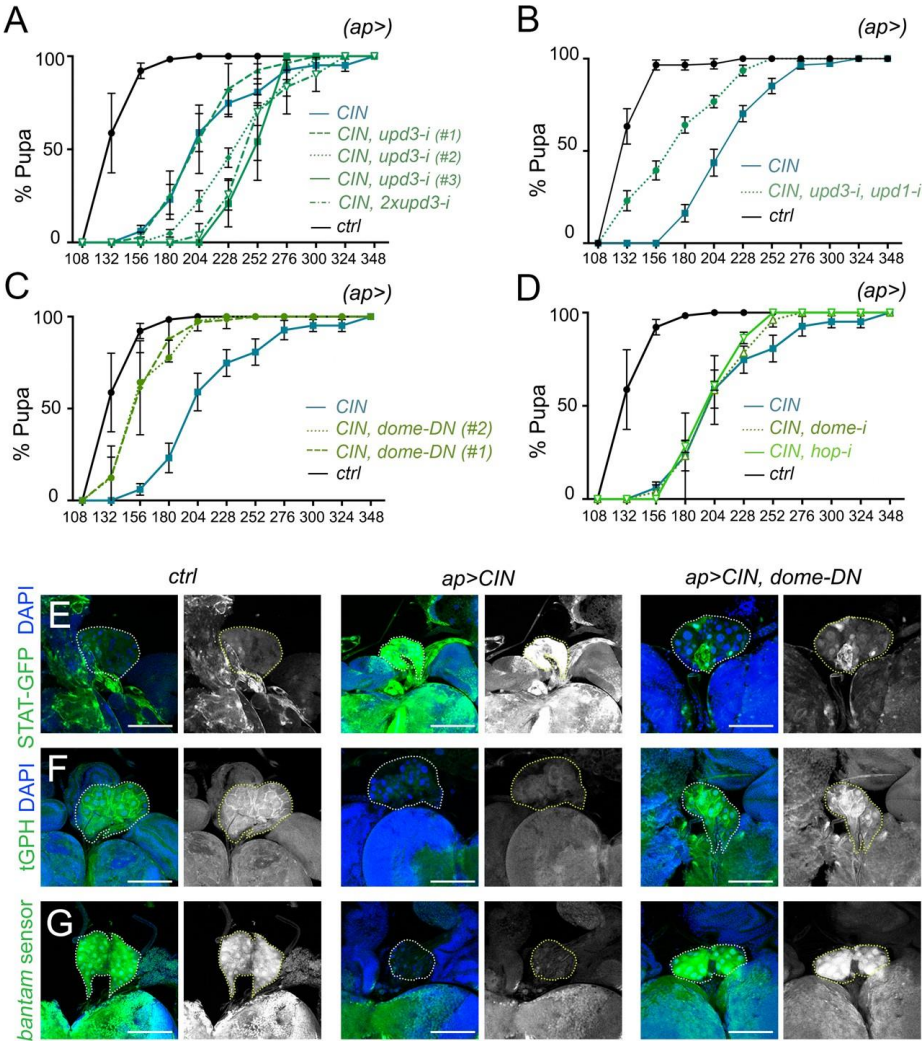
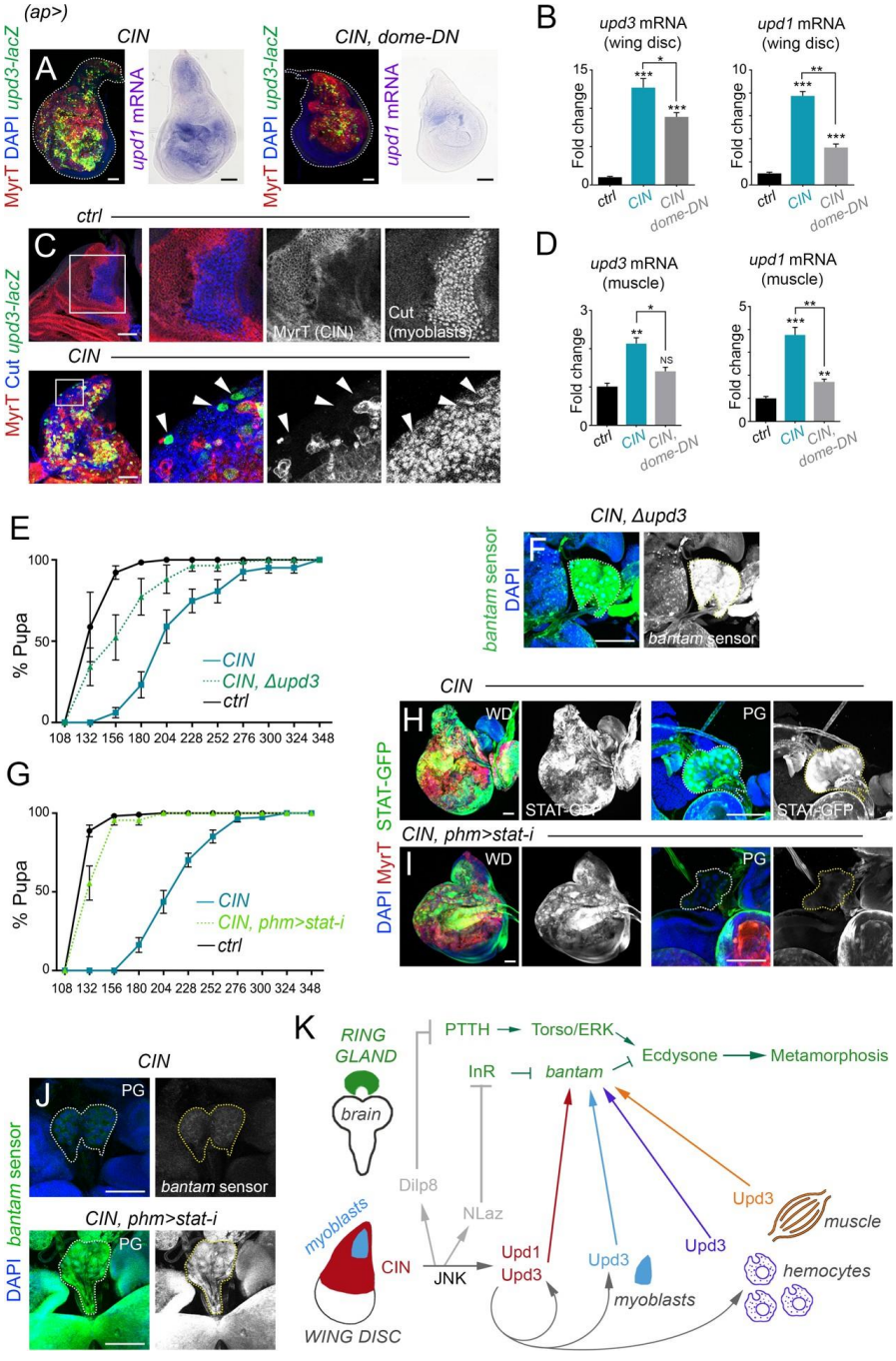


