The ETS transcription factor Pointed represents a point of crosstalk between the EGFR and JAK/STAT signalling pathways to establish posterior fate the follicular epithelium of the Drosophila

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Table of Content

List of Figures

Abstract

The generation of the many diverse cell types that arise during development is achieved by a surprisingly small number of signalling pathways, implying that the same pathway can generate multiple outcomes depending on the signalling context. As a model, we study the follicular epithelium of the Drosophila ovary, where localized activation of the epidermal growth factor receptor (EGFR) leads to one of two different outcomes depending on the timing and location of signalling. Early in oogenesis, posteriorly localized EGFR activity induces expression of the transcription factors Midline (Mid) and H15. Later, EGFR activity translocates to the dorsal anterior, where it instead induces the transcription factor Mirror (Mirr). EGFR output thus depends on the signalling context, ultimately allowing this one localized signal to define both the anterior-posterior and dorsal-ventral axes.

The choice between these two alternative EGFR signalling outcomes is mediated by additional positional information provided by opposing gradients of JAK/STAT and BMP activity, each of which promotes EGFR-mediated activation of one target while independently repressing the other. At the posterior, JAK/STAT signalling promotes Mid and represses Mirr expression. At the anterior, BMP signalling promotes Mirr and represses Mid expression. In addition, mutual repression between Mid and Mirr presumably stabilizes these outcomes. We have proposed that this regulatory network generates a bistable switch ensuring robust patterning of this tissue, but how these signals are captured and interpreted by the cells remains unknown.

Here we address this question by studying the mechanisms by which the JAK/STAT signalling pathway influences the outcome of EGFR signalling to promote expression of Mid. By characterizing putative cis-regulatory regions (CRRs) controlling Mid expression, we ask whether Mid expression is directly activated through binding of STAT92E. Contrary to our hypothesis, we

found that disruption of putative STAT92E binding sites in a putative CRR regulating expression of *mid* and capturing inputs from JAK/STAT signalling did not affect expression of a reporter gene, suggesting an indirect regulation. Instead, I found that the EGFR effector Pointed (Pnt) is regulated by JAK/STAT and that it can rescue the loss of Mid expression seen in cells lacking JAK/STAT signalling. This indicates that the JAK/STAT signalling pathway influences EGFR outcome by controlling expression of its effector Pnt.

Finally, I studied the mechanisms by which the EGFR signalling pathway controls expression of Pnt in the posterior domain, by focusing on the role of another EGFR effector Capicua (Cic). I found that Pnt expression in the posterior, unlike in dorsal-anterior is not regulated by Cic. Instead, I found that Cic plays a role in establishing the anterior limit of Mid expression, potentially through regulation of Mirr and Pnt in the dorsal-anterior. If I did not identify the exact mechanism by which the EGFR signalling pathway controls expression of Pnt in the posterior, I found that Pnt requires inputs from JAK/STAT and EGFR signalling independently of Cic.

Together, this data describes how the JAK/STAT signalling pathway influences outcome of EGFR signalling to induce expression of Mid and posterior fate. In one way by increasing the levels of the EGFR effector Pnt and in another way by repressing the alternative target Mirr. However, the mechanisms by which the EGFR signalling pathway regulation expression of Pnt remain unknown.

Résumé

La génération des nombreux types de cellules qui sont spécifiés pendant le développement requiert un nombre étonnement petit de signalisations cellulaires, cela implique qu'un même signal peut avoir deux issues différentes selon le contexte. Nous utilisons l'épithélium folliculaire de l'ovaire de la Drosophile comme modèle. Dans ce tissu, l'activation localisée du récepteur du facteur de croissance épidermique (EGFR) mène à deux issues différentes selon le moment et la localisation du signal. Dans les premières phases de l'ovogenèse, l'activation de l'EGFR dans le domaine postérieur induit l'expression des facteurs de transcription Midline (Mid) et H15. Ensuite, EGFR est activé dans le domaine antérieur-dorsal où est exprimé le facteur de transcription Mirror (Mirr). L'issue du signal de l'EGFR dépend donc du contexte cellulaire, conduisant ce seul signal à définir les axes antérieur-postérieur et dorsal-ventral.

Ce choix entre les deux issues du signalement de l'EGFR est déterminé par d'autres signaux localisés venant de gradients opposés des signaux JAK/STAT et BMP, chacun favorisant l'activation d'un des gènes cibles de l'EGFR et la répression de l'autre. Dans le domaine postérieur, la voie de signalisation JAK/STAT favorise l'expression de Mid et represse l'expression de Mirr. Dans le domaine antérieur, la voie de signalisation BMP favorise l'expression de Mirr et represse l'expression de Mid. De plus, la répression mutuelle entre Mid et Mirr stabilise l'issue du signalement de l'EGFR. Nous avons proposé que ce réseau de régulation génère une issue bistable permettant une formation de patterns robustes dans ce tissu. Cependant, comment ces signaux sont intégrés et interprétés par les cellules reste mal compris.

Dans cette thèse, j'approche cette problématique en étudiant les mécanismes par lesquels la voie de signalisation JAK/STAT détermine l'issue du signal de l'EGFR pour favoriser l'expression de Mid. En caractérisant une région cis-régulatrice (CRR) de Mid, nous avons testé si

l'expression de Mid est directement activée par liaison de STAT92E à cette CRR. Contrairement à notre hypothèse, nous avons trouvé qu'en mutant un site de liaison putatif de STAT92E dans une CRR contrôlant l'expression de Mid n'a pas d'effet sur l'activité de la CRR, suggérant une régulation indirecte. A la place, j'ai trouvé que l'effecteur de la voie de signalisation de l'EGFR Pointed (Pnt) est régulé par JAK/STAT et qu'il peut secourir l'expression de Mid dans les cellules mutantes n'ayant pas la capacité de recevoir des signaux de JAK/STAT. Cela indique que la voie de signalisation JAK/STAT influence l'issue des signaux de l'EGFR en contrôlant l'expression de son effecteur Pnt.

Enfin, j'ai étudié les mécanismes par lesquels la voie de signalisation de l'EGFR contrôle l'expression de Pnt dans le domaine postérieur, en testant l'hypothèse que Pnt est régulé par un autre effecteur de l'EGFR, Capicua (Cic). J'ai trouvé que l'expression de Pnt dans le domaine postérieur ne requiert pas Cic, contrairement au domaine antérieur-dorsal. Cependant, mes données indiquent que Cic joue un rôle dans l'établissement de la limite antérieure du domaine d'expression de Mid, potentiellement par la régulation de Mirr et Pnt dans le domaine antérieur-dorsal. Si je n'ai pas pu caractériser le mécanisme exact par lequel la voie de signalisation de l'EGFR régule Pnt dans le domaine postérieur, j'ai trouvé que Pnt requiert des signaux venant des voies JAK/STAT et de l'EGFR pour être exprimé dans le domaine postérieur et que cela est indépendant de Cic.

Dans l'ensemble, ces données décrivent comment la voie de signalisation JAK/STAT détermine l'issue des signaux de l'EGFR pour favoriser l'expression de Mid et l'identité postérieure : en régulant l'expression de l'effecteur de l'EGFR Pnt et réprimant l'expression du gène cible alternatif Mirr. Cependant, les mécanismes par lesquels la voie de signalisation de l'EGFR régule l'expression de Pnt reste inconnus.

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Contribution of Authors

Chapter 2

The *mid*^{up} *lacZ* reporter construct shown in Figure 2.1, the *mid*$^{up.1}$ and *mid*$^{up.1B\ MUT}$ *lacZ* reporter constructs shown in Figure 2.2 were made by Scott De Vito. The experiments shown in Figure 2.2 were designed by Scott De Vito and Figure 2.2 was made from panels made by Scott De Vito. I generated the *mid*$^{up1.B}$ and *mid*$^{up.1B2}$ derivative constructs. Images shown in Figure 2.1F-G", and Figure 2.2 (except 2.2D-D") were taken by Scott De Vito. Laura Nilson provided guidance and revision to the writing. I performed the rest of the experiments and wrote the chapter.

Chapter 3

The *pnt*^{45D11} and *pnt*^{43H01} *lacZ* reporter constructs were generated by Scott De Vito. Images in Figure 3.2A-A' and Figure 3.8A-A',C-C' were taken by Kenzie Melvill under my supervision. I designed and performed all the other experiments. Laura Nilson provided guidance and revision to the writing. I performed the rest of the experiments and wrote the chapter.

Chapter 4

Images in Figure 4.2B-B" were taken by Scott De Vito. Laura Nilson provided guidance and revision to the writing.

Chapter 1 – Introduction

1.1 Overview

Development of multicellular organisms requires cells to be aware of their location to adopt the correct fate. This decision must be coordinated among cells to produce specific patterns of cell fates and organize the body plan. Therefore, there must exist systems ensuring that each cell receives information about its position within a tissue and to communicate its fate to its neighbours.

Such information can be conveyed by localized signals present in the cellular environment. However, there only exists a small number of signalling cues for the wide variety of different cellular fates, suggesting that the same signalling pathways are reused during development to lead to different outcomes. These outcomes are determined by the context in which cells receive these signals. Factors such as duration of signalling, intensity, or interactions with other pathways can affect the outcome of signalling (Housden and Perrimon, 2014; Perrimon et al., 2012). However, if numerous factors have been identified, the mechanisms by which they influence signalling outcome remain poorly understood.

In this dissertation, I use the patterning of the follicular epithelium of the *Drosophila melanogaster* ovary as a model to address this question. In this tissue, the Epidermal Growth Factor Receptor (EGFR) signalling pathway establishes two mutually exclusive fates, posterior and dorsal-anterior fates. We have previously identified that the JAK/STAT and BMP signalling pathways determine the outcome of EGFR signalling to establish posterior and dorsal-anterior fates respectively. The objective of my project is to characterize the mechanisms used by the EGFR

signalling to induce posterior fate and how the outcome of EGFR signalling in this tissue is influenced by the interaction with the JAK/STAT signalling pathway.

1.2 Mechanisms of tissue patterning by localized signals

1.2.1 Overview

A classic example of tissue patterning is the *Drosophila* embryo at the blastoderm stage. The initial step in patterning requires localized factors that are maternally loaded into the oocyte (Jaeger et al., 2012; Kugler and Lasko, 2009). The anterior-posterior axis is set up by a gradient of Bicoid (Bcd) originating in the anterior and a gradient of Nanos (Nos) in the posterior. These gradients originate from localized mRNAs of *bcd* and *nos* in the anterior and posterior of the embryo respectively (Driever and Nüsslein-Volhard, 1988a; b; Gavis and Lehmann, 1992). When it is translated, the Bcd protein diffuses along the anterior-posterior axis and induces different fates along the axis. In addition, the Nos protein, when translated from the posterior mRNA, diffuses and induces different fates along the anterior-posterior axis. This step sets up a cascade of events that will pattern the embryo through the localized expression of gap , pair-rule , and segment polarity genes which define the segments making up the body of the adult fly and their identity (St Johnston and Nüsslein-Volhard, 1992).

Establishment of the dorsal-ventral axis is also initiated by maternally loaded factors. During oogenesis, expression of the *pipe* gene is restricted to the ventral side of the follicle cells (Sen et al., 1998). The action of the Pipe protein, through unknown mechanisms produces a chemical asymmetry in the extra-cellular space surrounding the early embryo. This asymmetry activates a protease cascade in the ventral side, leading to the activation of Spätzle, the ligand of the Toll receptor. Activation of the Toll receptor triggers a series of intracellular events leading to the nuclear localization of the transcription factor Dorsal which sets up a gradient of Dorsal activity

along the dorsal-ventral axis (Anderson, 1998; Moussian and Roth, 2005; Reeves and Stathopoulos, 2009).

The fact that the *Drosophila* embryo is organized as a syncytium makes the patterning unique because the localized signals can be transcription factors, as seen with Bcd. Nonetheless, the idea that localized positional information present only in a defined region and functioning to specify a domain, is widely present during development.

1.2.2 Localized activation of signalling pathways patterns tissues

1.2.2.1 Binding of a ligand to its receptor activates signalling pathways at different ranges

Cells in a multicellular organism must coordinate and communicate to correctly pattern tissues. A key mechanism by which cell-cell communication occurs is through the use of signalling pathways. In most cases, a ligand, produced by a cell, binds to a receptor expressed by a receiving cell. This binding event leads to a series of cellular responses that can include change in cell fate, proliferation, or apoptosis (Perrimon *et al.*, 2012).

Signalling pathways use different modes of communication which affect their range of effect. Juxtacrine signalling are mediated by membrane bound ligands and receptors and can only act between cells in direct physical contact. On the other hand, paracrine signalling work through the diffusion of factors and affect cells on a longer range (Perrimon *et al.*, 2012). Here, the range depends on several factors. Diffusion can be hindered by the presence of obstacles such as cells or extracellular components. Alternatively, there also exist factors increasing the range of the signalling. Ligands can use shuttle to facilitate their diffusion, or they can use cell-based mechanisms to extend their range. For example, ligands can move through cells in a process called transcytosis or through cytonemes that are cellular extensions that can release and catch ligands from a distance (Müller et al., 2013).

1.2.2.2 Signal transduction

Binding of a ligand to its receptor triggers a series of intracellular reactions that lead to changes in gene expression (Housden and Perrimon, 2014). How the signal is transduced varies between signalling pathways. Pathways working through hormones represent the simplest case where the ligands cross the membrane and bind to nuclear receptors that also act as transcription factors (King-Jones and Thummel, 2005). The simple organization of these pathways makes them key regulators of biological processes needed for homeostasis but they also play an important role in development (Pardee et al., 2011).

The Notch signalling pathway is a juxtacrine pathway showing a higher level of complexity. Here, when the ligand binds to the receptor Notch, the intracellular domain of Notch is cleaved and travels to the nucleus where it associates with the transcription effector CSL to regulates gene expression (Kovall and Blacklow, 2010). The extra steps of transduction such as cleavage and transport to the nucleus provide more opportunities for regulation, hence increasing the complexity of the pathway (Bray, 2006).

Paracrine signalling pathways, such as the Janus Kinase and Signal Transducer and Activator of Transcription (JAK/STAT) signalling pathway have higher levels of transduction complexity. Here, binding of the ligand to the receptor leads to the activation of the Janus Kinase (JAK) which in turn, phosphorylates the transcriptional effector STAT. Phosphorylation of STAT allows it to dimerize and to translocate to the nucleus where it regulates gene expression (Rawlings et al., 2004). The additional components in the signal transduction compared to the Notch pathway also allows for more levels of regulation. For example, there are negative regulator of the JAK/STAT signalling pathway (Greenhalgh and Hilton, 2001; Yasukawa et al., 2000). Suppressor

of cytokine signalling (SOCS) are proteins whose expression is activated by STAT and that inhibit JAK/STAT signalling in a negative-feedback loop.

Finally, signalling pathways mediated by receptor tyrosine kinase (RTK) represent the most complex case of signalling pathways where a single ligand-receptor pair can make use of multiple signal transduction cascades. This complexity comes from the large number of different components downstream of the receptor compared to the other types of signalling pathways (Housden and Perrimon, 2014). For example, in *Drosophila*, there exists more than 20 RTKs, each having its own unique downstream effectors (Sopko and Perrimon, 2013).

1.2.2.3 Cis-regulatory regions capture inputs from signalling pathways

Despite all the differences in the transduction of signalling pathways, they all work by controlling the activity of transcription factors to control gene expression. Transcription factors bind to DNA regulatory sequences and recruit co-factors to activate or repress gene expression. These sequences, called cis-regulatory regions (CRRs) can be bound by transcription factors acting downstream of different signalling pathways and represent therefore a point of crosstalk between pathways (Levine, 2010).

Gene expression is often controlled by numerous CRRs, and their interaction is important to generate diverse patterns of gene expression. CRRs can cooperate in an additive or synergistic mode to increase levels of expression within a domain or to be expressed in different domain. They can also repress activity of other CRRs or compete between each other (Long et al., 2016).

The CRR controlling expression of the second stripe of *even-skipped (eve)* in the Drosophila embryo is a very well studied example. Eve is expressed in seven stripes along the posterior-anterior axis and expression of these stripes are controlled by five distinct CRRs (Frasch

et al., 1987; Harding et al., 1986; Vincent et al., 2016). The CRR controlling expression of the second stripe is directly bound and activated by Bcd and Hunchback and repressed by Giant and Krüppel. The localized action of these transcription factors is integrated at the level of this CRR that is only expressed in a stripe where only Bcd and Hunchback are expressed (Bothma et al., 2014). This integration of the activating and repressive action of the different transcription factors is widely used to define patterns during development.

1.2.2.4 Opposite gradients generate pairs of alternative outcomes and stabilize patterning of tissues

As explained above, diffusible signals can give positional information, however, it is difficult for diffusion to consistently reproduce pattern in development because change in environmental factors such as temperature affect the range of diffusion (Anderson, 1998). Therefore, we observe that tissue patterning rarely depends on a single but pairs of anti-parallel gradients. In addition, target genes of signalling pathways often include other transcription factors that prevent expression of targets of the other anti-parallel gradient. This combination of induction and mutual repression creates a regulatory network allowing the formation of sharp boundaries and ensuring robustness in the patterning (Briscoe and Small, 2015).

In the Drosophila blastoderm-stage embryo, the gradient of Bcd in the anterior is complemented by an antiparallel gradient of Caudal (Cad) whose translation is repressed by Bcd (Chan and Struhl, 1997; Niessing et al., 2002). Downstream targets of Bcd, *sloppy-pair 1*, *giant*, and *hunchback* are expressed in the anterior and are all transcription repressors. They prevent expression of *runt*, *kruppel*, and *knirps* respectively. These three pairs of mutually exclusive transcription factors generate segments in the anterior and refine the pattern broadly established by Bcd (Clyde et al., 2003; Jaeger et al., 2004; Kraut and Levine, 1991).

Taken together, the positional information given by the localized signals is transduced to activate transcription factors and captured by CRRs of target genes. These target genes often include transcriptional repressor preventing expression of the target of the opposite gradient. This mutual repression creates a regulatory network converting positional information into a bistable switch providing robustness and proper patterning to the developing tissues (Briscoe and Small, 2015).

1.3 The follicular epithelium of the Drosophila ovary is patterned by different signalling pathways

1.3.1 Overview of oogenesis

Drosophila is a well established model organism to study development, notably because of the wide variety of tools available to researchers (Bellen et al., 2010; Bilder and Irvine, 2017). I use the patterning of the follicular epithelium of the Drosophila ovary to study how signalling pathways interact with each other to influence their outcome. The follicular epithelium has been widely used as a model to study signalling pathways and patterning (Pyrowolakis et al., 2017) because of the large number of genetic techniques available (Bratu and McNeil, 2015).

The Drosophila ovary is composed of ovarioles each containing a line of developing eggs. Oogenesis is divided into 14 stages based on changes in morphology. At the anterior of each ovariole is the germarium in which the germline stem cells and follicle stem cells are located. The germline stem cells divide once to produce a cystoblast and a new stem cell. The cystoblast then divides 4 times producing 16 cells through mitosis with incomplete cytokinesis, linking all the cells with ring canals. One of the cells becomes the oocyte and is positioned at the posterior of the future egg chamber. The 15 others differentiate into nurse cells that become polyploid through multiple rounds of endoreplication and support the maturation of the developing oocyte. The oocyte and the

nurse cells are covered by an epithelium of follicle cells produced by the follicle stem cells. This epithelium will secrete the eggshell at the end of oogenesis. Egg chambers within an ovariole stay connected through stalk cells.

When leaving the germarium, at stage 1 of oogenesis, the epithelium is composed of approximately 80 follicle cells covering the oocyte and the nurse cells. Until stage 5, follicle cells undergo mitosis to reach a population of around 650 cells. Then, until stage 10B, follicle cells stop dividing and execute three endocycles. Finally, at stage 10B, follicle cells stop endocycling and undergo gene amplification to replicate specific loci necessary for eggshell formation. At the end of oogenesis at stage 14, the follicle cells have secreted the eggshell and die by apoptosis.

During oogenesis, different populations of follicle cells are determined. After stage 8, 8-10 follicle cells in the anteriormost region, delaminate from the epithelium and migrate between the nurse cells to be in contact with the oocyte where they will form the structure for sperm entry, the micropyle (Ruohola et al., 1991; Silver and Montell, 2001). At the same time, some cells at the anterior become finer and stretch to cover the nurse cells while the rest of the anterior cells migrate toward the posterior. At stage 10B, follicle cells covering the anterior margin of the oocyte migrate centripetally between the nurse cells and the oocyte to encapsulate the oocyte. Finally, two appendages are produced by a group of follicle cells in the dorsal-anterior domain that will be necessary for gas exchange (Berg, 2005).

The formation of localized structures from an initially uniform tissue such as the dorsal appendages reflects the formation of an asymmetry and patterning during oogenesis. This phenomenon is mediated by signalling pathways.

1.3.2 The follicular epithelium is patterned by EGFR signalling

One of the major signalling pathways required for patterning of the egg is the EGFR signalling pathway, which is mediated in this tissue by its ligand Gurken (Grk). EGFR is expressed in all follicle cells and Grk is produced by the germline (Neuman-Silberberg and Schüpbach, 1993; 1996; Schüpbach, 1987). Grk mRNA is produced by the nurse cells and transported to the oocyte where it associates with the nucleus of the oocyte and where it is translated (Cáceres and Nilson, 2005; Saunders and Cohen, 1999). In early stages, when the oocyte is still small and its nucleus is located in the posterior of the egg chamber, posterior follicle cells are exposed to Grk which activates EGFR signalling, making cells adopt a posterior fate (González-Reyes et al., 1995; Gonzalez-Reyes and St Johnston, 1998; Roth et al., 1995).

Posterior follicle cells signal back to the oocyte leading a microtubule mediated migration of the oocyte's nucleus toward the dorsal-anterior side (González-Reyes *et al.*, 1995; Roth *et al.*, 1995; Steinhauer and Kalderon, 2006; Zhao et al., 2012). In this region, follicle cells exposed to Grk adopt dorsal-anterior fate. As a result, these cells lose expression of *pipe*. As previously explained, this will be important for the establishment of the dorsal-ventral axis in the embryo (Nilson and Schüpbach, 1998a; b; Sen *et al.*, 1998).

1.3.3 Mid and Mirr are two targets of EGFR signalling

An outstanding question about the patterning of this tissue came from the fact that both posterior and dorsal-anterior fates were established by the same ligand-receptor pair. In the posterior, in response to EGFR signalling, follicle cells express a pair of paralog T-box transcription factors Mid and H15 (Fregoso Lomas et al., 2013). Mid and H15 had previously been shown to be involved in the patterning the larval cuticle (Buescher et al., 2004; Nüsslein-Volhard et al., 1984), larval heart (Qian et al., 2005), leg imaginal disc (Svendsen et al., 2009), and larval

nervous system (Buescher et al., 2006) but their role in oogenesis was unknown. In fact, it is still currently unknown whether Mid and H15 directly induce posterior fate by inducing expression of other posterior determinants or if their role is only to prevent early EGFR activation to induce dorsal-anterior fate in the posterior (Fregoso Lomas *et al.*, 2013).

In the dorsal-anterior, EGFR signalling does not induce expression of Mid and H15 but of the homeobox transcription factor Mirror (Mirr) (Jordan et al., 2000; McNeill et al., 1997; Zhao et al., 2000). Mirr expression is graded along the DV axis with highest levels at the dorsal midline which is the point where the levels of EGFR activity are the highest. Mirr in turn plays an important role in the establishment of dorsal-anterior fate by regulating expression of Broad (Atkey et al., 2006; Boisclair Lachance et al., 2009; Cheung et al., 2013; Fuchs et al., 2012) which is necessary for the formation of the dorsal appendages (Deng and Bownes, 1997). High levels of Mirr correspond to dorsal midline fate and low Broad expression and lower levels of Mirr correspond to dorsal-lateral fate and high Broad expression (Fregoso Lomas et al., 2016). In addition, Mirr directly represses expression of *pipe*, which is important to establish the dorsal-ventral axis in the embryo (Andreu et al., 2012b).

1.3.4 Opposite gradients of JAK/STAT and BMP activity determine outcome of EGFR signalling

The discovery of the role of Mid and H15 and Mirr in the establishment of posterior and dorsal-anterior fate respectively raised the question of their regulation: how the cells know which target to express upon receiving EGFR signalling ? Previous lab members previously found that opposite gradients of JAK/STAT and BMP signalling determine the outcome of EGFR signalling (Fregoso Lomas *et al.*, 2016). The ligand for the JAK/STAT signalling, Unpaired (Upd) is produced by the polar cells that are present in the extremities of the egg chamber (Harrison et al.,

1995; McGregor et al., 2002; Xi et al., 2003) and posterior cells that are exposed to both Grk and Upd adopt posterior fate and express Mid (Figure 1.1). In the anterior, Decapentaplegic (Dpp), the ligand of the BMP signalling is produced by the anterior follicle cells (Twombly et al., 1996) and follicle cells exposed to Dpp and Grk adopt dorsal-anterior fate and express Mirr (Figure 1.1). Mid and Mirr are also repressing expression of the other, making the choice between posterior or dorsal-anterior mutually exclusive. Finally, Dpp and Upd repress Mid and Mirr independently of their mutual repression. Clones lacking *mirr* and unable to respond to JAK/STAT signalling still lose expression of *mid*, indicating that *mid* expression is not lost in clones unable to respond to JAK/STAT signalling because of induction of Mirr. In addition, clones lacking *mirr* and experiencing ectopic BMP activity still lose *mid* expression, indicating that Dpp repress *mid* expression independently of Mirr (Figure 1.1) (Fregoso Lomas *et al.*, 2016).

Together, these inputs generate a regulatory network integrating localized signals to produce a bistable system in which the JAK/STAT and BMP signalling pathways determine the outcome of EGFR signalling to induce two mutually exclusive fate. However, we do not know how the inputs from these signalling pathways are integrated and by which mechanisms they interact with each other.

1.3.5 Pointed and Capicua are downstream effectors of EGFR in the dorsal-anterior

Two important downstream effectors of EGFR signalling in the follicular epithelium are Pointed (Pnt) and Capicua (Cic). Pnt is an ETS transcription factors Its locus encodes 3 isoforms that come from alternative promoters, PntP1, PntP2, and PntP3 (Klambt, 1993). However, PntP2 and PntP3 only differ by a few exons that do not contain any known domains and the PntP3 isoform has only recently been described (Wu et al., 2020). Most previous studies have not made the distinction between the PntP2 and PntP3 isoforms and most reagents specific to PntP2 also affect

PntP3. All isoforms share their C-terminal ETS DNA binding domain and it is hypothesized that they are targeting the same targets (Klambt, 1993; Scholz et al., 1993). However, the two isoforms do not share the same N-terminal, the PntP2 isoform contain the Pnt domain, which is needed for MAPK mediated activation (Brunner et al., 1994; O'Neill et al., 1994). PntP1 lacks this domain and does not require phosphorylation by MAPK to be active. Instead, PntP1 is regulated at the level of transcription by transcription factors requiring MAPK (Gabay et al., 1996; Shwartz et al., 2013). Both isoforms of Pnt are general downstream effectors of EGFR signalling and they have been found to have a role in the development of many tissues such as the eye (Baonza et al., 2002; Flores et al., 2000; O'Neill *et al.*, 1994; Shwartz *et al.*, 2013; Xu et al., 2000), brain (Klaes et al., 1994; Klambt, 1993), mesoderm (Alvarez et al., 2003; Halfon et al., 2000; Schwarz et al., 2018), or different stem cells populations (Jin et al., 2015; Li et al., 2015; Liu et al., 2010; Ren et al., 2013; Xie et al., 2016; Xie et al., 2014; Zhu et al., 2011). It has also been found to have a role in oogenesis where both isoforms are expressed in the dorsal-anterior (Morimoto et al., 1996; Yakoby et al., 2008). Loss of *pnt* in this domain leads to a loss of the dorsal midline fate, potentially through regulation of *mirr* (Boisclair Lachance *et al.*, 2009; De Vito, 2021; Morimoto *et al.*, 1996). Interestingly, after stage 10B, *pnt* expression is lost in the dorsal midline and gets expressed in two patches of cells surrounding the dorsal midline (Morimoto *et al.*, 1996), this pattern of expression follow closely the pattern of MAPK activity that gets repressed in the dorsal midline (Zartman et al., 2009) which indicates that Pnt expression in the dorsal-anterior domain is dependent on the gradient of EGFR activity.

Another downstream effector of EGFR signalling is Capicua (Cic), which is an HMG-box transcriptional repressor. Cic was initially identified in Drosophila for its role in the formation terminal structure during embryogenesis (Jiménez et al., 2000). Cic’s activity is controlled by

MAPK phosphorylation which is controlled by RTK signalling. When cells experience RTK signalling, Cic is phosphorylated and silenced. In the follicular epithelium, phosphorylation leads to exclusion from the nucleus, preventing Cic from repressing targets (Jin *et al.*, 2015). However, in the embryo, repression of Cic by MAPK also lead to export to the cytoplasm where it is then degraded (Grimm et al., 2012). In the embryo, it was also shown that Cic is repressed before being excluded from the nucleus (Keenan et al., 2020; Lim et al., 2013) suggesting two modes of RTK mediated repression of Cic. This RTK mediated repression requires the C2 domain which is the docking site for MAPK to bind to and removal of the C2 domain causes Cic to be unresponsive to RTK signalling. Finally, the *cic* locus encodes two isoforms generated through alternative promoters and splicing sites (Forés et al., 2015; Jiménez et al., 2012; Lam et al., 2006). The two isoforms produced are named CicS and CicL for short and long isoforms. Each isoform shares a common C-terminus region containing the HMG DNA binding domain and the C2 domain. However, the CicL isoform contains the Tudor-like and N1 domains whose functions are unknown and the CicS isoform contains the N2 domain, which is necessary for Groucho (Gro) mediated repression of Cic target in the embryo (Forés *et al.*, 2015).

In the ovary, Cic has been shown to be important in the establishment of dorsal-anterior fate (Goff et al., 2001). Females mutant for *cic* produce dorsalized eggs. This phenotype is due to ectopic expression of Mirr in *cic* mutant cells causing anterior-dorsal fate (Goff *et al.*, 2001). Interestingly, this phenotype is independent of EGFR signalling, as *cic* mutant cells express Mirr even in the absence of Grk (Atkey *et al.*, 2006). In the dorsal-anterior, repression of Cic by EGFR, requires the C2 domains, clones expressing a mutant transgene of Cic lacking the C2 domain exhibit loss of Mirr expression and ectopic *pipe* expression (Andreu et al., 2012a; Astigarraga et al., 2007) due to lack of repression by Mirr (Andreu *et al.*, 2012b).

Together, these data showed that Pnt and Cic are two important effectors of EGFR signalling and a play major role in the establishment of dorsal-anterior fate. However, it is unknown whether they have a similar role in the establishment of posterior fate and in the regulation of Mid and H15.

1.4 Description of the aims of the thesis

The broad objective of this project is to understand how the inputs from the EGFR, JAK/STAT and BMP signalling pathways are integrated to generate the bistable outcome leading to the mutually exclusive expression of Mid and Mirr in the posterior and the dorsal-anterior domains respectively. My part in this project is to characterize the mechanisms by which the JAK/STAT signalling pathway promotes expression of Mid in the presence of EGFR signalling. My second objective is to describe the mechanisms by which EGFR establishes posterior fate.

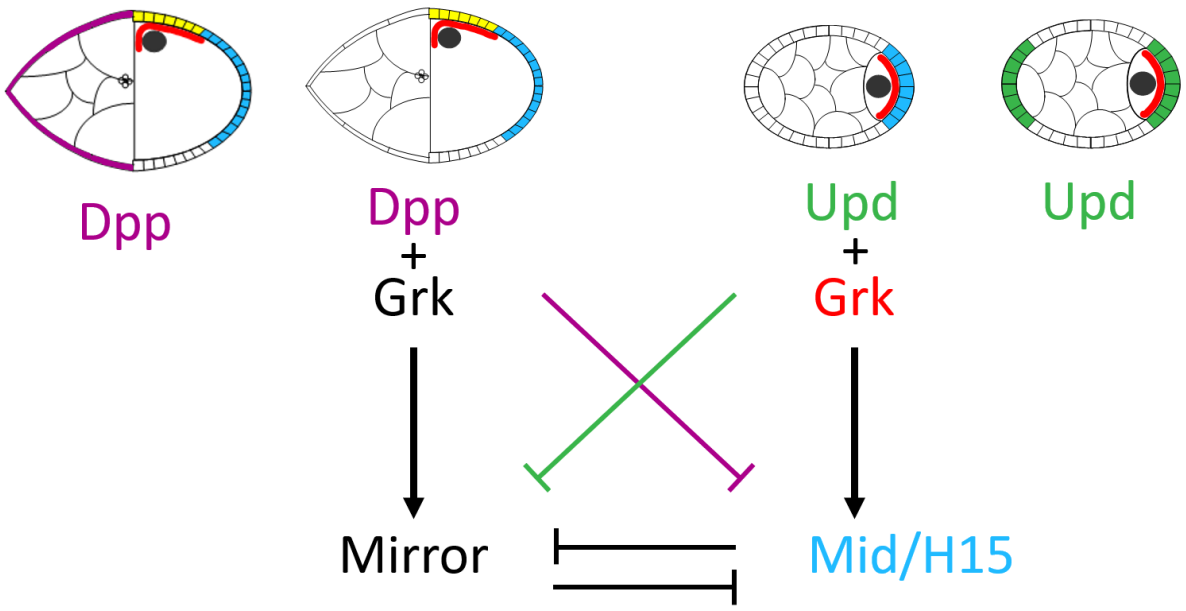
Dpp
Dpp
+
Grk
Upd
+
Grk
Upd
Mirror
Mid/H15

Figure 1.1 – Opposing gradients of JAK/STAT and BMP activity determine outcome of EGFR signalling.

In the posterior, the joint action of the EGFR ligand Grk and the JAK/STAT signalling ligand Upd determines posterior by inducing expression of Mid and H15. In the dorsal-anterior, the joint action of Grk and the BMP signalling ligand Dpp determines dorsal-anterior fate by inducing expression of Mid. In addition, Upd promotes posterior fate by repressing expression of Mirr and Dpp promotes dorsal-anterior fate by repressing expression of Mid and H15. Finally, the outcome of EGFR signalling is stabilized by the mutual repression between Mid/H15 and Mirr.

Chapter 2 - The JAK/STAT signalling pathway influences EGFR signalling outcome by controlling expression of its effector Pointed in the posterior region of the Drosophila ovary

2.1 Introduction

During development of multicellular organisms, cells need to be aware of their location within tissues to adopt the correct fate. Such positional information can be conveyed by localized signals present in the cellular environment that activate intracellular signalling pathways. These signalling pathways are activated upon binding of ligands to their receptor expressed by the receiving cell. This binding event causes an intracellular response leading to the activation or repression of target genes and to the differentiation of the cell. Localized activation of signalling pathways leads to the formation of domains in which cells adopt different fates. This initiates patterning of the tissue by regulating activity of transcription factors that control expression by binding to CRRs of target genes (Levine, 2010). These target genes include transcription factors generating a regulatory network leading to patterning of the tissue (Briscoe and Small, 2015). However, characterization of the signalling pathways led to the observation that the same pathways are reused over and over during development, indicating that the same signalling pathway can have different outcomes depending on the context (Perrimon *et al.*, 2012). The outcome of signalling depends on a large number of factors including duration of signalling, amplitude, or interaction with other pathways (Housden and Perrimon, 2014). However, the molecular mechanisms by which the cellular context affects the outcome of signalling remains poorly understood.

To address this question, we use the follicular epithelium of the Drosophila ovary as a model. The follicular epithelium is a single layer of cells covering the developing oocyte and

producing the eggshell. In this tissue, localized activation of the EGFR signalling pathway specifies different follicle cell fates, depending on the timing and location of the signals (González-Reyes *et al.*, 1995; Gonzalez-Reyes and St Johnston, 1998) indicating that the same signalling pathway leads to two different outcomes. The EGFR ligand, Grk is produced and secreted by the oocyte (Schüpbach, 1987). Grk colocalizes with the nucleus of the oocyte and follows its dynamic location. Early in development, when the oocyte is small, the nucleus of the oocyte is located at the posterior of the egg chamber, hence, only posterior follicle cells are exposed to Grk (Neuman-Silberberg and Schüpbach, 1993). In this domain, cells experiencing EGFR activity adopt a posterior fate (Gonzalez-Reyes and St Johnston, 1998) and express the transcription factors Mid and H15 (Fregoso-Lomas 2013, 2016). In later stages, when the oocyte grows, the nucleus and the gradient of Grk translocate to the dorsal-anterior. At this stage, follicle cells experiencing EGFR activity adopt a dorsal-anterior fate and express the transcription factor Mirr (Jordan, 2000; Zhao, 2000, Fregoso-Lomas, 2016). Therefore, the same inductive signal from EGFR induces both posterior and dorsal-anterior fate and patterns the epithelium along the anterior-posterior and dorsal-ventral axes. This is an example of a signalling pathways having two different outcomes within the same tissue

The choice between the alternative EGFR targets is determined by other localized factors. In the posterior region, cells are also exposed to Upd, an extracellular ligand that regulates its transcriptional targets through activation of the JAK/STAT signalling pathway (Harrison et al., 1998; McGregor *et al.*, 2002; Xi *et al.*, 2003). In the presence of JAK/STAT activity, EGFR signalling induces expression of Mid and H15. In the anterior region, cells are exposed to Dpp, the ligand activating the BMP signalling pathway (Twombly *et al.*, 1996). In the presence of BMP activity, Grk/EGFR signalling induces expression of Mirr. Mid and Mirr also repress expression

of the other. In addition, activation of the JAK/STAT signalling pathway represses expression of Mirr, independently of the regulation of Mid, and activation of the BMP signalling pathways represses of Mid, independently of the regulation of Mirr. Together, the mutual repression between Mid and Mirr and the opposite regulation by the JAK/STAT and BMP signalling pathways generates a switch-like decision ensuring that the correct EGFR target is expressed and proper patterning of the tissue.

This regulatory network was identified using genetic techniques and we do not know whether the different inputs are directly captured through binding of transcription factors to CRRs controlling expression of *Mid/H15* and *Mirr*. We approach this question by identifying CRRs controlling expression of *mid/H15* and *mirr* and by characterizing the responses of these CRRs to the inputs regulating expression of *mid/H15* and *mirr*. We can then test whether these sequences are directly capturing the regulatory inputs by searching and mutating binding sites for transcription factors acting downstream of the different signalling pathways.

Previously, another lab member had identified and characterized a putative CRR recapitulating the repression of *mirr* by JAK/STAT signalling. Disruption of a putative STAT92E binding site relieves the repression of JAK/STAT signalling and leads to ectopic activity of the putative CRR, suggesting that *mirr* is directly repressed through binding of STAT92E to this binding site present in the CRR (De Vito, 2021).

In this chapter, we use the same approach to ask how the activation of JAK/STAT signalling in posterior follicle cells positively regulates the expression of Mid. We identified and characterized a putative CRR recapitulating expression of Mid in posterior follicle cells that is responsive to JAK/STAT signalling. However, disruption of putative STAT92E binding sites did not affect activity of this putative CRR, suggesting that *mid* is not regulated through direct binding

of STAT92E to this putative CRR. Instead, we found that JAK/STAT signalling regulates the EGFR effector Pnt in posterior follicle cells which in turn regulates expression of Mid. We propose that JAK/STAT signalling influences outcome of EGFR signalling by increasing levels of its EGFR effector Pnt and by repressing its alternative target Mirr to promote expression of Mid and posterior fate.

2.2 Results

2.2.1 The *mid^{up}* cis-regulatory region recapitulates expression of Mid in the posterior domain and is responsive to EGFR and JAK/STAT signalling.

2.2.1.1 Rationale

To understand how the inputs regulating Mid expression in posterior follicle cells are captured, we looked for CRRs that recapitulate expression of Mid and therefore would capture these inputs. Previous lab members had screened two genome-wide collections of CRR reporters in which 2-3kb of genomic sequences were cloned upstream of a minimal promoter and a *Gal4* reporter gene (Kvon et al., 2014; Pfeiffer et al., 2008) for reporter annotated to the *mid* and *H15* genes. As explained above, we also looked for the reporter attributed to the *mirr* gene. We are interested in the mechanisms by which the JAK/STAT signalling influences EGFR signalling to promote expression of Mid. Here, we test the hypothesis that *mid* is directly activated by the JAK/STAT signalling through binding of STAT92E to CRRs controlling expression of Mid.