Figure 3. Local co-depletion or trapping of secreted cytokines rescues the CIN-delay.

(**A-D**) Developmental timing of larvae expressing the indicated transgenes in wing discs with the *ap-gal4* driver. n=12-151, data represent mean ± SEM. (**E**-**G**) Ring glands of larvae expressing the indicated transgenes in wing discs (*ap-gal4*) and stained for DAPI (blue), *STAT-GFP* (green or white, **E**), and tGPH (green or white, **F**), or *bantam* sensor (green or white, **G**). Contour of ring glands is marked by a dotted line. Scale bars, 100 µm. See also Figure S3.


(ap>)
CIN
CIN, dome-DN
MyrT DAPI upd3-lacZ
upd1 mRNA
A
B
upd3 mRNA (wing disc)
upd1 mRNA (wing disc)
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CIN
CIN dome-DN
C
ctrl
CIN
MyrT Cut upd3-lacZ
MyrT (CIN)
Cut (myoblasts)
D
upd3 mRNA (muscle)
upd1 mRNA (muscle)
NS
CIN, dome-DN
E
% Pupa
CIN
CIN, Δupd3
ctrl
108 132 156 180 204 228 252 276 300 324 348
CIN, Δupd3
bantam sensor DAPI
F
bantam sensor
G
CIN, phm>stat-i
H
WD
PG
STAT-GFP
DAPI MyrT STAT-GFP
I
J
bantam sensor
DAPI bantam sensor
K
RING GLAND
brain
PTTH
Torso/ERK
Ecdysone
Metamorphosis
InR
bantam
Dilp8
NLaz
myoblasts
CIN
JNK
Upd1
Upd3
muscle
hemocytes
WING DISC

Figure 4. A feed-forward loop spreads *upd3* expression to nearby tissues. (**A**, **C**, **F**, **H**-**J**) Wing discs (**A**, **C**, **H**, **I**) and ring glands (**F**, **J**) of larvae expressing the indicated transgenes in wing discs with the *ap-gal4* driver and stained for MyrT (red or white, **A**, **C**, **H**, **I**), *upd3-lacZ* (green, **A**, **C**), *upd1* mRNA (purple, **A**), Cut (blue or white, **C**), *bantam* sensor (green or white, **F**, **J**) and *STAT-GFP* (green or white, **H**, **I**). Scale bars, 50 μm (wing discs), 100 μm (ring glands). (**B**, **D**) *upd1* and *upd3* mRNA levels by qRT-PCR of wing discs (**B**) and muscle (**D**) of larvae expressing the indicated transgenes with the *ap-gal4* driver. ***p < 0.001, **p < 0.01, *p< 0.05, NS, not significant, data represent mean ± SEM. (**E**, **G**) Developmental timing of larvae expressing the indicated transgenes in wing discs with the *ap-gal4* driver. n=44-194, data represent mean ± SEM. In **F** and **E**, larvae were also mutant for a deletion allele of *upd3*. In **G**, **I**, **J**, the ring gland was expressing an RNAi of *stat*. (**K**) Schematic representation of the local and systemic contribution of secreted cytokines in the CIN model and their impact on the ecdysone production regulatory network. See also Figure S4.

STAR Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
mouse anti-dMMP1 (14A3D2)	Developmental Studies Hybridoma bank	RRID:AB_579782
rabbit polyclonal anti-GFP (A6455)	Molecular Probe	RRID: AB_221570
mouse polyclonal anti-GFP (A11120)	Molecular Probe	RRID: AB_221568
rabbit anti-β-galactosidase (0855976)	Cappel (MP Biochemicals)	Code: 0855976
mouse anti-Cut (2B10)	Developmental Studies Hybridoma bank	RRID: AB_528186
rabbit polyclonal anti-p-ERK (4370)	Cell Signaling Technology	RRID:AB_2315112
rat anti-Ci	Developmental Studies Hybridoma bank	RRID:AB_2109711
mouse anti-NimC1	(Honti et al., 2013)	N/A
Cy2 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	Code: 711-225-152
Cy2 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Code: 711-225-151
Cy5 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Code: 715-175-150
Cy5 AffiniPure Donkey Anti-Rat IgG (H+L)	Jackson ImmunoResearch	Code: 712-175-153
Chemicals, Peptides and Recombinant Proteins		
DAPI	Sigma Aldrich	Code: 28718-90-3
20-Ecdysone	Sigma Aldrich	Code: 5289-74-7
Experimental Models. Organisms/Strains		
ap-GAL4	Bloomington Drosophila Stock Center	RRID:BDSC_3041
UAS-myristoylated-Tomato (myrT)	Bloomington Drosophila Stock Center	RRID:BDSC_32222
UAS-gfp^{RNAi}	Bloomington Drosophila Stock Center	RRID:BDSC_35786

elav-Gal80	Bloomington Drosophila Stock Center	RRID:BDSC_98193
UAS- wg^{RNAi} (#1 in the text)	Bloomington Drosophila Stock Center	RRID:BDSC_32994
UAS- wg^{RNAi}(#2 in the text)	Bloomington Drosophila Stock Center	RRID:VDRC_104579
dilp8mimic-GFP	Bloomington Drosophila Stock Center	RRID:BDSC_33079
UAS-p35	Bloomington Drosophila Stock Center	RRID:BDSC_5073
UAS-BskDN	Bloomington Drosophila Stock Center	RRID:BDSC_6409
en-GAL4	Bloomington Drosophila Stock Center	RRID:BDSC_1973
∆upd3	Bloomington Drosophila Stock Center	RRID: BDSC_55728
UAS-upd1^{RNAi}	Bloomington Drosophila Stock Center	RRID: BDSC 28722
UAS-upd3^{RNAi} (#1 in the text)	Bloomington Drosophila Stock Center	RRID: BDSC_28575
UAS-upd3^{RNAi} (#2 in the text)	VDRC Stock Center	RRID: VDRC_27136
UAS-upd3$^{RNA\ I}$ (#3 in the text)	VDRC Stock Center	RRID: VDRC_27134
phm-gal4	Bloomington Drosophila Stock Center	RRID: BDSC_80577
UAS-bub3^{RNAi}	VDRC Stock Center	RRID:VDRC_21037
UAS-dilp8^{RNAi} (#1 in the text)	VDRC Stock Center	RRID: VDRC_9420
UAS-dilp8^{RNAi} (#2 in the text)	VDRC Stock Center	RRID: VDRC_102604
UAS-rod^{RNAi}	VDRC Stock Center	RRID:VDRC_16152
UAS-NLaz^{RNAi} (#1 in the text)	VDRC Stock Center	RRID:VDRC_107553
UAS-NLaz^{RNAi} (#2 in the text)	VDRC Stock Center	RRID:VDRC_35558
UAS-NLaz	Bloomington Drosophila Stock Center	RRID:BDSC_76608
NLaz^{NW5}/CyO	Bloomington Drosophila Stock Center	RRID:BDSC_76609
UAS-avalanche^{RNAi}	VDRC Stock Center	RRID:VDRC_107264
UAS-puc	(Martin-Blanco et al., 1998)	N/A
bantam sensor (II)	(Brennecke et al., 2003)	N/A
bantam sensor (III)	(Brennecke et al., 2003)	N/A
tGPH sensor	(Britton et al., 2002)	N/A
UAS-dilp8	(Colombani et al., 2012)	N/A