2.2.1.2 The *mid^{up}* CRR reporter recapitulates expression of Mid in posterior follicle cells.

One of the reporters identified (R86G04, renamed *mid^{up}*) is located upstream of the transcriptional start site of *mid* and contains 3.7kb of genomic sequence (Figure 2.1A). This reporter is driving expression of a reporter gene in a similar pattern as *mid* in posterior follicle cells. It starts being active around stage 7 of oogenesis in posterior follicle cells and its expression pattern,

despite being uneven, overlaps completely with the expression pattern of *mid (*Figure 2.1B-B', arrowhead). In later stages of oogenesis, the mid^{up} reporter expression exhibits an extension toward the dorsal-anterior, similar to what is observed with endogenous Mid (Figure 2.1C-C'). In addition, the mid^{up} reporter is also expressed sparsely in centripetal cells at stage 10B (Figure 2.1C, arrowhead) which is not observed with endogenous Mid. Because none of the line attributed to *H15* were active in the follicular epithelium, for the rest of the thesis, I will only focus on *mid* regulation.

2.2.1.3 The mid^{up} CRR reporter is regulated by EGFR signalling.

Given that this expression pattern closely resembles endogenous Mid, we wanted to test whether the mid^{up} reporter was responsive to EGFR signalling like the endogenous *mid* gene is. To do so, I generated clones homozygous for a null allele of *egfr* ($egfr^{CO}$) (Clifford and Schüpbach, 1989; James et al., 2002) making the cells unable to activate EGFR signalling. In clones lacking *egfr*, we observe loss of Mid expression, as previously reported (17/17, Figure. 2.1D-D''(Fregoso Lomas *et al.*, 2013)) and we also observe loss of mid^{up} expression. This loss is completely penetrant (17/17, Figure 2.1D') indicating that EGFR signalling is necessary for mid^{up} expression.

To further analyse the response of the mid^{up} reporter to EGFR signalling, I did the converse experiment in which I generated clones experiencing ectopic EGFR activity by expressing a constitutionally active form of EGFR ($EGFR^{ACT}$) (Queenan et al., 1997). In these clones, Mid and mid^{up} expression is induced (31/31, Figure 2.1E'-E") which can be observed all along the epithelium. These results are indicating that the mid^{up} reporter is responsive to EGFR, in a similar manner as the endogenous *mid* gene.

2.2.1.4 The *mid^{up}* CRR reporter is regulated by JAK/STAT signalling.

After finding that the *mid^{up}* reporter was responsive to EGFR signalling, we wanted to test whether it is also regulated by the other positive regulators of Mid expression, JAK/STAT signalling. To do so, we generated clones homozygous for a null allele of the transcription effector of the JAK/STAT signalling pathway, *Stat92E* (Hou et al., 1996). In clones lacking STAT92E, we observe loss of Mid expression, as previously reported (Figure 2.1F" (Fregoso Lomas *et al.*, 2016)), providing a positive control that confirms the loss of STAT92E activity in these clones. We also observe that expression of the *mid^{up}* reporter is lost in these clones (Figure 2.1F'). Similar to Mid, the penetrance of the loss of the *mid^{up}* reporter is not complete (moderate (4%) severe (24%), complete (68%), n=24 (De Vito, 2021)). This is nonetheless indicating that the *mid^{up}* reporter is responsive to JAK/STAT signalling.

To confirm this observation, we generated clones experiencing ectopic JAK/STAT signalling by overexpressing the JAK kinase, Hopscotch (Hop) (Harrison *et al.*, 1995). In these clones, we observe ectopic expression of both Mid and the *mid^{up}* reporter, but the induction is only occurring in the posterior and dorsal-anterior domains (Figure 2.1G'-G", arrow). There is no ectopic expression in the anterior-ventral side (Figure 2.1G'-G", arrowhead). This is consistent with our previous characterization of the regulation of *mid* by JAK/STAT signalling which showed that inputs from JAK/STAT signalling are not sufficient to induce ectopic Mid expression but require inputs from EGFR signalling (Fregoso Lomas *et al.*, 2016).

Together, these data are indicating that the *mid^{up}* CRR is regulated by both EGFR and JAK/STAT signalling and could represent the CRR by which inputs from these signalling are captured during regulation of the endogenous *mid* gene.

2.2.2 Disruption of putative STAT92E binding sites in the *mid^{up}* CRR does not affect expression of its reporter.

2.2.2.1 Rationale

We want to further understand the mechanism by which inputs from JAK/STAT signalling are captured by the CRR and how this influences EGFR signalling outcome. We hypothesized that the *mid^{up}* CRR would be directly activated by JAK/STAT signalling through binding of STAT92E to putative binding sites present in its sequence, analogous to what is observed with *mirr* (De Vito, 2021). We can test whether these sites are required by introducing mutations to the sequence in the reporter and observing the effect on the expression of the reporter.

2.2.2.2 Deletion mapping of the *mid^{up}* to further to define the CRR and narrow down the search for STAT92E binding sites.

A previous lab member had searched for putative STAT92E binding sites in the sequence of the *mid^{up}* CRR and found that the three best scoring sites were scattered around the sequence (Figure 2.2A, red squares (De Vito, 2021)). To further define the sequence of the CRR, we have generated derivative constructs by splitting the sequence and cloning each part upstream of a reporter. Two derivatives, *mid$^{up.1}$* and *mid$^{up.1B}$*, that contain 2267bp and 1193bp of the original sequence respectively, reproduced the exact same expression pattern as the original reporter (Figure 2.2B-C"), while the reporters containing the sequences not included in *mid$^{up.1}$* and *mid$^{up.1B}$* did not drive expression of a reporter in follicle cells. Another derivative, *mid$^{up.1B2}$*, made from *mid$^{up.1B}$*, also drove expression of the reporter in posterior follicle cells but also in the dorsal-anterior after stage 9 of oogenesis. This suggests that the expression pattern follows the gradient of EGFR activity and that it is missing an element responsible for repressing expression of the reporter in the dorsal-anterior. Interestingly, it is expressed in a domain that lack JAK/STAT signalling at

the moment when it is induced, suggesting that in the absence of a repressor element, Mid would be expressed in the dorsal-anterior in response to EGFR signalling.

This analysis identified a smaller region in *mid*$^{up.1B}$ which is recapitulating faithfully the original expression pattern of *mid*^{up} CRR reporter. We will use this derivative for the following analysis of STAT92E binding sites.

2.2.2.3 Disruption of a putative STAT92E binding site in the *mid*$^{up.1B}$ derivative construct does not affect expression of the reporter.

Out of the three best scoring putative STAT92E binding sites identified previously, only one is present in the *mid*$^{up.1B}$ derivative. To test whether this site is necessary for posterior expression of the reporter, we generated mutations in this site that would disrupt potential binding of STAT92E to this sequence (Figure 2.1F). The consensus binding site of STAT92E is TTCNNNGAA and the sequence was changed to TTCNNNGtt, which was previously shown to disrupt STAT92E binding in vitro and in vivo (Karsten et al., 2006; Rivas et al., 2008; Sotillos et al., 2010). Contrary to our hypothesis, in the mutant reporter, we observed that expression of the reporter is still present (Figure 2.1G') and was identical to the non-mutated reporter (compare with Figure 2.1C') which is indicating that the *mid*^{up} CRR is not regulated by JAK/STAT signalling through this binding site. Although we cannot rule out the possibility that *mid*^{up} is regulated directly by JAK/STAT signalling through a cryptic binding site(s), this observation suggests that the *mid*^{up} CRR and *mid* could be regulated by JAK/STAT signalling through an indirect mechanism.

2.3 The EGFR effector Pnt is regulated by JAK/STAT signalling in posterior follicle cells and regulates Mid expression.

2.3.1.1 Rationale

In the previous part, we found that disrupting putative STAT92E binding sites did not affect expression of the *mid*^{up} CRR reporter. This finding led us to the new hypothesis that *mid* could be regulated indirectly by JAK/STAT signalling. A good candidate for a factor through which JAK/STAT signalling would act on to regulate *mid* is Pnt which is an ETS transcription factor known to act downstream of EGFR signalling (Brunner *et al.*, 1994; O'Neill *et al.*, 1994). Previous studies have shown that *pnt* is expressed in the ovary (Morimoto *et al.*, 1996) and it has been shown to have an important role in the establishment of dorsal-anterior fate (Boisclair Lachance *et al.*, 2009; Morimoto *et al.*, 1996; Zartman *et al.*, 2009). Pnt is expressed in the posterior region around stage 6 and gets expressed in the dorsal-anterior after stage 9 (Figure 2.3A'A"). Pnt represents an excellent candidate for being the factor by which JAK/STAT signalling regulated *mid* expression because an enhancer trap from the *pnt* locus that recapitulates the posterior expression of *pnt* has been shown to require JAK/STAT signalling (Xi *et al.*, 2003). In addition, *pnt* has been shown to be regulated by JAK/STAT signalling the eye disc (Flaherty et al., 2009) and in the wing disc (Pascual et al., 2017). In the rest of this chapter, I ask whether *mid* is regulated by the JAK/STAT signalling pathway through Pnt.

2.3.1.2 Pnt is regulated by JAK/STAT signalling in the posterior region and its expression requires EGFR signalling.

As previously stated, a *pnt* enhancer trap was shown to be regulated by JAK/STAT signalling (Xi *et al.*, 2003), however, it has not been shown that the endogenous *pnt* gene is regulated by JAK/STAT signalling. To test whether *pnt* is regulated by JAK/STAT signalling in

the posterior, I generated clones unable to respond to JAK/STAT signalling by expressing an RNAi targeting the receptor of Upd, *domeless* (*dome*) (Brown et al., 2001) and looked at Pnt expression using an antibody targeting Pnt (Pascual *et al.*, 2017) as well as Mid as an internal control.

In clones lacking *dome*, we observe that *mid* expression is lost (Figure 2.3B"), as previously reported (Fregoso Lomas *et al.*, 2016), confirming the genotype of the clones and that the RNAi functioned to silence JAK/STAT signalling. For Pnt expression, we observe loss of expression in the posterior region (30/30, Figure 2.3B') which is consistent with the results from the *pnt* enhancer trap where expression of the reporter requires JAK/STAT signalling (Xi *et al.*, 2003). This is indicating that, in the posterior domain, *pnt* expression requires JAK/STAT signalling. However, no effect is observed on *pnt* expression in the dorsal-anterior region (0/22, Figure 2.3B'), which is consistent with the absence of JAK/STAT signalling in this region and shows the anterior and posterior domains of *pnt* expression are generated by different mechanisms.

To further define the response of Pnt to JAK/STAT signalling, we did the converse experiment by generating clones experiencing ectopic JAK/STAT signalling by overexpressing Hop and looking at expression of Mid and Pnt. We observe higher levels of *mid* expression in the posterior (80/87, Figure 2.3C"-E", arrowhead) and dorsal-anterior regions (11/24, Figure 2.3E", arrowhead) as previously shown (Fregoso Lomas *et al.*, 2016), again confirming the genotype of the clones. No expression in the ventral-anterior domain was detected (0/16, Figure 2.3D"-E", arrow). In early egg chambers, *mid* expression is induced in cells over the nurse cells but stays restrained in the posterior, cells in the anteriormost do not express *mid* (Figure 2.3C", arrow). For Pnt, we observe ectopic expression in the posterior domain (80/87, Figure 2.3C'-E') but clones in the dorsal-anterior do not exhibit higher levels of Pnt expression compared to the levels of

endogenous dorsal-anterior Pnt in adjacent wild type cells (2/21, Figure 2.3E', arrowhead). Clones in the anterior-ventral domain do not exhibit a gain of *pnt* expression (0/16 Figure 2.3D').

Lack of *pnt* expression in ventral clones is expected due to the requirement for EGFR signalling (Morimoto *et al.*, 1996) and because we know from the absence of Pnt in anterior follicle cells in early-stage egg chambers that Upd is not sufficient to induce Pnt expression. However, the absence of ectopic expression in the dorsal side is surprising, especially given that Mid is induced there. We would have expected to see levels of Pnt similar to the ones observed in the posterior, but we do not see a change in expression levels. This would suggest that something prevents Pnt from being expressed in response to JAK/STAT signalling in this domain. In this situation, JAK/STAT signalling seems unable to induce higher levels of Pnt expression but could still promote expression of Mid by repressing Mirr. In clones in the posterior, we observe a strong overlap in clonal cells expressing Pnt and Mid suggesting that Mid expression could require Pnt.

2.3.1.3 Mid is regulated by Pnt.

Next, we wanted to ask whether Pnt was regulating expression of Mid in the posterior with the hypothesis that JAK/STAT signalling would regulate Mid through Pnt. We generated clones homozygous for a null allele of *pnt* ($pnt^{\Delta 88}$) (Scholz *et al.*, 1993) which affects both Pnt isoforms. In these clones, we observe loss of Mid expression, but this loss is not completely penetrant: we observe a strong loss in the anterior domain of Mid expression and the posterior domain (Figure 2.4A-A', arrow), but in the domain in between, we observe a weaker loss (Figure 2.4A-A', arrowhead). We see this in 37/53 clones that span the whole domain of Mid expression from anterior to posterior. A similar, but weaker regional effect was also observed with a second null allele of *pnt* (pnt^{2}) (22/51; data not shown). This observation is consistent with a recent report that

showed that Mid is regulated by Pnt (Stevens et al., 2020). However, the authors of this study have only described the difference in the penetrance without explaining the regional effect.

We also looked at the effect of losing Pnt on the *mid*^{up} reporter and we observe loss of expression with a strong penetrance (Figure 2.4A"), and we do not observe this regional effect, suggesting that the regulation of *mid* by Pnt could be captured by this CRR.

To further characterize the response of *mid* to Pnt, we then generated clones gaining Pnt function. The *pnt* locus encodes for two isoforms (Klambt, 1993), PntP1 and PntP2. Both are ETS transcription factors, but PntP1 is constitutionally active (Gabay *et al.*, 1996) whereas PntP2 requires activation by MAPK to be active (Brunner *et al.*, 1994; O'Neill *et al.*, 1994). In the ovary, only PntP1 is reported to be expressed in posterior follicle cells and both isoforms are expressed in the dorsal-anterior (Morimoto *et al.*, 1996; Yakoby *et al.*, 2008). We hypothesized that *mid* is most likely regulated by PntP1 because it is the only isoform expressed in the posterior, but we nonetheless tested both isoforms. Clones experiencing ectopic PntP1 express Mid everywhere (148/150, Figure 2.4B-C'), regardless of the position of the clones. We observe the same phenotype with the *mid*^{up} reporter (57/59, Figure 2.4B"-C"). These results are similar to those observed with the clones expressing Egfr^{ACT}, which is consistent with the role of PntP1 as an effector of EGFR signalling and the fact that it is constitutionally active.

Additionally, in late-stage egg chambers, cells in the clones expressing PntP1 change their shape and appear more clustered (Figure 2.4C-C"). This change of morphology suggests a possible change in cell fate. The cells in the clones may be adopting an aeropyle fate (Dobens and Raftery, 2000). Cells in the posteriormost normally become clustered to form the aeropyle and the fact that formation of the aeropyle was shown to be influenced by JAK/STAT signalling (Wang et al., 2014) could suggest a role of PntP1.

For UAS-PntP2 clones, we observe a more complex phenotype. In early-stage egg chambers, we see ectopic expression of Mid in the posterior and in the cells covering the nurse cells in the posteriormost position (54/65, Figure 2.4D'), cells located anterior to this region do not exhibit ectopic expression. In later egg chambers, when Grk is located in the dorsal-anterior, we observe ectopic expression of Mid in the dorsal-anterior (19/23, Figure 2.4E', arrowhead) domain but not in the ventral domain (6/28, Figure 2.4D', arrow). These results are consistent with the fact that PntP2 requires phosphorylation by MAPK to be active (Brunner *et al.*, 1994) and therefore PntP2 would only be active in the region where Grk is present. It is nonetheless surprising to observe that despite not being expressed in the posterior (Morimoto *et al.*, 1996; Yakoby *et al.*, 2008) PntP2 is able to induce expression of Mid but could be explain by the fact that PntP1 and PntP2 share the same DNA binding domain and would target the same genes.

For the mid^{up} reporter, we observe similar results with a weaker induction. In early-stage egg chambers, the reporter is still induced in the posterior (14/19, Figure 2.4C") but its expression does not get induced as much in the anterior as Mid. In the dorsal-anterior domain of later egg chambers, we observe that the mid^{up} reporter is almost not induced past its endogenous expression domain (2/9, Figure 2.4D"). In the ventral-anterior domain, we observe no induction of expression (1/10, Figure 2.4D"). The mid^{up} reporter is therefore responsive to gain of PntP2 but is less sensitive compared to Mid. It is also more difficult to detect ectopic expression of the mid^{up} reporter because its expression is not uniform. Together, this data indicates that Pnt expression in posterior follicle cells requires JAK/STAT signalling and regulates Mid expression. Pnt therefore represents a good candidate for factor by which the JAK/STAT signalling pathway regulates expression of Mid.

2.3.2 Pnt expression rescues loss of Mid seen in clones unable to respond to JAK/STAT signalling.

2.3.2.1 Rationale

We have shown previously that Pnt expression in the posterior requires JAK/STAT signalling, and that Mid expression is regulated by Pnt. In addition, by studying a putative CRR recapitulating *mid* expression in the posterior, we were unable to find a direct a regulation through binding of STAT92E to this sequence. Together, these data led us to the hypothesis that JAK/STAT signalling regulates expression of Mid by regulating levels of Pnt.

To test this hypothesis, we asked whether Pnt acts downstream of JAK/STAT signalling to regulates Mid expression. To do so, we generated clones lacking the ability to respond to JAK/STAT signalling by expressing a RNAi against *dome,* which causes loss of Pnt and Mid expression, and restoring expression of Pnt using the UAS-GAL4 system. For technical reasons, I was unable to remove JAK/STAT signalling by making *Stat92E* mutant clones; because the locus of the insertion of the UAS-PntP1 transgene we used is too close to the locus of FRT82B, I could not generate a recombination between FRT82B and UAS-PntP1, which prevented me from making *Stat92E* clones expressing ectopic PntP1. Therefore, for this experiment I used the RNAi against *dome,* described above, which allowed me to generate clones that both lack expression of *dome* and express ectopic PntP1.

If we observe Mid expression in these clones, it will mean that ectopic expression of Pnt can rescue the loss of Mid seen in clones lacking JAK/STAT signalling and will indicates that Pnt acts downstream of JAK/STAT signalling to regulate Mid expression. Finally, we also tested the response of the mid^{up} reporter to test whether these inputs would be captured by this putative CRR.

2.3.2.2 Ectopic expression of PntP1 rescues loss of Mid in clones lacking JAK/STAT signalling.

First as a control, we generated clones expressing the RNAi against *dome* and looked at Mid and the *mid*^{up} reporter. Consistent with previous results, we still observe loss of Mid (Figure 2.5A-B') in the clones and we also observe loss of the *mid*^{up} reporter (35/38, Figure 2.5A"-B"). However, the penetrance of the loss is weaker than what is observed with the *Stat92E* null allele (compare Figure 2.5A" and 2.5B" to Figure 2.1F'). We observe nonetheless that both Mid and the *mid*^{up} reporter expression is lost in the clones expressing the RNAi against *dome*.

Next, we looked at clones expressing the RNAi against *dome* but also expressing PntP1. In these clones, we observe that expression of both Mid and the *mid*^{up} reporter is rescued in the posterior (176/183 for Mid, 26/26 for *mid*^{up}, Figure 2.5C-C") and we even observe ectopic expression everywhere in the epithelium (Figure 2.5D-D"), similar to the phenotype observed with clones expressing PntP1 alone. This is indicating that PntP1 is sufficient to induce expression of Mid in the absence of JAK/STAT signalling, thus presumably bypassing the requirement of Upd for Mid expression.

2.3.2.3 Ectopic expression of PntP2 partially rescues loss of Mid in clones lacking JAK/STAT signalling.

When we did the equivalent experiment with PntP2, we observe that in early egg chambers, we saw that Mid expression is mostly rescued in the posterior (27/38, Figure 2.5E'-E") and we see ectopic expression being restrained to the posterior domain, which is consistent with the phenotype observed with clones expressing PntP2. However, in some rare clones, levels of Mid expression are lower than in the neighboring wild type cells which was not observed with clones expressing PntP2 indicating that Mid expression is not completely rescued (11/38, Figure 2.5.F'). In the

anterior, Mid is mostly not induced (3/13 in the dorsal side and 0/8 in the ventral side, Figure 2.5E'-F', arrows).

We observe a similar phenotype with the *mid*^{up} reporter but with a weaker phenotype as compared to the PntP2 clones (46/55, Figure 2.5E"-F", arrowhead), and we also observe loss of expression in some clones (9/55). However, in the anterior, expression of the *mid*^{up} reporter is not induced (0/12 in the dorsal side and 0/11 in the ventral side, Figure 2.5E"-F", arrow). This shows that in the posterior, PntP2 is mainly able to rescue loss of JAK/STAT signalling but with a weaker effect than PntP1. In addition, we see that ectopic expression of Mid is almost not seen, compared to the PntP2 single clones.

2.4 Discussion

2.4.1 JAK/STAT regulates Mid expression through the EGFR effector Pnt and by repressing Mirr

Our data suggest that *mid* is not a direct target of STAT92E, instead, we found that the JAK/STAT signalling pathway regulates Pnt, whose expression in the posterior requires EGFR signalling (Morimoto *et al.*, 1996). This indicates that the JAK/STAT signalling pathway interacts with EGFR signalling through the control of its effector. Pnt had previously been shown to be regulated by the JAK/STAT signalling pathway in the eye disc (Flaherty *et al.*, 2009) and in the wing disc (Pascual *et al.*, 2017), suggesting that Pnt could be a common point of crosstalk between EGFR and JAK/STAT signalling in Drosophila.

Another role of the JAK/STAT signalling pathway in posterior follicle cells is to repress expression of the alternative EGFR target Mirr. Recent work in our lab has shown that a potential CRR recapitulating the early expression of Mirr requires a putative STAT92E binding site for JAK/STAT mediated repression in the termini of the egg chamber (De Vito, 2021; Fregoso Lomas

et al., 2016). This is suggesting that the endogenous Mirr could be repressed by the JAK/STAT signalling pathway through this putative STAT92E binding site.

This is describing the mechanism by which the JAK/STAT signalling pathway influences the outcome of EGFR signalling in follicular epithelium to establish posterior fate. In one way, JAK/STAT signalling increases the levels of the EGFR effector Pnt, which in turn promotes expression of Mid. In another way, JAK/STAT signalling also favors induction of *mid* expression by EGFR signalling by directly repressing the alternative EGFR target Mirr. It is, however, still unknown whether Pnt is directly activated by binding of STAT92E to a CRR in the *pnt* locus.

2.4.2 Repressive action of STAT92E

As explained above, recent studies have shown a role for STAT92E as a direct repressor of Mirr in the termini region of the early egg chamber (De Vito, 2021). It was surprising to observe, that in the regulatory network establishing posterior fate in the follicular epithelium, a direct repression of STAT92E was observed where we could not find a direct activation, despite the role of STAT92E as an activator of transcription (Levy and Darnell, 2002; Yan et al., 1996).

Studies in mammals and Xenopus have identified a role of STAT proteins as repressor. STAT1 was shown to repress *Skp2* in Ras human colon cancer cells (Wang et al., 2010). STAT3 was shown to repress expression of tumor-suppressor genes when acetylated in cancer cells (Lee et al., 2012). STAT5 was shown to represses BCL6 expression in lymphomas (Walker et al., 2007) and in Xenopus to regulate early embryonic erythropoiesis (Schmerer et al., 2006). Finally, a recent study has shown the role of STAT6 as a repressor in macrophage (Czimmerer et al., 2018). Interestingly, this study showed that CRRs of gene repressed by STAT6 exhibit lower STAT occupancy and canonical STAT6 binding sites. Repression of BCL6 by STAT5 was shown to

happen through a canonical binding site (Walker *et al.*, 2007), similar to what we observe with Mirr (De Vito, 2021).

Our evidence showing a repressive role of STAT92E is especially interesting when we observe that many of the mammalian STAT proteins also seem to have a repressive function which could indicate that the repressive function of STAT protein is well conserved. Many studies have shown the JAK/STAT signalling pathway is well conserved between flies and humans (Arbouzova and Zeidler, 2006). More studies will be needed to further understand how STAT protein repress transcription.

2.4.3 How are repressive inputs of Mid captured?

We have described a mechanism by which positive inputs from the JAK/STAT and EGFR signalling pathways are integrated into Pnt to regulate Mid, but Mid is also repressed by Mirr and the BMP signalling pathway (Fregoso Lomas *et al.*, 2016). Other lab members have looked at the response of the mid^{up} CRR to Mirr and found that ectopic expression of Mirr represses expression of the reporter (De Vito, 2021). We are now interested in finding whether this repression happen directly through binding of Mirr to the CRR sequence. Interestingly, one of the derivatives of mid^{up} presented above, $mid^{up.1B2}$ (Figure 2.2), is not repressed in the dorsal-anterior domain, suggesting that it is no longer repressed by Mirr. Therefore, the fragments present in the $mid^{up.1B}$ derivative but absent in the $mid^{up.1B2}$ derivative represent good candidate for sequence containing binding site of Mirr. Andreu et al. have described how Mirr directly represses expression of *pipe* in the follicular epithelium and the framework described in their study could be replicated to test whether *mid* is directly regulated by Mirr (Andreu *et al.*, 2012b).

We are also interested in understanding how the inputs from the BMP signalling pathway are captured to repress expression of Mid; as previously reported, BMP signalling can repress Mid

expression independently of the repression of Mid by Mirr. The *mid*^{up} CRR reporter is not responsive to BMP signalling (data not shown), but previous lab member had identified another CRR recapitulating the expression pattern of Mid that is responsive to BMP signalling. This CRR, named *mid*^{intron} because its sequence is from the first intron of the *mid* gene, is a good candidate to test whether Mid is directly regulated by the BMP signalling pathway.

2.4.4 Is Pnt directly regulating *mid* expression ?

We have identified that Mid is regulated by Pnt, which together with other data implies that Pnt represents the point of crosstalk between the EGFR and JAK/STAT signalling pathways. However, we do not know if Mid is directly regulated by Pnt. In heart progenitor cells, a putative CRR controlling expression of *mid* has been shown to require a Pnt binding site (Schwarz *et al.*, 2018). In the ovary, two putative CRRs have been identified to recapitulate expression of Mid, *mid*^{up} and *mid*^{intron}, and both are responsive to Pnt (De Vito, 2021). Interestingly, the expression of *mid*$^{up.1B2}$ reporter in the dorsal-anterior is extremely similar as the expression pattern of Pnt at the same stage (compare Figure 2.2D' to 3A'). If Mid is directly regulated through direct binding of Pnt, the sequence contained in the *mid*$^{up.1B2}$ reporter would represent a good candidate for the search of putative Pnt binding sites. However, the fact that the *mid*^{intron} CRR also requires Pnt, could suggest that more than one Pnt binding sites are regulating expression of Mid. Alternatively, it is possible that Pnt does not directly regulate *mid* expression by binding to one of its CRRs. In that case, Pnt would most likely activate another regulator of *mid* expression that we have not identified.

Finally, we observed that if Pnt is sufficient to induce Mid expression, it is not necessary. Clones lacking *pnt* still express Mid at lower levels, the loss is stronger at the anteriormost and posteriormost domains of Mid expression. The cells in between are less affected. A similar observation was made by (Stevens et al., 2020) but they have not described the regional differences

and not shown the effect in the posteriormost cells. This would suggest that there exist other regulators of Mid that we have not identified.

Understanding how all the inputs from the different signalling pathways are integrated to regulate *mid* and *mirr* expressions will provide lots of insight to how signalling pathways interact to pattern tissues and generate diversity in the outcome of signalling.

2.5 Material and Methods

2.5.1 Drosophila strains

The following Drosophila stocks were used: FRT42D $egfr^{CO}$ (Clifford and Schüpbach, 1989; James *et al.*, 2002), UAS-$Egfr^{ACT}$ (Queenan *et al.*, 1997), FRT82B $Stat92^{85C9}$ (Silver and Montell, 2001), UAS-hop (Harrison *et al.*, 1995), UAS-$dome^{RNAi}$ P{TRiP.HMS00647}, $pnt^{\Delta 88}$ (Scholz *et al.*, 1993), UAS-PntP1 (Klaes *et al.*, 1994), UAS-PntP2 (Klaes *et al.*, 1994). CRR lacZ reporters and derivatives were made for this study but original Gal4 reporter were produced by (Pfeiffer *et al.*, 2008).

2.5.2 Mosaic analysis

Negatively marked loss-of-function were generated as described in (Laplante and Nilson, 2006) through FLP/FRT-mediated mitotic recombination between a wild-type or mutant chromosome bearing P{neoFRT42D} and a marker chromosome bearing the same FRT and a P{piM} insertion of the same chromosome arm. The MARCM system (Lee and Luo, 2001) was used to generate clones of cells positively marked GAL4-driven expression of either GAL4-driven expression of either P{UAS-*mCD8::GFP.L*} or P{UAS-*GFP.nls*}. In some experiments, the GAL4 expressed in these clones also drove the expression of a UAS-$Egfr^{ACT}$, a UAS-*hop*, a UAS-*PntP1* or a UAS-*PntP2* transgene or an RNAi construct targeting *dome*. For generation of clones, females of the appropriate genotype were heat shocked for 1 hr at 37°C as late pupae or newly

eclosed adults, either once or on 3 consecutive days, then aged at 18 or 22°C and supplemented overnight or two days with dry yeast before dissection. Ovaries were harvested 10 days after first heat shock, with shorter intervals between clone induction and dissection enriching the sample for clones generated later in oogenesis.

2.5.3 Immunohistochemistry

Dissection and immunostaining were carried out as described previously (Laplante and Nilson, 2006) Antibodies used were guinea pig anti-Mid (1:2,000) (Fregoso Lomas *et al.*, 2013), rabbit anti-Pnt (Pascual *et al.*, 2017), rabbit anti-β-gal (1:10,000, MP Biomedicals-Cappel), mouse anti-β-gal hybridoma supernatant 40A-1A-s (1:200, Developmental Studies Hybridoma Bank), rabbit anti-c-Myc supernatant sc-789 (1:200, Santa Cruz Biotechnology). All secondary antibodies were highly cross-adsorbed Alexa Fluor-conjugated anti-IgG (1:2,000, Invitrogen). All secondary antibodies, as well as anti-Mid, were diluted 1:10 in PBS and pre-adsorbed by incubation with fixed wild-type ovaries or embryos prior to use.

2.5.4 Microscopy and image analysis

Images were acquired using a Zeiss Axioplan 2 wide-field fluorescence microscope. Apart from minor uniform linear adjustments to brightness and contrast, no image manipulations were performed.

2.5.5 Reporter cloning and generation of fly constructs

For generation of lacZ constructs sequences were amplified from the endogenous gene using primers containing restriction enzyme sites so that they could be cloned into a linearized placZ.attB vector (Bischof et al., 2013). For derivative constructs regions were amplified from a plasmid containing the original complete insert. Conservation of DNA regions was determined from the UCSC genome browser using 124 insects basewise conservation by PhyloP and

breakpoints were designed in regions of low conservation where possible. Individual colonies were picked and tested for the presence of the insert by miniprep, restriction digest, and sequencing. Constructs were amplified by midiprep (Qiagen) and integrated into the Drosophila genome using the PhiC31 integrase and the attP landing site at cytological position 58A. Fly transformants were used to generate a stable stock and dissected to determine expression pattern in the ovary. DNA from transformant fly lines was extracted and plasmid DNA was amplified and genotyped by either restriction digestion or sequencing.

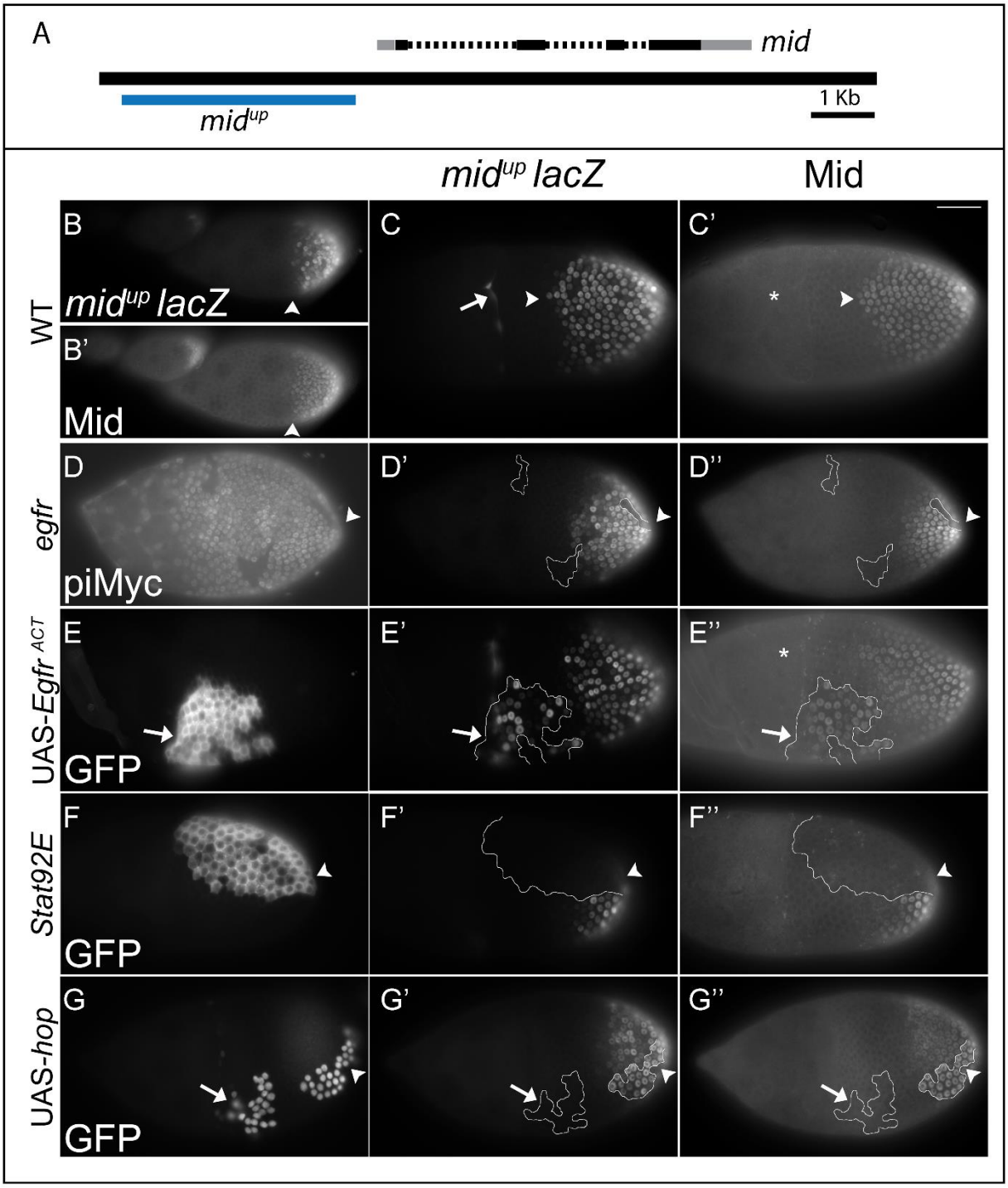
A
mid
1 Kb
mid^up
mid^up lacZ
Mid
WT
egfr
UAS-Egfr ACT
Stat92E
UAS-hop
B
mid^up lacZ
B'
Mid
C
C'
D
piMyc
D'
D''
E
GFP
E'
E''
F
GFP
F'
F''
G
GFP
G'
G''

Figure 2.1 – The *mid*^{up} CRR recapitulates Mid expression and capture inputs from EGFR and JAK/STAT signalling

(A) Map of the *mid* locus showing the *mid*^{up} in blue. Exons of *mid* are shown in plain line, introns in dotted lines, and untranslated region in gray. (B-B') Expression of the *mid*^{up} *lacZ* reporter and endogenous Mid in a Stage 8 wild type (WT) egg chamber. Expression of endogenous Mid and of the *mid*^{up} *lacZ* reporter overlap in the posterior (arrowheads) but the reporter expression is uneven. (C-C') WT stage 10B egg chamber also exhibits overlap of the *mid*^{up} *lacZ* reporter and endogenous Mid, both have a dorsal bias expression at this stage (arrowheads). At this stage, the *mid*^{up} *lacZ* reporter is also expressed in some centripetal cells (arrow) which is not observe with endogenous Mid expression. (D-D") Loss-of-function *egfr*^{CO} clones exhibit loss of the *mid*^{up} *lacZ* reporter and endogenous Mid (arrowheads), negatively marked with piMyc. (E-E") Clones expressing UAS-*Egfr*^{ACT} exhibit ectopic expression of the *mid*^{up} *lacZ* reporter and endogenous Mid (arrows). Positively marked with GFP (F-F") Loss-of-function *Stat92E*^{85C9} clones exhibit loss of the *mid*^{up} *lacZ* reporter and endogenous Mid (arrowhead). Positively marked with GFP. (G-G") Clones expressing UAS-*hop* exhibits ectopic expression of the *mid*^{up} *lacZ* reporter and endogenous Mid in the posterior domain (arrowheads) but not in the ventral-anterior (arrows). Dorsal side is indicated by an asterisk. Scale bar = 50µm for all panels.

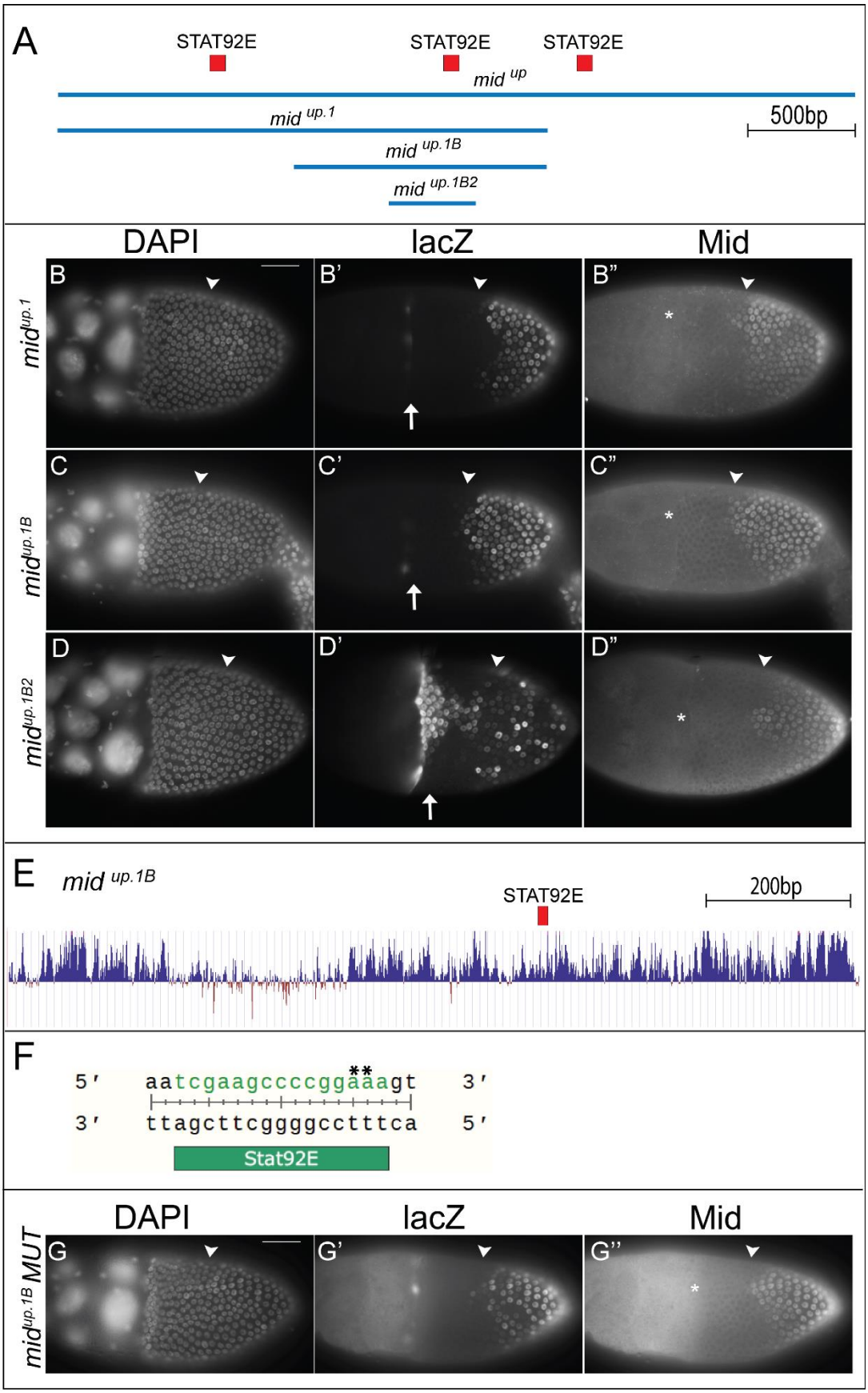
A
STAT92E
STAT92E
STAT92E
mid up
mid up.1
mid up.1B
mid up.1B2
500bp
DAPI
lacZ
Mid
B
B'
B"
C
C'
C"
D
D'
D"
mid up.1
mid up.1B
mid up.1B2
E
mid up.1B
STAT92E
200bp
F
5' aatcgaagccccggaaagt 3'
3' ttagcttcggggcctttca 5'
Stat92E
DAPI
lacZ
Mid
G
G'
G''
mid up.1B MUT