upd3-lacZ (II)	(Bunker et al., 2015)	N/A
STATGFP10x (II)	(Bach et al., 2007)	N/A
STATGFP10x (III)	Bloomington Drosophila Stock Center	RRID:BDSC_26198
UAS-hop	(Sotillos et al., 2008)	N/A
UAS-upd3	(Wang et al., 2014)	N/A
UAS-domeDN (III) (#1 in the text)	(Brown et al., 2001)	N/A
UAS-domeDN (II) (#2 in the text)	(Brown et al., 2001)	N/A
P0206-gal4	kindly given by H. Jasper	N/A
UAS-hop^{RNAi}	Bloomington Drosophila Stock Center	RRID:BDSC_31319
UAS-dome^{RNAi}	VDRC Stock Center	RRID:VDRC_106071
mef2-gal4	VDRC Stock Center	RRID:VDRC_50742
GMR15B03-gal4	Bloomington Drosophila Stock Center	RRID:BDSC_49261
UAS-upd1	(Ayala-Camargo et al., 2013)	N/A
hemese-gal4	Bloomington Drosophila Stock Center	RRID:BDSC_8699
croquemort-gal4	Bloomington Drosophila Stock Center	RRID:BDSC_25041
cg-gal4	Bloomington Drosophila Stock Center	RRID:BDSC_7011
phm-lexA	kindly given by Gary Struhl	N/A
LexAop stat-i	generated in this work	N/A
Software and Algorithms		
Fiji	Fiji	https://fiji.sc/
Excel	Microsoft Excel 2016	N/A
GraphPad Prism 7 Project	GraphPad	RRID:SCR_002798

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marco Milán (marco.milan@irbbarcelona.org).

Experimental Model and Subject Details

Fly strains

The strains used were kindly provided by the following sources: (1) Bloomington Drosophila Stock Center (BDSC): *ap-GAL4;* UAS-*myristoylated-Tomato; UAS-gfp^{RNAi}; elav-Gal80; UAS-wg^{RNAi} #1; UAS-wg^{RNAi} #2; dilp8mimic-GFP;* UAS-*NLaz^{NW5}/CyO; UAS-p35; UAS-bskDN; en-GAL4; 10XSTAT-GFP (III); ∆upd3; UAS-upd1^{RNAi}; UAS-upd3^{RNAi} #1*;; *UAS-hop^{RNAi}*; *phm-GAL4*; *hem-GAL4; crq-GAL4; GMR15B03- GAL4; cg-GAL4;* (2) Vienna Drosophila RNAi Center (VDRC): *UAS-bub3^{RNAi}; UAS-dilp8^{RNAi} #1; UAS-dilp8^{RNAi} #2; UAS-rod^{RNAi}; UAS-NLaz^{RNAi} #1; UAS-NLaz^{RNAi} #2; UAS-NLaz*; *UAS-upd3^{RNAi} #2*; *UAS-upd3^{RNAi} #3; mef2-gal4*; UAS-*avalanche^{RNAi}*; UAS-*dome^{RNAi}* and (3) UAS-*puc* (J. P. Vincent)*; bantam* sensor (S. Cohen)*; tGPH* sensor (H. Stocker); *UAS-dilp8* (P. Leopold)*; upd3-lacZ* (H. Jiang)*; 10XSTAT-GFP (II)* (M. Dominguez)*, UAS hop* (J. Castelli)*, UAS-upd3* (D. Harrison)*, P0206-gal4* (H. Jasper), *UAS-domeDN* #1 and #2 (J. Castelli), *UAS-upd1* (E. Bach) and *phm-lexA* (G. Struhl). Flies were allowed to lay eggs on fly food for 12 h at 25°C for dissection assays and 5h at 25°C for pupariation assays. Larvae were then switched to 29ºC for 4 days before dissection or pupa counts took place.