Figure 2.2 – Disruption of putative STAT92E binding site does not affect expression of the *mid^{up}* CRR reporter

(A) Map of the *mid^{up}* CRR sequence and its derivatives constructs. Red boxes indicate putative STAT92E binding sites. (B-B") WT expression pattern of the midup.1 reporter. Expression is similar to the original midup reporter in the posterior and still overlap with Mid expression (arrowheads). Expression of the reporter is still detected in centripetal cells (arrow). (C-C") WT expression pattern of the *mid$^{up.1B}$* reporter. Expression is similar to the original *mid^{up}* and the *mid$^{up.1}$* reporters in the posterior and still overlap with Mid expression (arrowheads). Expression of the reporter is still detected in centripetal cells (arrow). (D-D") WT expression pattern of the *mid$^{up.1B2}$* reporter. Expression in the posterior is still present but is more uneven than the original derivatives (arrowhead) and this derivative reporter is expressed in the dorsal-anterior domain (arrow). Expression in the centripetal cells is still present. (E) Conservation of the sequence of the *mid$^{up.1B}$* CRR and a putative STAT92E binding site. (F) Mutagenesis of the putative STAT92E binding site in the *mid$^{up.1B}$* CRR. Asterisks indicate mutated nucleotides. (G-G") Expression of the mutated *mid$^{up.1B}$* reporter is not different compared to the original *mid$^{up.1B}$*. The expression of the reporter still overlaps with endogenous Mid (arrowhead). Dorsal side is indicated by an asterisk. Scale bar = 50µm for all panels. Adapted from De Vito 2021

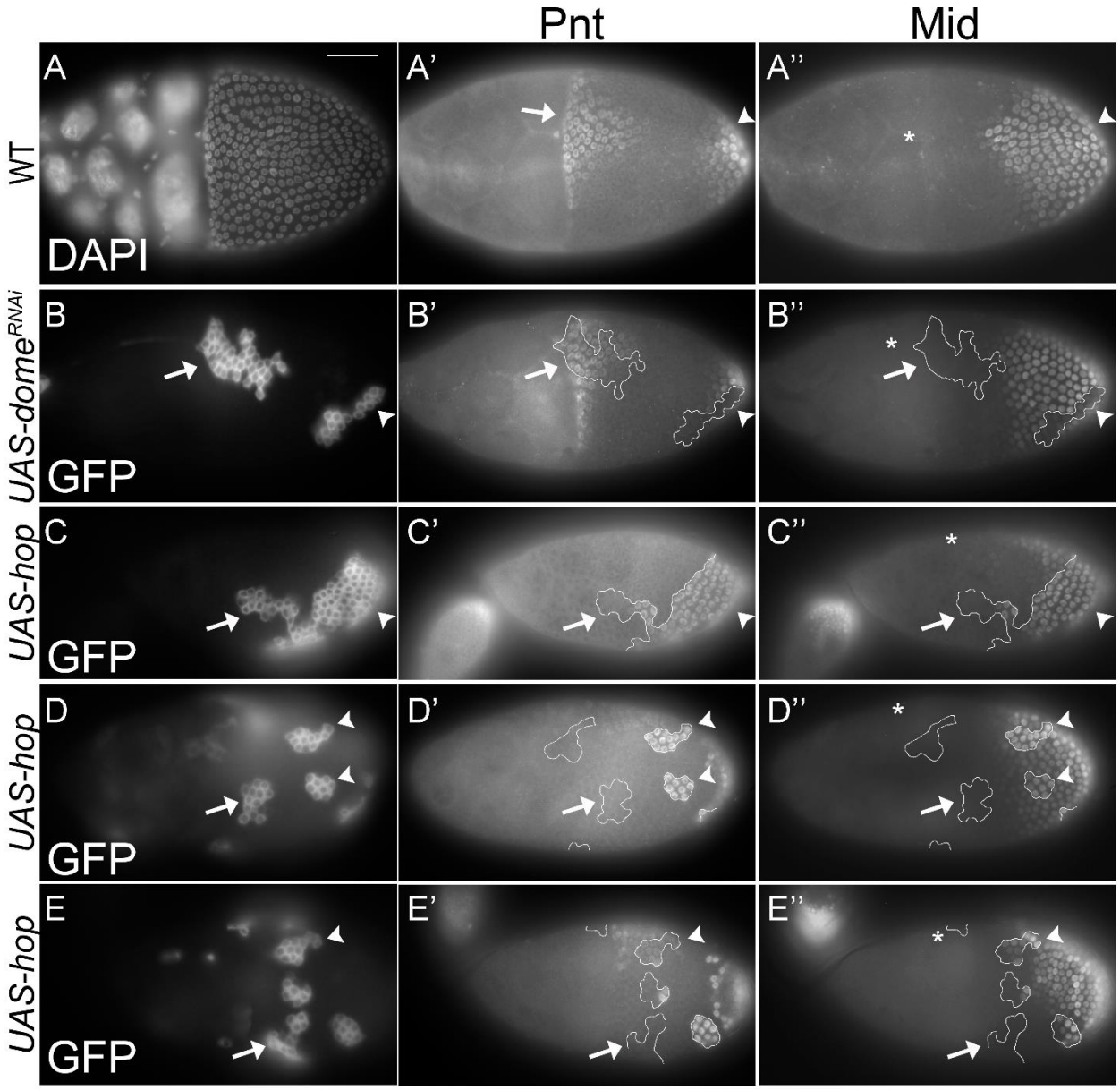
Pnt
Mid
WT
A
A'
A''
DAPI
UAS-dome^RNAi
B
B'
B''
GFP
UAS-hop
C
C'
C''
GFP
UAS-hop
D
D'
D''
GFP
UAS-hop
E
E'
E''
GFP

Figure 2.3 – Pnt is regulated by JAK/STAT signalling

(A-A") WT expression pattern of Pnt and Mid. Pnt is expressed in the posterior, where Mid is expressed (arrowhead). Pnt is also expressed in the dorsal-anterior (arrow). (B-B") Clones expressing a RNAi construct targeting *dome* exhibit loss of Pnt and Mid expression in the posterior (arrowhead). Expression of Pnt in the dorsal-anterior is not affected (arrow). Positively marked by GFP. (C-E") Clones expressing UAS-*hop* exhibit ectopic expression of Mid and Pnt in the posterior (arrowheads). Pnt and Mid are not induced in the anterior (arrow in C'-C"). Pnt and Mid are not induced in clones in the ventral-anterior (arrow in D'-E"). Mid is induced in clones in the dorsal-anterior but expression of Pnt is not affected (arrowhead in E'-E"). Dorsal side is indicated by an asterisk. Scale bar = 50μm for all panels.

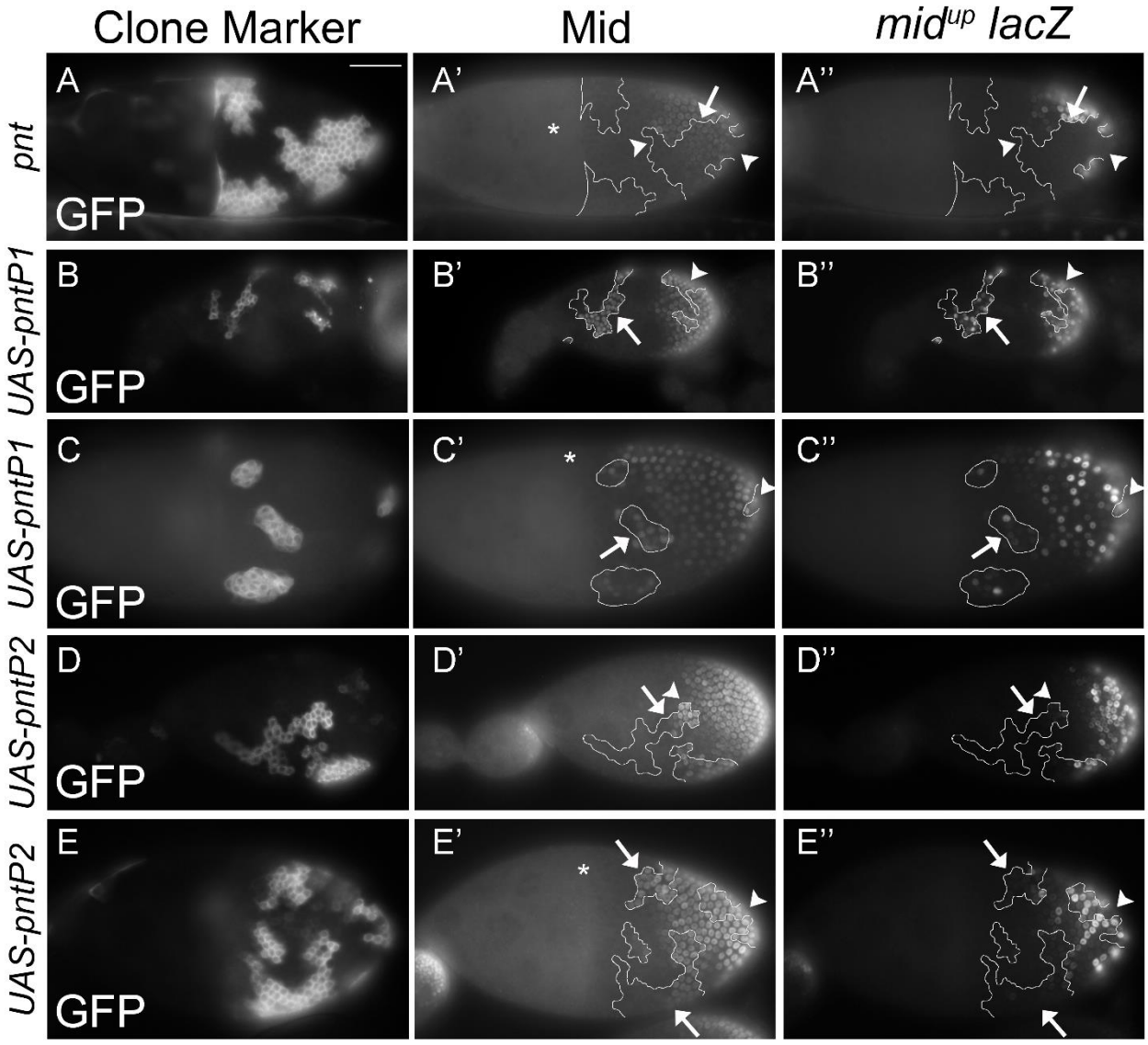
Clone Marker
Mid
mid^up lacZ
pnt
UAS-pntP1
UAS-pntP1
UAS-pntP2
UAS-pntP2
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''
GFP
GFP
GFP
GFP
GFP

Figure 2.4 – Mid and the *mid*^{up} CRR are regulated by Pnt

(A-A") Loss of function *pnt*$^{\Delta 88}$ clones exhibit loss of Mid and the *mid*^{up} reporter. Loss in the cells in the anterior and the posteriormost regions of Mid show a strong loss (arrow). Cells in between show a weak loss (arrowhead). This regional difference is not detected with the *mid*^{up} reporter (B-C"). Clones expressing UAS-*pntP1* exhibit induction of Mid and of the *mid*^{up} reporter expression both in the posterior (arrowhead) and in the anterior (arrow). Note the change in the cell shape in late egg chambers (C-C"). (D-E") Clones expression UAS-*pntP2* exhibit induction of Mid and of the *mid*^{up} reporter expression both in the posterior (arrowhead). Mid and the *mid*^{up} reporter are not expressed in the anterior of early egg chamber (arrow in D'-D"). Induction also occurred in the dorsal-anterior of late egg chambers (top arrow in E'-E") but not the ventral-anterior (bottom arrow in E'-E"). All clones are positively marked with GFP. Dorsal side is indicated by an asterisk. Scale bar = 50μm for all panels.

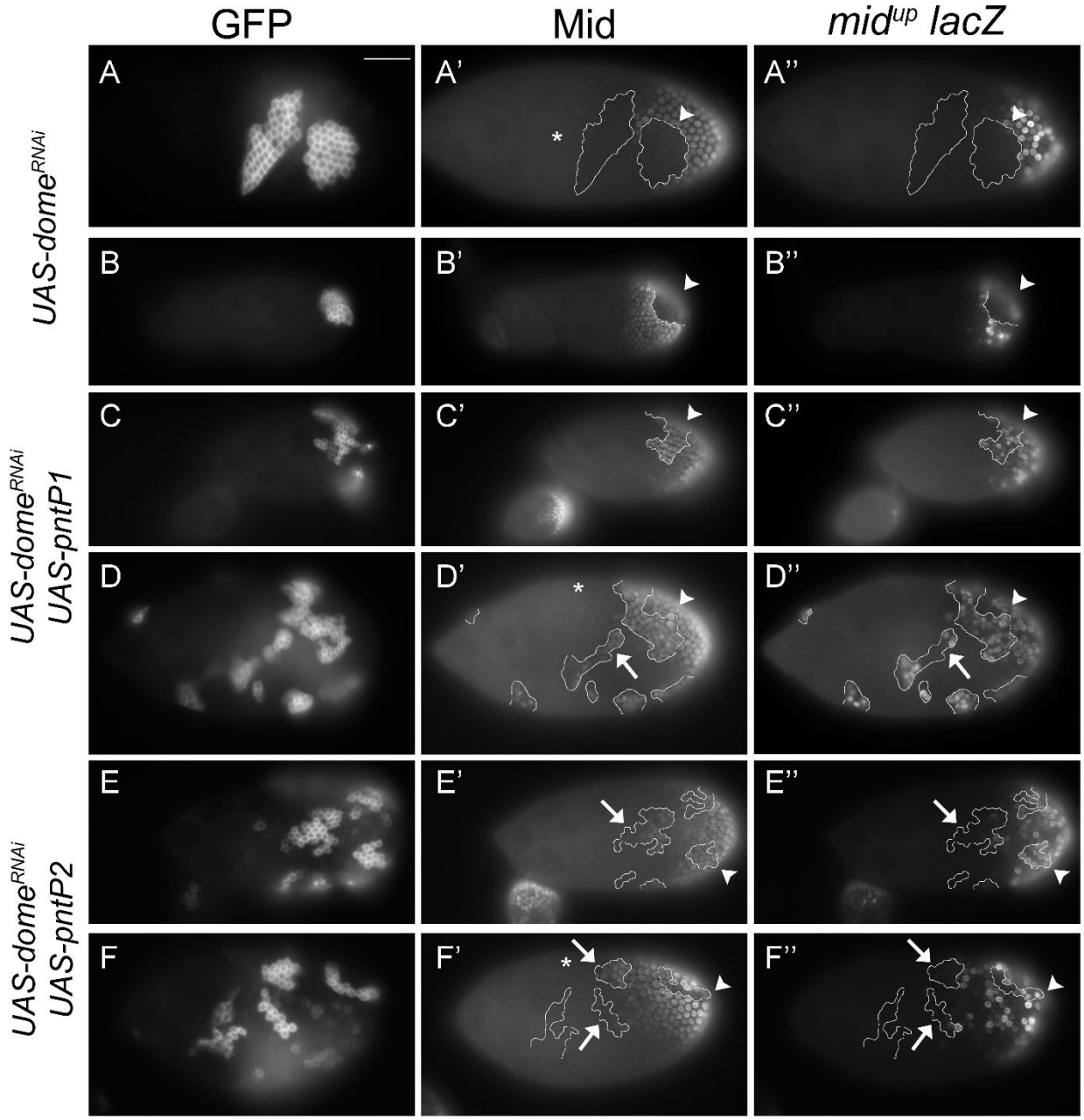
GFP
Mid
mid^up lacZ
UAS-dome^RNAi
UAS-dome^RNAi UAS-pntP1
UAS-dome^RNAi UAS-pntP2
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''
F
F'
F''

Figure 2.5 – Expression of Pnt rescues loss of Mid in clones unable to respond to JAK/STAT signalling

(A-B") Clones expressing a RNAi construct targeting *dome* exhibit loss of Mid and of the *mid*^{up} reporter (arrowhead). (C-D") Clones expressing UAS-*pntP1* and a RNAi construct targeting *dome* show a rescue of Mid and of the *mid*^{up} reporter expression (arrowhead). Ectopic expression in the anterior is also observed (arrow). (E-E") Clones expressing UAS-*pntP2* and a RNAi construct targeting *dome* exhibit a rescue of Mid and of the *mid*^{up} reporter expression (arrowhead). Clones in the anterior do not exhibit ectopic expression of Mid and of the *mid*^{up} reporter expression (arrow). (F-F") In some clones expressing UAS-*pntP2* and a RNAi construct targeting *dome*, expression of the *mid*^{up} reporter is rescued but not of Mid (arrowhead). Clones in the dorsal-anterior exhibit ectopic expression (top arrow) but not clones in the ventral-anterior (bottom arrow). All clones are positively marked with GFP. Dorsal side is indicated by an asterisk. Scale bar = 50µm for all panels.

Chapter 3: The ETS transcription factor Pointed is regulated by two different EGFR mediated mechanisms in the follicular epithelium

3.1 Introduction

In the previous chapter, we have shown that the JAK/STAT signalling pathway regulates Mid expression by controlling the levels of the EGFR effector Pnt. However, it is unknown whether *pnt* is a direct target of JAK/STAT signalling in the posterior domain of the follicular epithelium. To further understand the mechanisms by which the JAK/STAT signalling influences the outcome of EGFR signalling, it is important to determine whether *pnt* is a directly regulated by the JAK/STAT signalling pathway through the binding of STAT92E to a CRR controlling *pnt* expression. The first part of this chapter aims to address this hypothesis.

In addition to require inputs from JAK/STAT signalling, *pnt* expression in posterior follicle cells also requires EGFR signalling (Morimoto *et al.*, 1996). The mechanism by which EGFR regulates *pnt* expression in the posterior is unknown. Understanding this mechanism will provide insight on how the JAK/STAT signalling pathway interacts with the EGFR signalling pathway.

In this tissue, the role of Pnt has been well studied in the dorsal-anterior (Boisclair Lachance *et al.*, 2009; Morimoto *et al.*, 1996; Zartman *et al.*, 2009) and the previous chapter described a function in establishment of posterior fate. However, if we know that expression of *pnt* in this tissue requires EGFR signalling, the mechanism by which *pnt* expression is controlled is unknown. Early studies on *pnt* hypothesized that its expression is under the control of transcription factors whose activity depends on MAPK (Gabay *et al.*, 1996) and Forés et al. hypothesized that this factor could be Capicua (Cic) (Forés et al., 2017). Cic is a transcriptional repressor working downstream of the EGFR signalling pathway (Ajuria et al., 2011; Jiménez *et al.*, 2000; Jiménez *et al.*, 2012).

Repression of Cic by EGFR signalling is necessary for the establishment of dorsal-anterior fate in the follicular epithelium through repression of *mirr*. The role of Cic has mostly been studied in the context of *mirr* regulation (Andreu *et al.*, 2012a; Astigarraga *et al.*, 2007; Atkey *et al.*, 2006; Goff *et al.*, 2001). A Pnt-GFP fusion construct that reproduces endogenous expression of Pnt (Boisclair Lachance et al., 2014) has been shown to be regulated by Cic in the dorsal-anterior (De Vito, 2021). However, whether Cic regulates *pnt* in the posterior and if repression of Cic is necessary to establish posterior fate is unknown.

In this chapter, we characterized two overlapping CRR reporters recapitulating expression of *pnt* in posterior follicle cells. These CRR reporters are responsive to JAK/STAT signalling. I found that the overlap sequence shared between the CRR contains a strong putative STAT92E binding site and hypothesized that this site was necessary for *pnt* expression in the posterior. However, a derivative reporter construct containing only this overlap region is not expressed in the follicle cells. This suggests that inputs from JAK/STAT signalling could be captured through other binding sites present in the non-overlapping sequences of the CRRs. Next, I found that *pnt* and *mid* in the posterior are not regulated by Cic but found that Cic plays a role of setting up the anterior boundary of the domain of Mid expression. This suggests that *pnt* is regulated through another unknown mechanism by EGFR.

3.2 Results

3.2.1 Two cis-regulatory region (CRRs) reporters recapitulate *pnt* expression in posterior follicle cells and are regulated by JAK/STAT signalling

3.2.1.1 Rationale

In the previous chapter, we have shown that JAK/STAT signalling indirectly regulates *mid* expression by controlling the levels of Pnt in posterior follicle cells. However, we do

not know whether *pnt* is directly regulated through binding of STAT92E to CRRs controlling its expression in posterior follicle cells or if this regulation occurs indirectly through an unidentified factor. Positive regulation of *pnt* expression through direct binding of STAT92E would be particularly interesting, given the previous observation that STAT92E binding mediates repression of a *mirr* CRR reporter in posterior follicle cells. Such a result would imply that STAT92E binding can lead to activation or repression of different targets in the same cell.

To address this question, we looked for CRRs that control expression of *pnt* in posterior follicle cells and onto which STAT92E would bind. With the same approach used for *mid* and *mirr* (De Vito, 2021), previous lab members have screened a collection of CRR GAL4 reporters from the *pnt* locus (Jenett et al., 2012; Pfeiffer et al., 2008) and looked for those whose expression in follicle cells resembles the endogenous *pnt* gene. We then tested whether these reporters are responsive to JAK/STAT signalling and then further characterized their sequences to map putative STAT92E binding sites.

3.2.1.2 Two overlapping CRRs reporters recapitulate *pnt* expression in posterior follicle cells

Two of the lines screened drove expression of a GAL4 reporter in posterior follicle cells. The reporters contain sequences from an intron of the *pnt* gene (Figure 3.1A). The two sequences, named pnt^{45D11} and pnt^{43H01}, are 3631bp and 2227bp long respectively and share an overlap sequence of 602bp. In the presence of a UAS-GFP transgene, each of these reporters drives GFP expression in posterior follicle cells, but the reporter expression expands more toward the anterior than endogenous *pnt* for both lines (Figure 3.1B-C, arrowhead). The fact that these reporters are expressed in the posterior domain suggests that they contain *pnt* regulatory elements. Unlike endogenous Pnt, the CRRs reporters are not expressed in the dorsal-anterior domain (Figure 3.1B'-

C', arrow). In addition to being expressed in posterior follicle cells, *pnt*^{45D11} is also expressed the border cells (Figure 3.1B, arrow) and *pnt*^{43H01} is expressed in anterior follicle cells (Figure 3.1C, arrow). Border cells expression of Pnt has been observed with a Pnt-GFP fusion, but this expression is not maintained past stage 10A of oogenesis (Boisclair Lachance *et al.*, 2014) and endogenous Pnt is not detected in border cells (data not shown). The *pnt*^{45D11} reporter stays expressed in the border cells past stage 10A. These results were also observed in another study that looked at CRR reporters in the follicular epithelium (Revaitis et al., 2017) and suggests that, individually, these elements lack response elements preventing expression of *pnt* in the anterior. Because our lab is interested in the regulation of *pnt* by JAK/STAT signalling, previous lab members had tested the response of these two reporters to loss of JAK/STAT signalling and observed that expression of each of these reporters was lost in these clones (De Vito, 2021).

To further characterize these putative CRRs, we cloned the sequences into a transgenic lacZ reporter construct, allowing us to further characterize the response of the reporter to signalling pathways using the MARCM system, which generates clones cells expressing GAL4 as a driver of a positive clone marker as well as various UAS transgenes of interest (Lee and Luo, 2001). Similar to the GAL4 versions described above, the *pnt*^{45D11} and *pnt*^{43H01} *lacZ* reporters drive expression in posterior follicle cells (Figure 3.1D-E, arrowhead) but the expression is more contained in the posterior and less uniform. In addition, expression of *pnt*^{45D11} in the border cells and expression of *pnt*^{43H01} is absent. These differences between the *lacZ* and GAL4 reporters may be explained by the lower detection threshold of the *lacZ* reporter compared to the GAL4. Nonetheless, *pnt*^{45D11} and *pnt*^{43H01} both drive expression of reporter genes in posterior follicle cells and therefore represent good candidates for CRRs capturing inputs from JAK/STAT signalling.

3.2.1.3 The overlap region in the *pnt* CRR reporters is not necessary for expression

Given the fact that the two reporters are active in the posterior domain and share an overlap sequence, I hypothesized that this overlapping region contain the response elements necessary for posterior expression. This hypothesis predicts that this "overlap sequence" contains binding sites for STAT92E. The consensus binding sequence of STAT92E is TTCNNNGAA (Hou et al., 1996; Yan *et al.*, 1996). Using a STAT92E binding site matrix (Fornes et al., 2019), I screened the sequences of pnt^{45D11} and pnt^{43H01} for putative STAT92E binding sites. The lab had previously successfully identified a STAT92E binding site in a CRR regulating *mirr* expression (De Vito, 2021), therefore validating this approach. This search identified a cluster of STAT92E binding sites in the overlap region (Figure 3.1F-G, red squares), having a close sequence similarity with the site found in the *mirr* CRR (Figure 3.1H) (De Vito, 2021).

To test my hypothesis that the overlap region is sufficient to produce posterior expression in the follicle cells, I generated a lacZ reporter construct ($pnt^{overlap}$) containing the overlap sequence; I also included 605bp contained in pnt^{45D11} to keep conserved sequence intact (Figure 3.1F). As a control, I also generated two constructs containing the sequences not included in the overlap construct ($pnt^{45D11.1}$ and $pnt^{43H01.1}$). Unexpectedly, the $pnt^{overlap}$ reporter is not expressed in the follicle cells (Figure 3.1I, arrowhead), while $pnt^{45D11.1}$ and $pnt^{43H01.1}$ each have similar expression patterns as their parent constructs (Figure 3.1J-K'). This is indicating that the $pnt^{overlap}$ sequence does not contain element responsible for posterior expression of *pnt* and that these elements are present in the sequences of $pnt^{45D11.1}$ and $pnt^{43H01.1}$.

3.2.1.4 The *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$ derivative constructs are responsive to JAK/STAT signalling

Next, I wanted to test the hypothesis that the *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$ reporter constructs are responsive to JAK/STAT signalling. To do so, I have generated clones lacking *Stat92E* function or experiencing ectopic JAK/STAT signalling activity by overexpressing Hop (Harrison *et al.*, 1995). In clones lacking a functional *Stat92E* gene, we observe loss of expression of both *pnt*$^{45D11.1}$ (22/22) and *pnt*$^{43H01.1}$ (23/23) reporters (Figure 3.2A-B'). Conversely, in clones overexpressing *hop*, we observe ectopic expression of both *pnt*$^{45D11.1}$ (20/28) and *pnt*$^{43H01.1}$ (34/38) (Figure 3.2C-D', arrowheads). This shows that *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$ are responsive to JAK/STAT, that they contain response elements for JAK/STAT.

Together, this is indicating that the overlap sequence shared between *pnt*^{45D11} and *pnt*^{43H01} does not contain elements sufficient for expression of *pnt* in posterior follicle cells and that these elements are scattered around the sequences present in *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$. I have identified putative STAT92E binding sites present in *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$. Future work should focus on continuing to characterize these reporters and map putative STAT92E binding sites.

3.2.2 Role of Cic in the regulation of *mid* and *pnt* in posterior follicle cells

3.2.2.1 Rationale

Another important regulator of *pnt* expression in the posterior is EGFR signalling. Indeed, egg chambers mutant for *grk* lose expression of Pnt (Morimoto *et al.*, 1996) showing that EGFR signalling is required for Pnt expression. However, the molecular mechanisms by which EGFR regulates *pnt* expression in the posterior are unknown. Because *pnt* expression is absent in the absence of Grk, I hypothesized that *pnt* would be under transcriptional control in the posterior follicle cells.

A good candidate for a regulator of *pnt* expression is Cic which is a transcriptional repressor acting downstream of EGFR signalling (Jiménez *et al.*, 2000). EGFR regulates Cic by controlling its nuclear localization. In the absence of EGFR signalling, Cic is nuclear and can repress the expression of its target genes. Activation of EGFR causes Cic to be excluded from the nucleus leading to derepression of EGFR target genes (Grimm *et al.*, 2012; Jiménez *et al.*, 2012). The role of Cic as a downstream effector of EGFR signalling in the establishment of dorsal-anterior fate has been well studied and characterized (Andreu *et al.*, 2012a; Andreu *et al.*, 2012b; Astigarraga *et al.*, 2007; Atkey *et al.*, 2006; Goff *et al.*, 2001). In addition, Cic has also been shown to regulate *pnt* expression in the gut (Jin *et al.*, 2015) and in the developing wing disk (Pascual *et al.*, 2017). Preliminary data from our lab also showed that a Pnt-GFP fusion protein is induced in *cic* clones suggesting that *pnt* is also regulated by Cic in the ovary (De Vito, 2021). However, whether Cic regulates *pnt* in posterior follicle cells and if it is required for establishment of posterior fate is unknown.

Based on the data from my previous chapter showing the requirement of JAK/STAT signalling in the expression of Pnt in the posterior domain and the known requirement for EGFR signalling for Pnt expression, I hypothesize that the joint action of JAK/STAT signalling and repression of Cic by EGFR signalling activates expression of Pnt in posterior follicle cells, which in turn leads to the expression of Mid and establishment of posterior fate.

3.2.2.2 Cic is repressed in posterior follicle cells in early stages egg chambers

To test this hypothesis, I first asked whether Cic localization is regulated by EGFR signalling in the posterior. Because Cic is excluded from the nucleus in response to EGFR signalling (Grimm *et al.*, 2012; Jiménez *et al.*, 2012), we can look at the nuclear localization of Cic to monitor its activity. To do so, I dissected and stained ovaries expressing a CicS transgene fused

with a HA-tag (Astigarraga *et al.*, 2007) which allow to co-stain the tissue with Cic and Pnt because both antibodies targeting endogenous Pnt and Cic are raised in rabbits.

As a control, I first looked at stage 10A or 10B egg chambers when Grk is present in the dorsal-anterior. Consistent with previous observations (Astigarraga *et al.*, 2007), I observe that nuclear localization of Cic is lost in the dorsal-anterior and present in other domains (Figure 3.3A,B). We also observe that Pnt is expressed in the cells lacking nuclear Cic in the dorsal-anterior (Figure 3.3A',B', arrow), suggesting that expression of Pnt in this domain requires repression of Cic. This confirms that Cic is excluded from the nucleus in the domain where EGFR activity is present.

Next, I looked at early-stages egg chambers in which Grk is still present in the posterior of the oocyte. Similar to what is observed with later egg chambers, nuclear localization of Cic is lost in the posterior and present in the anterior (Figure 3.3C,D, arrowhead) indicating that Cic is repressed in posterior follicle cells at these stages. In addition, Pnt is expressed in the posterior at this stage but not the anterior (Figure 3.3C',D'). This time however, the expression domain of Pnt does not perfectly overlap with the domain where Cic is repressed (Figure 3.3C',D') but we see a much closer overlap with the expression domain of Mid (Figure 3.3C",D"). This difference could be explained by the fact that Pnt expression requires JAK/STAT signalling in the posterior (See chapter 2). Because inputs from both Grk and Upd are required for posterior *pnt* expression, the expression domain may closely follow the range of Upd diffusion, since at this stage, it is more restricted to the posterior than Grk, which we infer from the domain lacking nuclear Cic staining and expressing Mid. This data shows that Cic is repressed in posterior follicle cells in early-stages egg chambers and suggest that repression of Cic by EGFR could lead to expression of Pnt and Mid in the posterior.

3.2.2.3 Cic represses *pnt* but not *mid*

To test this hypothesis, I generated clones lacking *cic*, either by being homozygous for a *cic* null allele (cic^{2}) (Klein and Campos-Ortega, 1992; Roch et al., 2002) or by expressing a RNAi construct targeting *cic* transcripts (UAS-cic^{RNAi}). In these clones, expression of Pnt is induced in every clone regardless of the position or stage of the egg chamber (161/163 for cic^{2}, 96/100 for cic^{RNAi}, Figure 3.4A'-K"), indicating that *pnt* is regulated by Cic in the follicular epithelium and consistent with previous observation with a Pnt-GFP fusion protein (De Vito, 2021). The levels of expression of Pnt in the *cic* clones are lower than the endogenous levels seen in the posterior and are similar to the endogenous levels observed in the dorsal-anterior. In fact, we observe that *cic* clones in the dorsal-anterior have no effect on the levels of Pnt expression. This would suggest that *pnt* expression in the dorsal-anterior is only regulated by Cic and the differences of levels between the posterior and the *cic* clones are due to the JAK/STAT mediated regulation of Pnt in addition to the regulation by Cic.

The phenotype observed with Mid is more complex, here, we do not observe ectopic expression in the anterior (1/75 for cic^{2}, 0/49 for cic^{RNAi} , Figure 3.4B-L', arrow). However, there is a weak induction of Mid expression at the anterior limit of the endogenous domain of Mid expression (Figure 3.4B-H'). In addition, induction of Mid expression is more common in the ventral side (17/23 for cic^{2}, 16/19 for cic^{RNAi}, Figure 3.4B-D', arrowhead) compared to the dorsal side (23/33 for cic^{2}, 0/11 for cic^{RNAi}, Figure 3.4F-H'), arrowhead) for posterior clones. The difference is more visible in clones expressing cic^{RNAi} in which there is no induction in the dorsal side.

Since I showed previously (Figure 2.4) that clonal expression of ectopic Pnt is sufficient to induce ectopic Mid expression, it is surprising to observe that despite expressing Pnt, clones lacking

cic do not exhibit Mid expression. This absence of Mid expression could be caused by Mirr. Indeed, derepression of Mirr by Cic has been shown to be sufficient to induce Mirr expression, even in the absence of EGFR signalling (Atkey *et al.*, 2006). We have recently shown that a *mirr* CRR is directly repressed by Cic (De Vito, 2021), suggesting that *mirr* is a direct target of Cic. Therefore, induction of Mirr in these clones would repress Mid expression (Fregoso Lomas *et al.*, 2016).

Although this hypothesis can explain why Mid is not induced in clones located in the center of the egg chamber, it cannot account for why Mid is also not induced in anterior clones of early egg chambers (0/23 for cic^{2}, 0/17 for cic^{RNAi}, Figure 3.4J-L'). Cells in this region at these stages are exposed to Upd (McGregor *et al.*, 2002; Xi *et al.*, 2003) and therefore would not express Mirr. In this region, Pnt is expressed at higher levels compared to the cells more in the posterior (Figure 3.4I-K"), highlighting the effect of JAK/STAT signalling on *pnt* regulation.

Therefore, this is indicating that Mirr cannot solely explain why Mid is not induced in the clones lacking *cic* despite the induction of Pnt expression. In the previous chapter, we have shown that ectopic expression of PntP1 is sufficient to induce expression of Mid but expression of PntP2 is not sufficient to induce Mid expression in the absence of Grk. Therefore, it is possible that the Pnt isoform induced in clones lacking *cic* is PntP2, which could explain the absence of Mid induction in the clones, despite the presence of Pnt.

3.2.2.4 Is the Pnt isoform regulated by Cic PntP2 ?

In support of the hypothesis that Cic specifically regulates the PntP2 isoform, we can observe the similarities between the phenotype of Mid in clones lacking *cic* and clones expressing ectopic PntP2. Indeed, similar to clones lacking *cic*, cells expressing ectopic PntP2 exhibit induction of Mid expression only in the posterior. In early stages egg chambers, clones expressing PntP2 exhibit ectopic expression of Mid only in the posterior and in the most posterior cells

covering the nurse cells (Figure 3.5A, see chapter 2). In later stage egg chambers, Mid is only induced slightly to the anterior of the endogenous Mid expression domain, with a stronger effect in the dorsal side (Figure 3.5B). These similarities are consistent with my hypothesis that the isoform induced in clones lacking *cic* was PntP2 .

Unfortunately, due to the absence of reagent specific to PntP2, I could not directly look at whether PntP2 was induced in clones lacking *cic*. Instead, to test this hypothesis, I generated clones expressing a RNAi construct targeting *cic* while being homozygous for a *pnt* allele specific to the *pntP2* isoform ($pnt^{\Delta 78}$) (O'Neill *et al.*, 1994) and look at whether Pnt is expressed in these clones. If I observe Pnt expression, it will suggest that the isoform being induced is PntP1, as PntP2 would not be expressed. First, as a control, I generated clones homozygous for the $pnt^{\Delta 78}$ allele and look at Mid and Pnt expression. In these clones, I observe that Mid expression is lost but only in the anterior domain of Mid expression (30/35, Figure 3.5.C', arrowhead), expression in the posterior remains mainly unaffected (8/44, Figure 3.5.C', arrow), suggesting that only the anterior portion of the Mid expression domain requires PntP2.

Unexpectedly, we observe that Pnt expression is not lost in the $pnt^{\Delta 78}$ clones (Figure 3.5.C") even in the dorsal-anterior. This is surprising because Shwartz et al., have shown that $pnt^{\Delta 78}$ specifically affect the *pntP2* transcript by using *in situ* hybridization on mutant embryos and have observed lost of expression (Shwartz *et al.*, 2013). In the ovary, it could be possible that a truncated protein is still being formed because the antibody against Pnt was raised to target the C-terminal two Pnt isoforms, which is not affected in the deletion causing the $pnt^{\Delta 78}$ allele, expression could still be detected. Alternatively, it is possible that Pnt staining is still detected because of the presence of PntP1 which is also expressed in the dorsal-anterior (Morimoto *et al.*, 1996; Yakoby *et al.*, 2008). A potential reason of why I have expected to see complete loss of Pnt staining in

pnt$^{\Delta 78}$ is because it is possible that PntP1 is regulated by PntP2 activity in the dorsal-anterior. A similar situation has been described in the eye disc where loss of PntP2 also leads to loss of PntP1 (Shwartz *et al.*, 2013). In support of this hypothesis, it was reported that loss of PntP2 is equivalent to loss of both Pnt isoform in establishment of dorsal-anterior fate (Boisclair Lachance *et al.*, 2009) which suggest that PntP1 is not sufficient to induce dorsal-anterior fate or that loss of PntP2 also leads to loss of PntP1 in the follicular epithelium as well as in the developing eye. Nevertheless, it important to note that there are different Mid phenotypes between the clones lacking *cic* and the clones lacking *pntP2*.

Although this analysis of the *pnt*$^{\Delta 78}$ allele calls into question whether it does in fact eliminate expression of PntP2, I nevertheless analyzed the phenotype of *pnt*$^{\Delta 78}$ clones expressing an RNAi against *cic*, I found that in these *pnt* expression is induced (91/93, Figure 3.5D"-E"), which is consistent with the results from clones lacking *cic* and the fact that we still detect Pnt staining in *pnt*$^{\Delta 78}$ clones. Clones in the posteriormost do not exhibits lost of Mid expression (2/12, Figure 3.5D'-E', arrowhead) which is what is observed in both single clones. Interestingly, there is a difference between clones in the ventral side and the dorsal side. In some clones in the dorsal side, we observe loss of Mid expression (18/33, Figure 3.5D'-E', arrowhead) which is seen in clones lacking *pntP2*. In the ventral side, however, few clones exhibit ectopic expression (9/38, Figure 3.5D'-E', arrowhead), which is seen in clones lacking *cic*. In both cases, the phenotype is not very penetrant. Therefore, in the ventral side, the Mid phenotype is similar to a *cic* clone and in the dorsal side to a *pntP2* clone. It is therefore not possible to conclude with certainty that the isoform induced in clones lacking *cic* is PntP2.

To further test which Pnt isoform is controlled by Cic, I took advantage of PntP1 specific antibody (Alvarez *et al.*, 2003). This antibody detects PntP1 staining in the posterior but not the

dorsal anterior (Figure 3.6A) where PntP1 is reported to be expressed (Morimoto *et al.*, 1996; Yakoby *et al.*, 2008). I tested the specificity of this antibody by generating clones ectopically expressing PntP1 and PntP2. As expected, there is PntP1 staining in clones expressing PntP1 (Figure 3.6B) and no staining in clones expressing PntP2 (Figure 3.6C), therefore, confirming the specificity of the antibody. I stained egg chamber with clones lacking *cic* for PntP1 staining and did not detect ectopic expression in the clones (Figure 3.6D). This would suggest that PntP1 is not induced in these clones but the lack of the staining in the dorsal-anterior could also suggest that, due to lower expression levels, PntP1 cannot be detected in the dorsal-anterior and potentially in the *cic* clones.