Method Details

Immunohistochemistry and confocal imaging

Wing imaginal discs, brains, ring glands, trachea and fat body tissues of third instar larvae were dissected in cold PBS, fixed in 4% formaldehyde for 20 min and immunolabeled in BBT (PBS with 0.3% BSA, 0.2% Triton X-100) with the following antibodies: mouse anti-MMP1 (1:20) (14A3D2, Developmental Studies Hybridoma Bank, DSHB); rat anti-Ci (1:10; 2A1, DSHB); rabbit and mouse anti-GFP (1:600) (Molecular Probe, A6455 and A11120, respectively); rabbit anti-β-galactosidase (1:600) (Cappel; 0855976); mouse anti-Cut (1:100) (2B10, Developmental Studies Hybridoma Bank, DSHB), rabbit polyclonal anti-p-ERK (1:100) (4370, Cell Signaling Technology), mouse anti-NimC1 (1:25) kindly provided by I. Andó. For the muscle tissues, the general procedure was the same as described previously except for an extended time of fixation of 30 min after filet dissection after which the *10xSTAT-GFP* reporter was analyzed. Secondary antibodies Cy2 and Cy5 (1:400) were obtained from Jackson ImmunoResearch. Leica TCS SP5 MP confocal microscope was used to perform high resolution images. *NLaz* digoxigenin (DIG)-labelled antisense probe was transcribed by T3 RNA polymerase from a XhoI-linearized plasmid containing the full-

length cDNA (clone RE67583 obtained from DGRC). *upd1* DIG-labelled antisense probe was transcribed by T7 polymerase from an EcoRI-linearized plasmid containing a 600bp fragment of *upd1* cDNA (nt 946 to 1547, this work). Probes were synthesized using the DIG RNA Labelling Kit (Roche) according to the manufacturer's instructions. In situ hybridization protocol was performed as in (Milan et al., 1996).

LexAop-stat-RNAi cloning

To generate the LexAop-statRNAi construct, the following oligos were used.

stat-i Fwd (EcoRI)

CTA GCA GTT CGC AGC TTT ATG TAC GAT AGT TAT ATT CAA GCA TAT CGT AGT ACA AGC TGC GAG CG

stat-i Rev (XbaI)

AAT TCG CTC GCA GCT TTA TGT ACT ACG ATA TGC TTG AAT ATA ACT ATC GTA CAT AAA GCT GCG AAC TG

9.5μl of each oligo (100μM) was used to mix with 1μl of annealing buffer (20X SSC). The mix was heated at 100°C for 10 minutes and let to cool down overnight for an efficient annealing. 1/200 dilution of the annealed mix was used to ligate in the pLOT plasmid (carrying the lexA operator), previously digested with EcoRI and XbaI endonucleases.. The plasmid was transformed in DH5alpha competent cells and 5 colonies were selected for checking. To assess for the correct generation of vector and oligos combined, the absence of the $XhoI_{3461bp}$ digestion site was checked. Plasmids with the correct size and absence of the XhoI site were sequenced and one positive clone was used to generate transgenic flies with standard protocols.

Pupariation Assays

Flies were allowed to lay eggs for 4 h (or a maximum of 5 h for difficult phenotypes, namely the mutants) at 25°C after which they were switched to 29ºC for a total of 4 days. After these 4 days of induction, tubes were taken from the bath and placed at room temperature to allow for counting from 108 to 358 h after egg deposition, accounting for a period of approximately referent from 5 to 15 days' time. Counts were performed always at the same time point and 24h apart. The resulting percentage of pupae was calculated accordingly with the total number of larvae capable of transitioning to the next developmental phase.

Larval Feeding Assays

For the blue food feeding assays, flies were allowed to lay eggs for a maximum of 5 h at 25°C and were then switched to 29ºC for 4 days. After this induction period larvae were transferred to plates with food supplemented with Bromophenol Blue (Sigma-Aldrich 114391_5G). Next, L3 larvae were isolated from each plate at different time points. The presented data refer to the highly relevant time points of 132h, 156h, 180h and 204h imaged using an Inverted Microscope Olympus IX71 SCMOS.

Ecdysone Feeding Assays

Flies were allowed to lay eggs on standard ecologic fly food for 4 h at 25°C and were then switched to 29°C. At 84 h they were selected for early L3 stage characteristics and switched to food supplemented with an activated form of ecdysone. Stock solution for the steroidal hormone at a concentration of 5 mg/ml was prepared from 5 mg of 20-ecdysone powder (Sigma) in 1mL of EtOH. Working solution was adjusted by diluting the initial stock solution in PBS until a final concentration of 0.2 mg/ml. Control solutions were prepared using the same amount of EtOH in PBS. The amount of hormone and control solution were calculated to allow for the dilution to add up to a total volume of 100 ml. Each dilution was prepared daily and 50 ml was added first thing in the morning and last thing in the afternoon, respecting a 10-h window. The food in each vial was stirred slightly prior to addition of the hormone or control solution to facilitate diffusion.

Quantitative RT-PCR

For the quantification of mRNA levels, total RNA was extracted from wing discs and muscles of at least 25 and 15 larvae, respectively, using TRIzol reagent (Invitrogent). A total of 1.5 μg of total RNA was treated with DNase and used as a template for cDNA synthesis using Maxima Reverse Transciptase (ThermoScientific). Maxima SYBR Green/ROX qPCR Master Mix (ThermoScientific) was used, and reactions were run in a QuantStudio 6 Pro Real-Time PCR Systems (Applied Biosystems). Samples were normalized to the levels of forkhead domain 68A (Mnf, for wing disc samples), or Actin42A (Act, for muscle samples) transcript levels and fold changes were calculated using the ΔΔCt method. Three separate

biological samples were collected for each experiment. The following primer pairs were used: Mnf-Fw: 5'-GAG CAG AAG AGC CCC TAC CT-3', Mnf-Rv: 5'-AAT GAA ACC CTG ACG TGG AC-3' (Ponton et al., 2011); Act-Fw: 5'-GCG TCG GTC AAT TCA ATC TT-3', Act-Rv: 5'-AAG CTG CAA CCT CTT CGT CA-3'(Ponton et al., 2011); Dilp8-Fw: 5'- GCA CCA CCA TCT GAA TCG AC-3', Dilp8-Rv: 5'- CTG AGG CGA TTG AAG TGC TC -3'; NLaz-Fw:5'- GCC AGA AGT AGA ACG GAT ACC A-3', NLaz-Rv:5'- ACT GGT GCA GCT GTA GAC GAC-3' (Hull-Thompson et al., 2009); Upd1-Fw: 5'- AGA CAG CCG TCA ACC AGA C-3', Upd1-Rv: 5'- AGA CAG CCG TCA ACC AGA C-3' [(Moskalev et al., 2019), wing disc samples)]; Upd1-Fw: 5'- TGT AAC CCC GTT CGC TGT AT-3', Upd1-Rv: 5'- GCT GAT GTT TCC GTT TCC GT-3' (muscle samples); Upd3-Fw: 5'- ATC CCA CCA ATC CCC TGA AG-3', Upd3-Rv: 5'-AGA TTG CAG GTG TTC TCC CA-3'.

Quantification and Statistical Analysis

Image Processing and Analysis

Fiji [National Institute of Health (NIH) Bethesda, MD] was used to measure the size of the anterior (a), dorsal (d) compartments (based on Ci and MyrT expression respectively), or the whole wing discs (based on DAPI staining), and to monitor reporter intensities (specifically bantam, tGPH, STATGFP and upd3-lacZ reporter lines). Image stacks were obtained using a Leica TCS SP5 MP confocal microscope, 20X dry objective with 1 µm per optical section to cover the entire thickness of each disc. Maximum intensity Z-projection was performed on the stacks prior to reporter analysis. Control wing discs grown in parallel and subjected to the same experimental conditions (temperature and time of transgene induction) were analyzed in parallel. At least 10 wing discs per genotype were scored. In the special case of the myoblast population, a 40x glycerol immersion objective was used to facilitate the acquisition of high-quality images of both tumor and myoblast cells.

Statistical Analysis

Statistical analysis was generally performed by unpaired equal-variance two-tail t-test. Differences were considered significant when p values were less than 0.001 (***), 0.01 (**), or 0.05 (*). All genotypes included in each histogram were analyzed in parallel. All data points were plotted in Prism 7.0 (Graphpad) statistical software.