Taken together, this data suggest that PntP2 is the isoform induced in *cic* clones, but a more direct mean of detection is needed to confirm this observation, with reagents able to directly detect PntP2, such an isoform specific fusion-protein or *in situ* hybridisation.

3.2.2.5 Pnt and Mid are not repressed by Cic in posterior follicle cells

The previous part has shown that Cic regulates *pnt* expression in the follicular epithelium and suggest that PntP2 is the isoform induced in clones lacking *cic*. However, although all *cic* deficient clones outside of the normal Pnt domain of expression exhibit ectopic Pnt, we still do not know whether Cic regulates *pnt* in the endogenous posterior domain of expression because Pnt is already expressed in this domain, and we do not observe an increase in the levels of expression. To answer this question, I generated clones expressing a derivative CicS transgene lacking the C2 domain (CicS$^{\Delta C2}$-HA) which contain the MAPK docking site required for EGFR mediated repression of Cic and therefore is not repressed by EGFR signalling (Andreu *et al.*, 2012a; Astigarraga *et al.*, 2007). In these clones, Pnt expression is lost in the dorsal-anterior (31/32, Figure 3.7A', arrow), which is consistent with the previous results showing loss of dorsal-anterior fate in

clones expressing this transgene (Andreu *et al.*, 2012a) and validate the gain of function phenotype. However, expression of Pnt is not affected posterior clones (7/40, Figure 3.7A', arrowhead) indicating that *pnt* is not regulated by Cic in the posterior.

In clones in the posterior end of the egg chamber, Mid expression is not affected (6/33, Figure 3.7A", arrowhead) but clones more in the anterior domain of Mid expression do lose expression (37/66, Figure 3.7A", arrow). This loss is stronger in the ventral side (16/23, Figure 3.7B", arrowhead) compared to dorsal side (21/43). This observation is consistent with the results showing that only the anterior portion of the Mid expression domain is affected by clones lacking *cic* or in clones gaining or losing *pntP2*. Finally, in early-stage egg chambers, Mid and Pnt expression in the posterior are not affected (Figure 3.7C-C").

Together, this data indicates that Pnt and Mid are not regulated by Cic in the posterior domain. However, the fact that Mid expression is expanded toward the anterior in loss of function *cic* clones and more restrained toward the posterior in gain of function *cic* clones suggest that Cic plays a role in establishing in the anterior boundary of Mid expression, potentially through regulation of *pntP2* and *mirr*.

3.2.2.6 The $pnt^{45D11.1}$ and $pnt^{43H01.1}$ CRRs are not regulated by Cic

Finally, we have tested the response of the $pnt^{45D11.1}$ and $pnt^{43H01.1}$ reporters to Cic to test whether *pnt* is regulated by Cic in the posterior using a second method. We first tested the response of the reporters to loss of *cic* by generating clones homozygous for the null allele cic^{2}. For both $pnt^{45D11.1}$ and $pnt^{43H01.1}$ reporters, no ectopic expression of the reporters is detected (0/15 for $pnt^{45D11.1}$, Figure 3.8A-A'; 0/23 for $pnt^{43H01.1}$, Figure 3.8B-B'). In addition, clones expressing $CicS^{\Delta C2}$-HA do not exhibit loss of reporter expression (0/11 for $pnt^{45D11.1}$, Figure 3.8C-C'; 0/27 for $pnt^{43H01.1}$, Figure 3.8D-D'). Together, these data indicates that the regulation of Pnt by Cic is not

captured through these CRRs and are captured through others unidentified CRRs. These results present more evidence showing that the establishment of the posterior domain of Pnt expression is not regulated by Cic.

3.2.3 Pnt is regulated by EGFR by two different mechanisms

3.2.3.1 Rationale

In the previous part, I have shown that if Cic does not regulate *pnt* expression in the posterior, it plays a role in the establishment of posterior fate by controlling the location of the anterior limit of Mid expression. This seems to be achieved through regulation of PntP2 whose expression is derepressed in the absence of Cic activity and in turn leads to a modest expansion of the anterior limit of Mid expression. In addition, we observed that in the posterior, regulation of Pnt by EGFR does not require Cic and is dependent on JAK/STAT signalling. It is surprising to see that Pnt is not regulated by Cic in the posterior even though we observe repression of Cic, which we can see with loss of nuclear staining in the posterior, and despite the fact that Pnt is induced everywhere in clones lacking *cic*. There seems to be two modes of regulation of Pnt in this tissue, one that is Cic dependent and JAK/STAT independent and another that is JAK/STAT dependent and Cic independent, both requiring EGFR. If in the dorsal-anterior, we can see that EGFR acts through Cic to regulates *pnt*, we still do not know how posterior Pnt is regulated by EGFR. The observation that Pnt seems to be completely lost in clones lacking JAK/STAT signalling (Figure 2.3B') also suggest that Cic repression is not sufficient to turn on expression of *pnt* there.

To further understand the EGFR mediated regulation of Pnt and Mid, and how regulation by JAK/STAT signalling and Cic affect Pnt expression, I generated clones expressing a

constitutively active form of EGFR ($EGFR^{ACT}$) (Queenan *et al.*, 1997) and $CicS^{\Delta C2}$-HA which would allow me to distinguish the effect of Cic on EGFR mediated regulation of Mid.

3.2.3.2 Upd and Cic regulate Pnt expression independently

First, as a control, I generated clones expressing $EGFR^{ACT}$. In these clones, as previously stated, Mid expression is induced (176/208, Figure 3.9A'-C', see chapter 2 and (Fregoso Lomas *et al.*, 2013)). We observe that Pnt expression is also induced (173/207, Figure 3.9A"-C"). However, the levels of Pnt expression are lower than expected especially if we compare the levels in the clones expressing $EGFR^{ACT}$ to the clones lacking *cic* (compared Figure 3.9A"-C" to Figure 3.4). This is surprising because EGFR activity should inhibit activity of Cic. In this case, clones expressing $EGFR^{ACT}$ or lacking *cic* should be equivalent regarding Cic function and therefore should exhibit similar levels of Pnt expression. This could suggest that Cic retains some function in the presence of EGFR signalling. If this is correct, we are seeing higher levels of Pnt induction in clones lacking *cic* because, in this situation, all function of Cic is lost. In early-stage egg chambers, clones in the anteriormost exhibit much higher levels of Pnt expression compared to clones in the main body follicle cells (Figure 3.9A", arrow). This difference in expression is most likely due to Upd which would increase levels of Pnt expression in the presence of EGFR.

Interestingly, in egg chambers after stage 10A, a third of the clones expressing $EGFR^{ACT}$ exhibit a decrease in Pnt expression (8/24, Figure 3.9C"). In wild-type egg chambers, Pnt expression is normally lost in the dorsal midline after stage 10B and Pnt gets expressed in the dorsal-lateral regions (Morimoto *et al.*, 1996) and has been proposed to be due a feedback mechanism, where cells exposed to higher levels of EGFR in the dorsal midline become irresponsive to EGFR signalling (Boisclair Lachance *et al.*, 2009; Ghiglione et al., 1999; Neuman-Silberberg and Schupbach, 1994; Nilson and Schüpbach, 1998a; Wasserman and Freeman, 1998;

Zartman *et al.*, 2009). In addition, at this stage, Cic nuclear localization can be seen in the dorsal midline (Astigarraga *et al.*, 2007), suggesting that loss of Pnt expression could be due to the repressive action of Cic.

In clones expressing $EGFR^{ACT}$ and $CicS^{\Delta C2}$-HA, Mid is induced in almost every clone, exactly like what is observed with clones expressing only $EGFR^{ACT}$ (160/187, Figure 3.9D'-E'). Consistent with clones expressing only $CicS^{\Delta C2}$-HA, this is indicating that the EGFR mediated regulation of Mid expression does not occur through Cic. Looking at Pnt, we observe that clones in the posterior do not have changes in expression, most clones retain expression (52/65, Figure 3.9D", arrowhead). In clones in the anterior, there is no induction of Pnt expression, and we even see loss of expression in the dorsal-anterior (0/92, Figure 3.9D"-E"). However, in clones in the anteriormost region of early-stage egg chambers, we see strong induction of Pnt (29/30, Figure 9.D", arrow). This supports the interpretation that regulation of Pnt in the posterior does not require Cic but requires Upd because Pnt expression is only induced where Upd is present.

3.2.3.3 Pnt is necessary for EGFR mediated ectopic expression of Mid but not in the endogenous domain

In the previous experiment, we observed that in clones expressing $EGFR^{ACT}$ and $CicS^{\Delta C2}$-HA, some clones exhibited ectopic expression of Mid while not expressing Pnt. This could suggest that EGFR signalling induces expression of Mid without requiring Pnt. To test if this is correct, I generated clones homozygous for the null allele $pnt^{\Delta 88}$ expressing $EGFR^{ACT}$. In these clones, we observe that Mid is rarely induced in the anterior domain (16/52, Figure 3.10A'-C', arrow) regardless of the stage. However, in clones in the posterior, Mid expression is reduced but not lost. This reduction in expression is not consistent between clones (21/41, Figure 3.10A'-C', arrowhead) with some clones showing no loss of Mid expression (20/41, Figure 3.10B', arrowhead).

This is indicating that, if Pnt is required for induction expression of Mid in response to EGFR signalling, it is not necessary for expression in the posterior. This is consistent with the observation made in the previous chapter, where we showed that loss of Pnt did not lead to complete loss of Mid expression. This result suggests that there are other regulators of Mid in the posterior domain working downstream that we have not yet identified.

3.3 Summary

In this chapter, I have characterized two overlapping CRRs recapitulating expression of Pnt in posterior follicle cells. These reporters are responsive to JAK/STAT signalling, and I had hypothesized that a cluster of putative STAT92E binding sites present in the overlap sequence shared between the CRRs was necessary for this response. However, I found that a reporter containing only the overlap sequence is not expressed in the follicle cells, indicating that the cluster of putative STAT92E binding sites is not required for expression. This is suggesting that JAK/STAT response elements are located in the sequences contained in $pnt^{45D11.1}$ and $pnt^{43H01.1}$ reporters and future studies should test whether putative STAT92E binding sites in these regions are required for expression.

Next, I tested whether Cic is regulating expression of Mid and Pnt in posterior follicle cells and found that regulation of Mid and Pnt in the posterior is independent of Cic. However, I found that Cic plays a role in the establishment of the anterior boundary of the domain of Mid expression, potentially through regulation of *mirr* and *pntP2*. Finally, I described that Pnt is differentially regulated in the posterior and the dorsal-anterior by EGFR. Dorsal-anterior expression requires repression of Cic by EGFR and is independent of JAK/STAT signalling. Posterior expression requires JAK/STAT signalling and is independent of Cic. This is suggesting that in the posterior and in the dorsal-anterior, *pnt* expression is not regulated by EGFR through the same mechanism.

Future works should continue to characterize how inputs from the EGFR signalling pathway are transduced in the posterior follicle cells. Understanding how inputs from a single signalling pathway can be transduced using different mechanisms within a tissue will give important insight in the ways by which single pathways can lead to different outcomes during development.

3.4 Material and Methods

3.4.1 Drosophila strains

The following Drosophila stocks were used: FRT82B *Stat92*^{85C9} (Silver and Montell, 2001), UAS-*hop* (Harrison *et al.*, 1995), CicS-HA (Astigarraga *et al.*, 2007), FRT82B *cic*^{2}*(Klein and Campos-Ortega, 1992; Roch et al., 2002), UAS-cic*^{RNAi} (VDRC40867), UAS-*pntP2* (Klaes *et al.*, 1994), FRT82B *pnt*$^{\Delta 78}$*(O'Neill et al., 1994),* UAS-*pntP1* (Klaes *et al.*, 1994), UAS-*cicS*$^{\Delta C2}$*-HA (Andreu et al., 2012a),* UAS-*Egfr*^{ACT}(Queenan *et al.*, 1997), *pnt*$^{\Delta 88}$ (Scholz *et al.*, 1993). CRR lacZ reporters and derivatives were made for this study but original Gal4 reporter were produced by (Pfeiffer *et al.*, 2008) and obtained from the Bloomington Drosophila Stock Center.

3.4.2 Mosaic analysis

The MARCM system (Lee and Luo, 2001) was used to generate clones of cells positively marked GAL4-driven expression of either GAL4-driven expression of either P{UAS-*mCD8::GFP.L*} or UAS-*cicS-HA*. In some experiments, the GAL4 expressed in these clones also drove the expression of a UAS-*Egfr*^{ACT} , a *UAS-cicS-HA*, a UAS-hop, a UAS-PntP1 or a UAS-PntP2 transgene or an RNAi construct targeting *cic*. For generation of clones, females of the appropriate genotype were heat shocked for 1 hr at 37°C as late pupae or newly eclosed adults, either once or on 3 consecutive days, then aged at 18 or 22°C and supplemented overnight or two days with dry yeast before dissection. Ovaries were harvested 10 days after first heat shock, with

shorter intervals between clone induction and dissection enriching the sample for clones generated later in oogenesis.

3.4.3 Immunohistochemistry

Dissection and immunostaining were carried out as described previously (Laplante and Nilson, 2006). Antibodies used were guinea pig anti-Mid (1:2,000) (Fregoso Lomas et al., 2013), rabbit anti-Pnt (Pascual et al., 2017), rabbit anti-β-gal (1:10,000, MP Biomedicals-Cappel), mouse anti-β-gal hybridoma supernatant 40A-1A-s (1:200, Developmental Studies Hybridoma Bank), rat anti-HA . All secondary antibodies were highly cross-adsorbed Alexa Fluor-conjugated anti-IgG (1:2,000, Invitrogen). All secondary antibodies, as well as anti-Mid and anti-Pnt, were diluted 1:10 in PBS and pre-adsorbed by incubation with fixed wild-type ovaries or embryos prior to use.

3.4.4 Microscopy and Image analysis

Images were acquired using a Zeiss Axioplan 2 wide-field fluorescence microscope except for images in Figure 3.3 which were acquired on Leica SP8. Apart from minor uniform linear adjustments to brightness and contrast, no image manipulations were performed.

3.4.5 Reporter cloning and Generation of fly constructs

For generation of lacZ constructs sequences were amplified from the endogenous gene using primers containing restriction enzyme sites so that they could be cloned into a linearized placZ.attB vector (Bischof et al., 2013). For derivative constructs regions were amplified from a plasmid containing the original complete insert. Conservation of DNA regions was determined from the UCSC genome browser using 124 insects basewise conservation by PhyloP and breakpoints were designed in regions of low conservation where possible. Individual colonies were picked and tested for the presence of the insert by miniprep, restriction digest, and sequencing. Constructs were amplified by midiprep (Qiagen) and integrated into the Drosophila genome using

the PhiC31 integrase and the attP landing site at cytological position 58A. Fly transformants were used to generate a stable stock and dissected to determine expression pattern in the ovary. DNA from transformant fly lines was extracted and plasmid DNA was amplified and genotyped by either restriction digestion or sequencing.

3.4.6 Search for STAT92E binding sites

Sites were search based on a previous protocol (De Vito, 2021). Sequences of the *pnt45D11* and *pnt43H01* were screened with MEME suite FIMO tool using a pair-wise motif for STAT92E (Fornes et al., 2020; Grant et al., 2011).

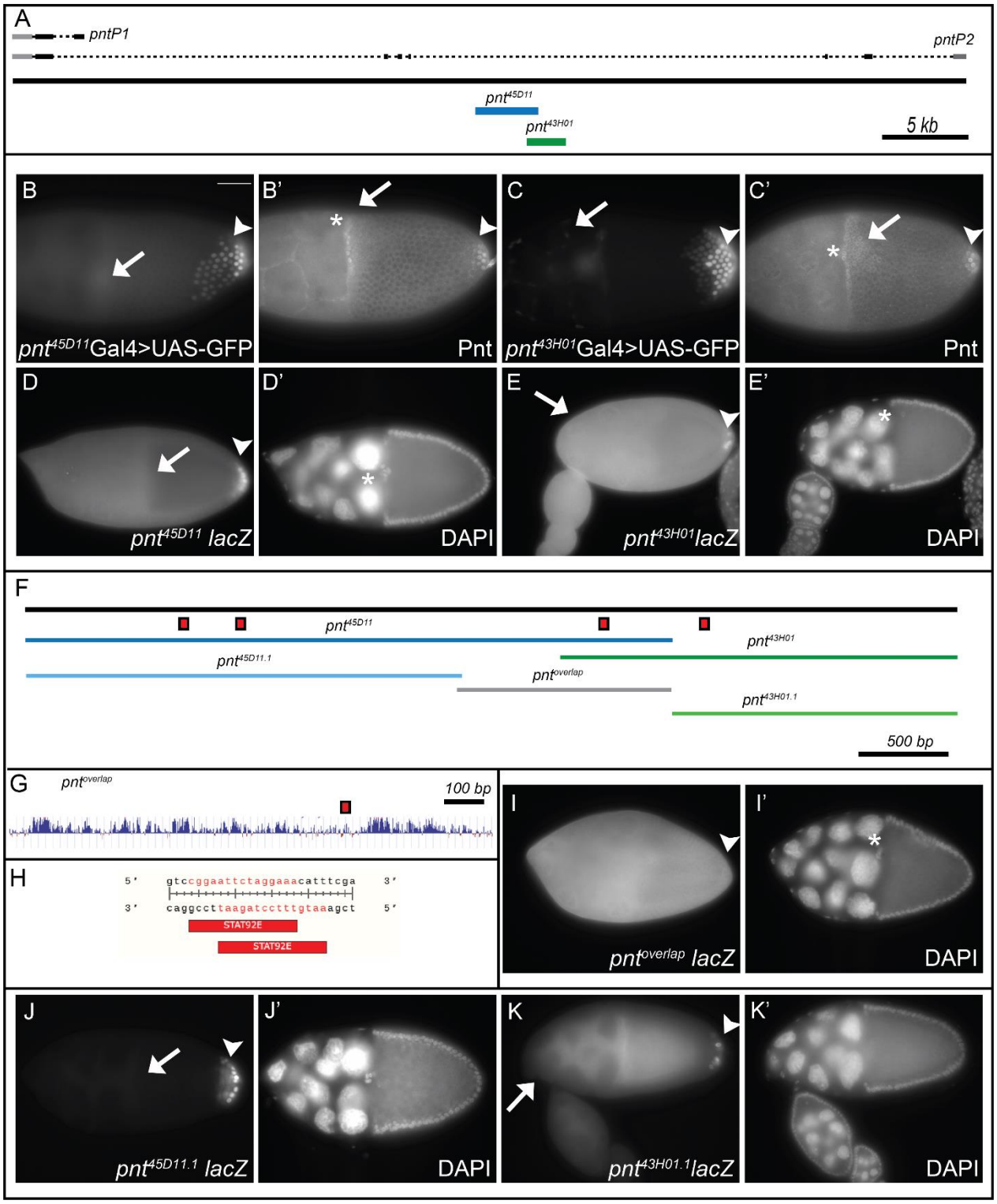
A
pntP1
pntP2
pnt45D11
pnt43H01
5 kb
B
pnt45D11Gal4>UAS-GFP
B'
Pnt
C
pnt43H01Gal4>UAS-GFP
C'
Pnt
D
pnt45D11 lacZ
D'
DAPI
E
pnt43H01lacZ
E'
DAPI
F
pnt45D11
pnt43H01
pnt45D11.1
pntoverlap
pnt43H01.1
500 bp
G
pntoverlap
100 bp
H
5' gtccggaattctaggaaacatttcga 3'
3' caggccttaagatcctttgtaagct 5'
STAT92E
STAT92E
I
pntoverlap lacZ
I'
DAPI
J
pnt45D11.1 lacZ
J'
DAPI
K
pnt43H01.1lacZ
K'
DAPI

Figure 3.1 – Two CRRs recapitulate posterior expression of Pnt.