Supplementary Figures

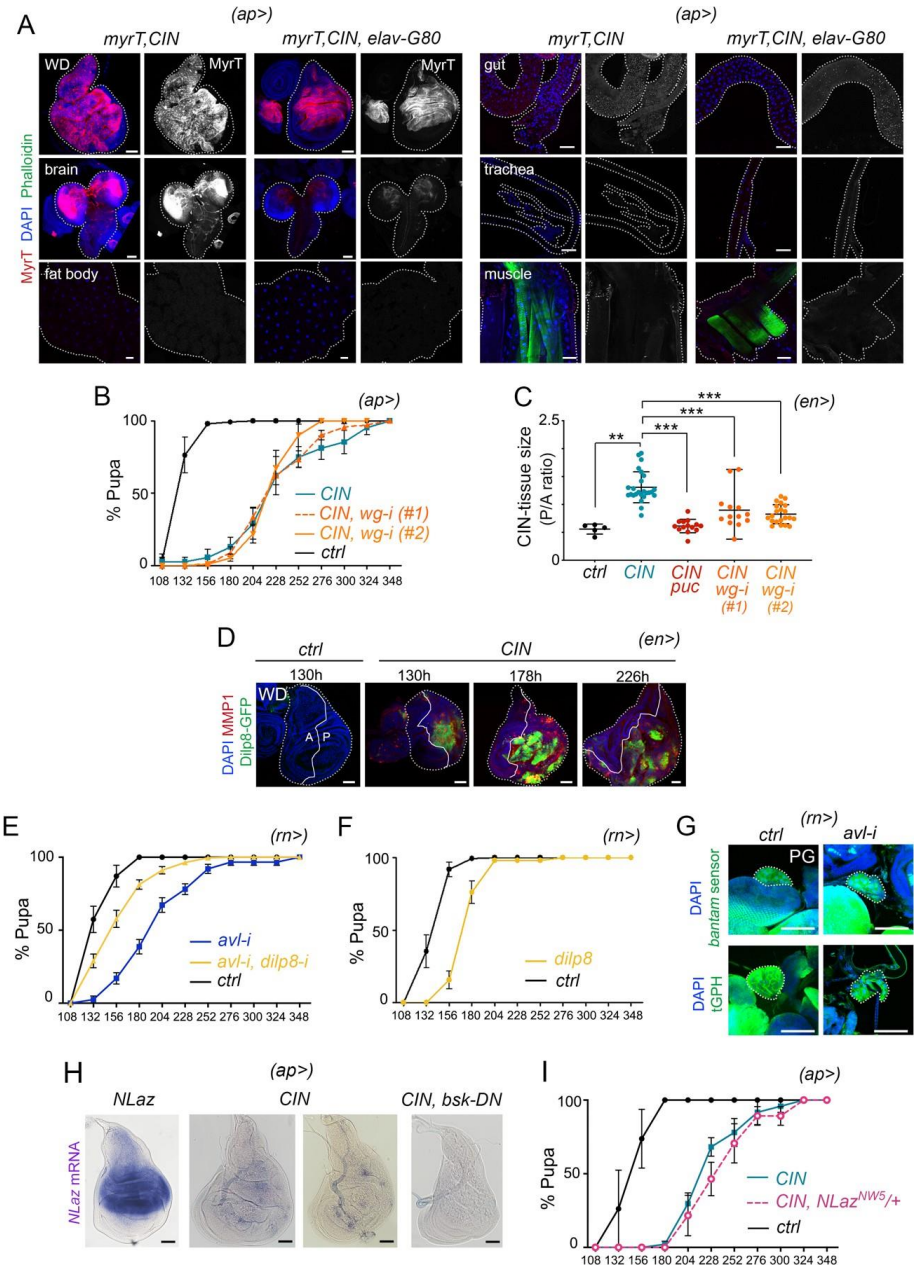

Figure S1. CIN induces a Dilp8-independent developmental delay (related to Figure 1)

(**A**) Wing discs, brain, fat body, gut, trachea and muscle tissues (contours depicted by a dotted line) expressing the indicated transgenes under the control of *ap-gal4* and stained for MyrT (red), DAPI (blue) and phalloidin (green). MyrT labels the *ap-gal4* domain (red). (**B**, **E**, **F**, **I**) Developmental timing of larvae expressing the indicated transgenes in wing discs with *ap-gal4* (**B**, **I**) or *rn-gal4* (**E**, **F**). In **I**, larvae were also heterozygous for *NLaz*. n=50-221, represent mean ± SEM. (**C**) Tissue size of wing discs (P/A ratio) subjected to expression of the indicated transgenes in the posterior (P) compartment. Data represent mean ± SD. ***p < 0.001, **p< 0.01, n = 5-25. (**D**) Wing discs expressing the indicated transgenes under the control of *en-gal4* driver and stained with DAPI (blue), MMP1 (red) and Dilp8-GFP (green). The anterior-posterior (AP) boundary is depicted by a white line and wing disc contours by a dotted line. (**G**) Ring glands of larvae expressing the indicated transgenes in wing discs with *rn-gal4* and stained for DAPI (blue) and tGPH or *bantam* sensors (green). (**H**) Wing imaginal discs expressing the indicated transgenes with *ap-gal4* and stained for *NLaz* mRNA expression (purple). Scale bars, 50 μm (wing discs), 100 μm (ring glands).

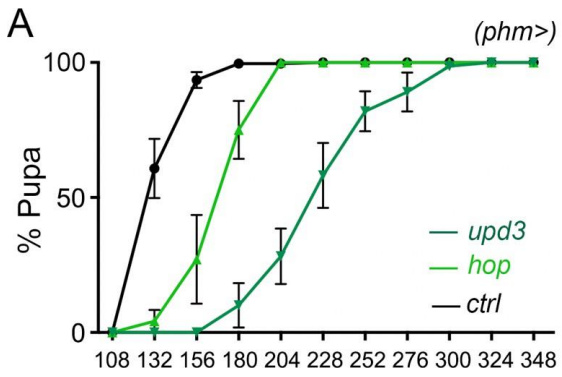

Figure S2. Local activation of JAK/STAT in the prothoracic gland induces a developmental delay (related to Figure 2)

(**A**) Developmental timing of larvae expressing the indicated transgenes in the prothoracic gland with *phm-gal4*. n=28-128, data represent mean ± SEM.

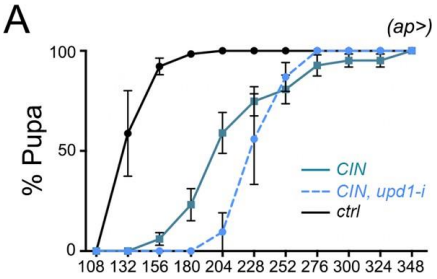

Figure S3. Depletion of *upd1* is not sufficient to rescue the CIN-developmental delay (related to Figure 3)

(**A**) Developmental timing of larvae expressing the indicated transgenes in wing discs with *ap-gal4*. n=12-146, several replicas, data represent mean ± SEM.

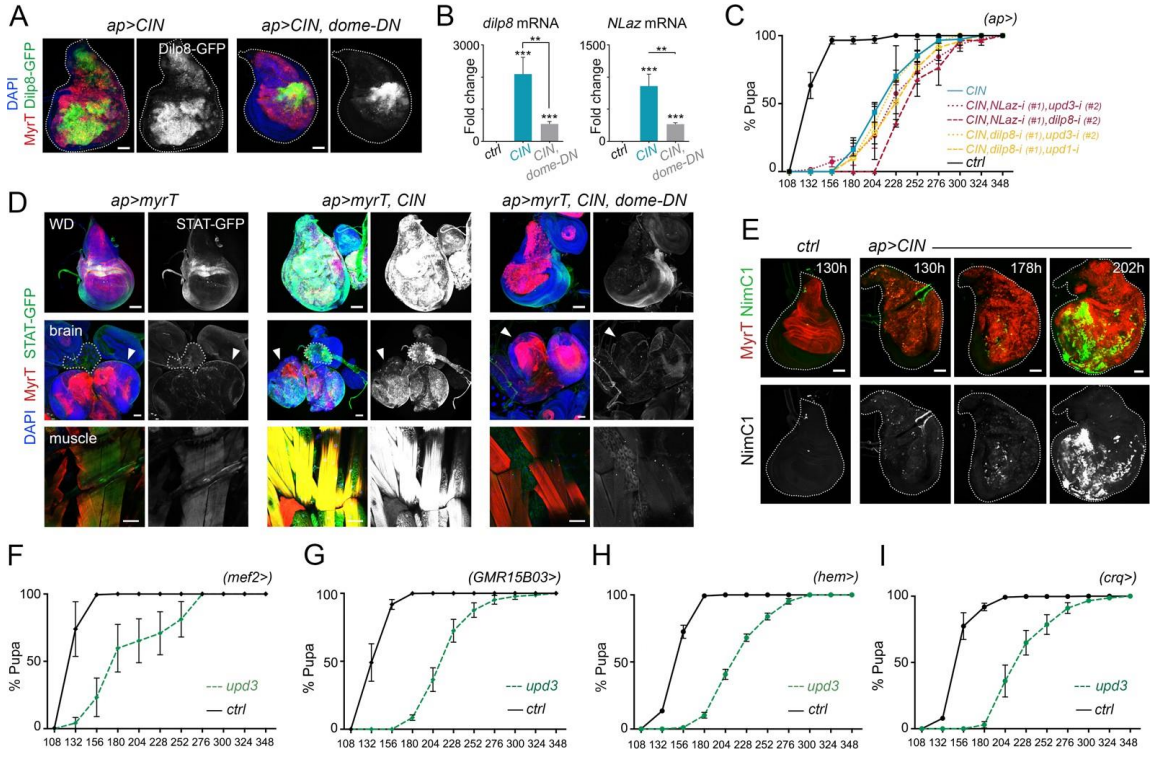

Figure S4. Different sources of Upd3 contribute to the CIN-developmental delay (related to Figure 4)

(**A**, **D**, **E**) Wing discs, brain (white arrowhead in **D**) and muscle (**D**) tissues of larvae expressing the indicated transgenes under the control of *ap-gal4* driver and stained for MyrT (red), DAPI (blue, **A**, **D**), Dilp8-GFP (green or white, **A**), STAT-GFP (green or white, **D**) and NimC1 (green or white, **E**). Scale bars, 50 µm. (**B**) *dilp8* and *NLaz* mRNA levels by qRT-PCR of wing discs expressing the indicated transgenes with the *ap-gal4* driver. Contour of wing discs and ring glands is marked by a dotted line. (**C, F-I**) Developmental timing of larvae expressing the indicated transgenes in wing disc (*ap-gal4*, **C**) or overexpressing Upd3 in muscles (*mef2-gal4*, **F**), myoblasts (*GMR15B03-gal4*, **G**), or hemocytes (*hem-gal4*, **H** and *crq-gal4*, **I**). Data represent mean ± SEM (n=20-447).

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