(A) Map of the *pnt* locus showing the pnt^{45D11} and pnt^{43H01} CRRs in blue and green respectively. (B-B') Expression of pnt^{45D11} Gal4 reporter in posterior follicle cells, where endogenous Pnt is also expressed (arrowhead). The reporter is also expressed in border cells (arrow in B). Expression in the dorsal-anterior is absent (arrow in B'). (C-C') Expression of pnt^{43H01} Gal4 reporter in posterior follicle cells, where endogenous Pnt is also expressed (arrowhead). The reporter is also expressed in anterior follicle cells (arrow in C). Expression in the dorsal-anterior is absent (arrow in C'). (D-D') Expression of the pnt^{45D11} *lacZ* reporter in posterior follicle cells (arrowhead). Expression in the border cells is not detected (arrow). (E-E') Expression of the pnt^{43H01} *lacZ* reporter in posterior follicle cells (arrowhead). Expression in the anterior is not detected (arrow). (F) Map of the pnt^{45D11} and pnt^{43H01} CRRs. Sequences of the $pnt^{45D11.1}$, $pnt^{overlap}$, and $pnt^{43H01.1}$ derivative constructs. Red boxes indicate putative STAT92E binding sites. (G) Conservation of the $pnt^{overlap}$ sequence, showing the putative STAT92E binding site. (H) Sequence of the cluster of putative STAT92E binding sites in the sequence of the $pnt^{overlap}$. (I-I') The $pnt^{overlap}$ *lacZ* reporter is not expressed in the posterior follicle cells (arrowhead). (J-J') The $pnt^{45D11.1}$ *lacZ* reporter is expressed in the posterior follicle cells (arrowhead) in a similar domain as the original pnt^{45D11}. Expression in the border cells is not detected (arrow). (K-K') The $pnt^{43H01.1}$ *lacZ* reporter is expressed in the posterior follicle cells (arrowhead) in a similar domain as the original pnt^{43H01}. Expression in the anterior follicle cells is not detected (arrow). Scale bar = 50μm for all panels.

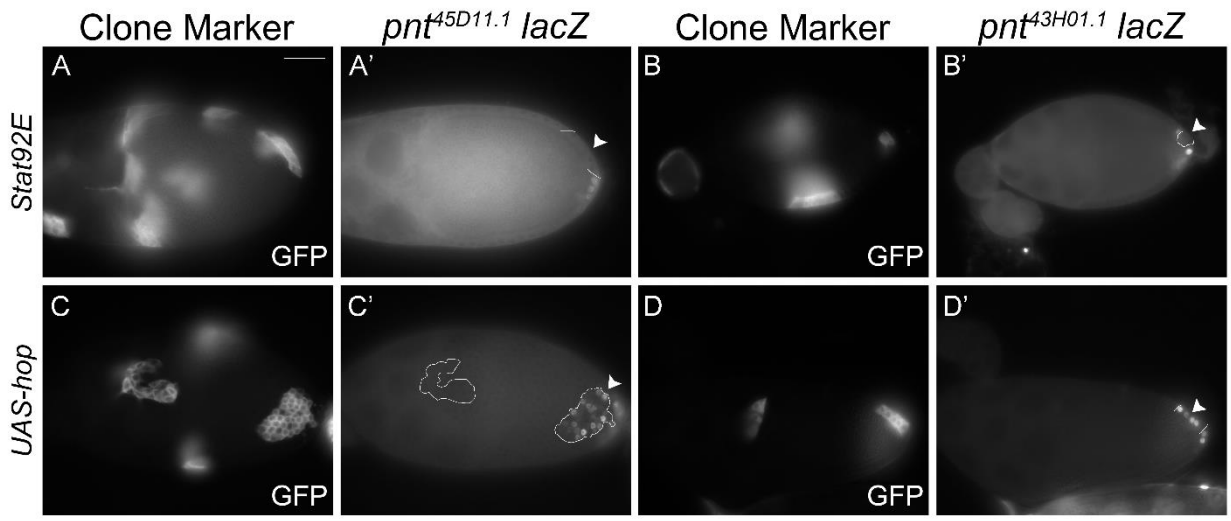
Clone Marker
pnt45D11.1 lacZ
Clone Marker
pnt43H01.1 lacZ
Stat92E
UAS-hop
A
A'
B
B'
C
C'
D
D'
GFP
GFP
GFP
GFP

Figure 3.2 – Response of the *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$ CRRs reporters to JAK/STAT signalling.

(A-A') Loss-of-function *Stat92E*^{85C9} clones exhibit loss of the *pnt*$^{45D11.1}$ *lacZ* reporter (arrowhead). (B-B') Loss-of-function *Stat92E*^{85C9} clones exhibit loss of the *pnt*$^{43H01.1}$ *lacZ* reporter (arrowhead). (C-C') Clones expressing UAS-*hop* exhibit gain of the *pnt*$^{45D11.1}$ *lacZ* reporter (arrowhead). (D-D') Clones expressing UAS-*hop* exhibit gain of the *pnt*$^{43H01.1}$ *lacZ* reporter (arrowhead). All clones are positively marked with GFP. Scale bar = 50μm for all panels.

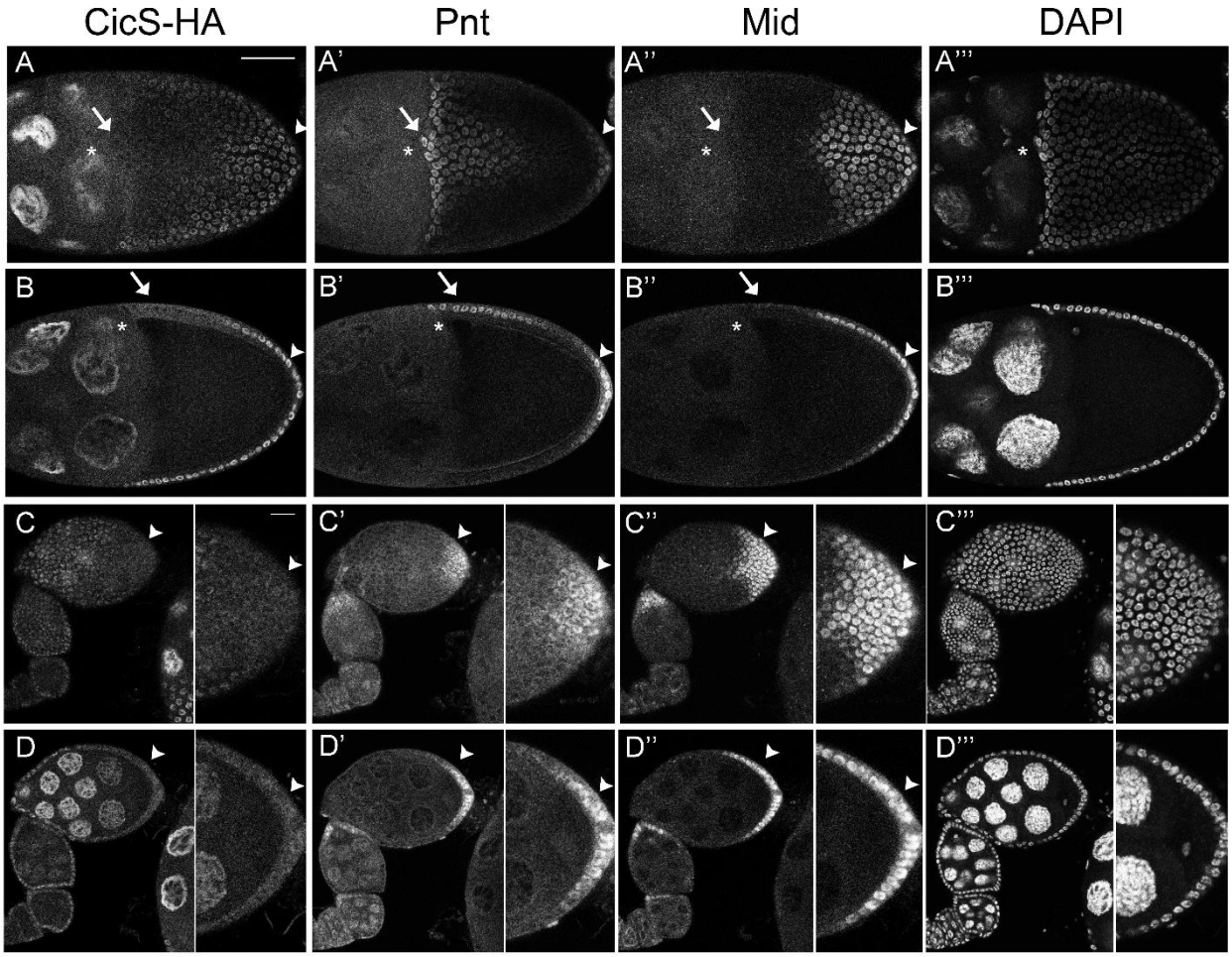
CicS-HA
Pnt
Mid
DAPI
A
A'
A''
A'''
B
B'
B''
B'''
C
C'
C''
C'''
D
D'
D''
D'''

Figure 3.3 – CicS nuclear localization is lost in response to EGFR signalling

(A-A’”). Dorsal view of a stage 10B egg chamber. Nuclear localization of CicS-HA is lost (arrow in A). Posterior CicS-HA is nuclear (arrowhead in A). Pnt is expressed in the dorsal anterior (arrow in A’) and in the posterior (arrowhead in A’). Mid is only expressed in the posterior (arrowhead in A”) but not in the dorsal-anterior (arrow in A”). (B-B”’) Cross section of a stage 10B egg chamber. (C-C”’) Surface view of a stage 8 egg chamber. Nuclear localization of CicS-HA is lost in the posterior where Pnt and Mid are expressed (arrowhead). (D-D”’) Cross section view of a stage 8 egg chamber. Dorsal side is indicated by an asterisk. Scale bar = 50µm for all panels except for the zoomed images in C-D”’ where scale bar = 10µm.

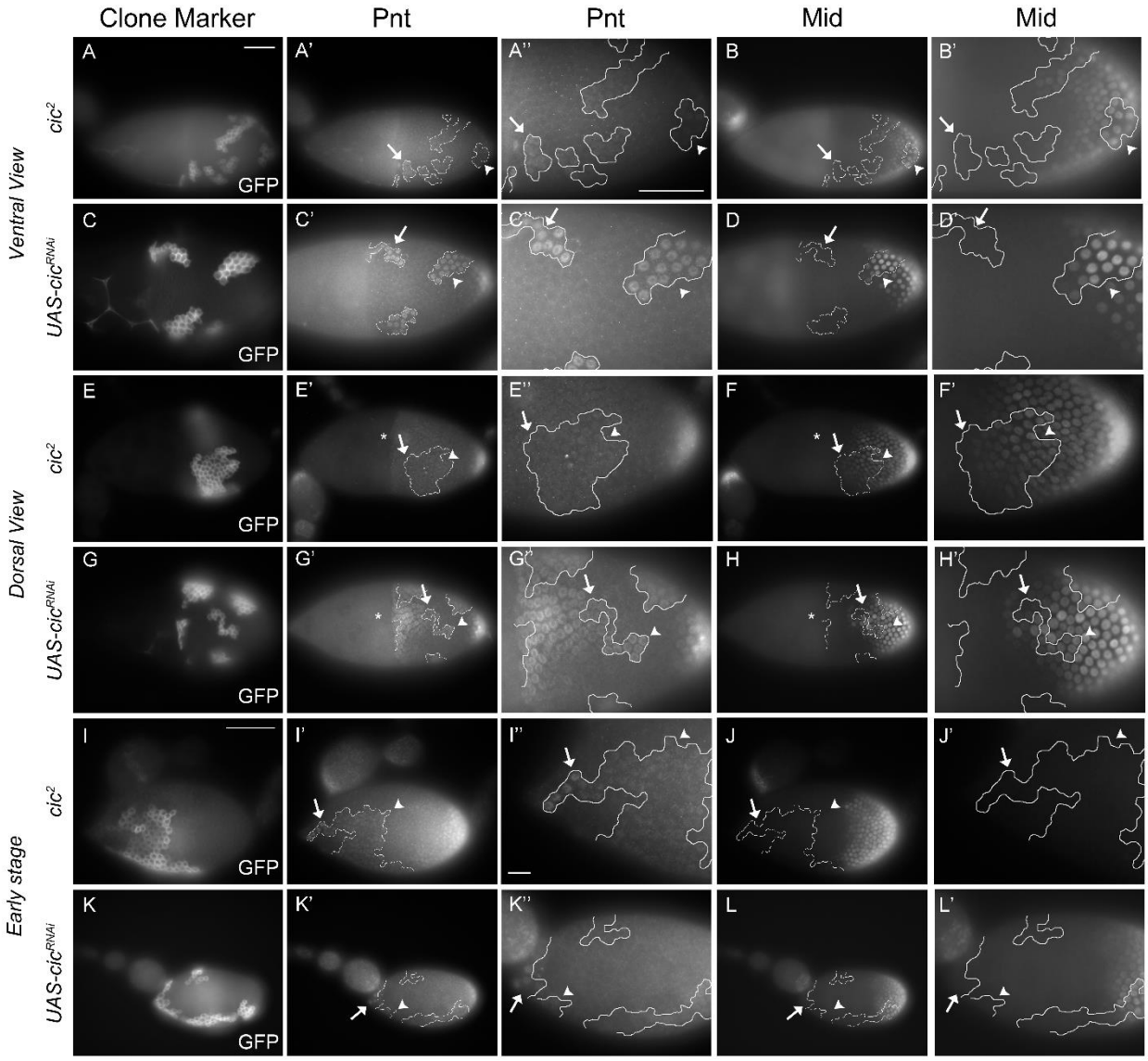
Clone Marker
Pnt
Pnt
Mid
Mid
Ventral View
Dorsal View
Early stage
cic2
UAS-cicRNAi
GFP

Figure 3.4 – Response of Pnt and Mid to loss of *cic*

(A-B") Loss-of-function cic^{2} clones in the ventral side exhibit ectopic expression of Pnt, both in the anterior (arrow) and in the posterior (arrowhead). Mid expression is induced in posterior clones (arrowhead) but not in the anterior (arrow). (C-D") Clones expressing a RNAi construct against *cic* in the ventral side exhibit ectopic expression of Pnt, both in the anterior (arrow) and in the posterior (arrowhead). Mid expression is induced in posterior clones (arrowhead) but not in the anterior (arrow). (E-F") Loss-of-function cic^{2} clones in the dorsal side exhibit ectopic expression of Pnt, both in the anterior (arrow) and in the posterior (arrowhead). Mid expression is not affected in posterior clones (arrowhead) or in the anterior (arrow). (G-H") Clones expressing a RNAi construct against *cic* in the dorsal side exhibit ectopic expression of Pnt, both in the anterior (arrow) and in the posterior (arrowhead). Mid expression is not affected in posterior clones (arrowhead) or in the anterior (arrow). (I-J") Loss-of-function cic^{2} clones in the anterior of early egg chamber side exhibit ectopic expression of Pnt, clones in the anteriormost exhibit higher levels of Pnt expression (arrow) compared to clones more in the posterior (arrow). Mid expression is not induced in the anterior (arrow and arrowhead). (K-L") Clones expressing a RNAi construct against *cic* in the anterior of early egg chamber side exhibit ectopic expression of Pnt, clones in the anteriormost exhibit higher levels of Pnt expression (arrow) compared to clones more in the posterior (arrow). Mid expression is not induced in the anterior (arrow and arrowhead). All clones are positively marked with GFP. Dorsal side is indicated by an asterisk. Scale bar = 50μm for all panels, except zoomed in panels where scale bar = 10μm.

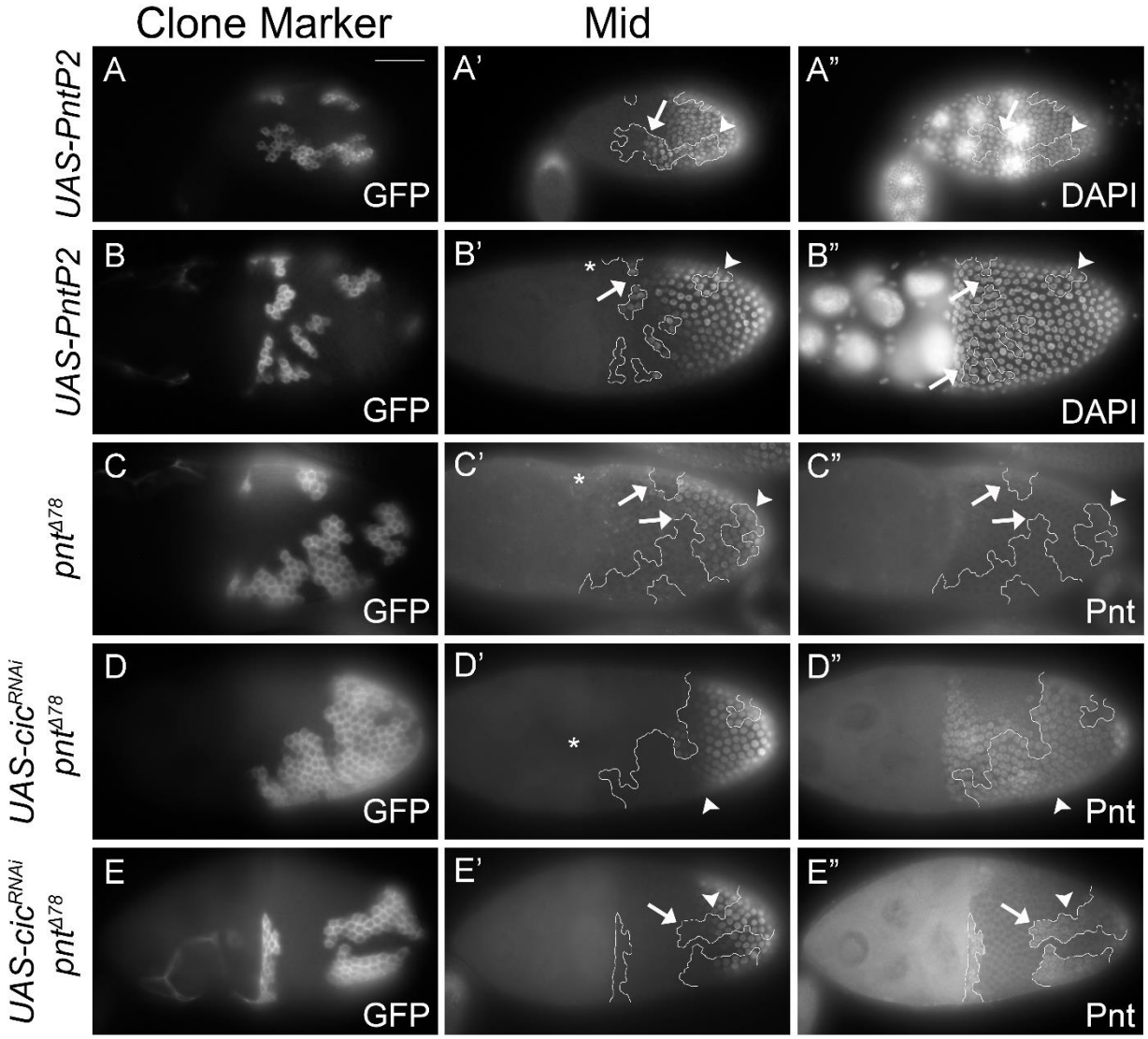
Clone Marker
Mid
UAS-PntP2
A
GFP
A'
A"
DAPI
UAS-PntP2
B
GFP
B'
B"
DAPI
pntΔ78
C
GFP
C'
C"
Pnt
UAS-cicRNAi pntΔ78
D
GFP
D'
D"
Pnt
UAS-cicRNAi pntΔ78
E
GFP
E'
E"
Pnt

Figure 3.5 – PntP2 may be the isoform induced in loss-of-function *cic* clones

(A-A") Clones expressing UAS-*pntP2* show a similar phenotype as clones lacking *cic*. Mid expression in induced only in the posterior (arrowhead) but not in the anterior (arrow) in early egg chamber. (B-B") Clones expressing UAS-*pntP2* exhibit ectopic expression of Mid in the posterior (arrowhead) and in the dorsal-anterior (top arrow). Mid is not induced in the ventral-anterior (bottom arrow). (C-C") Loss-of-function $pnt^{\Delta 78}$ clones exhibits loss of Mid in the anterior domain of Mid expression (arrow) but not in the posterior (arrowhead). Pnt expression is not lost in dorsal-anterior clones (arrow). (D-D") Loss-of-function $pnt^{\Delta 78}$ clones expressing a RNAi construct against *cic* in the dorsal side exhibits loss of Mid in the anterior domain of Mid expression (arrowhead) but not in the posterior. Pnt is induced everywhere in the clones. (E-E") Loss-of-function $pnt^{\Delta 78}$ clones expressing a RNAi construct against *cic* in the dorsal side exhibits ectopic expression of Mid in the posterior (arrowhead). Cells more in the anterior of the clones do not express Mid (arrow). Pnt is expressed everywhere in the clones. All clones are positively marked with GFP. Dorsal side is indicated by an asterisk. Scale bar = 50μm for all panels.

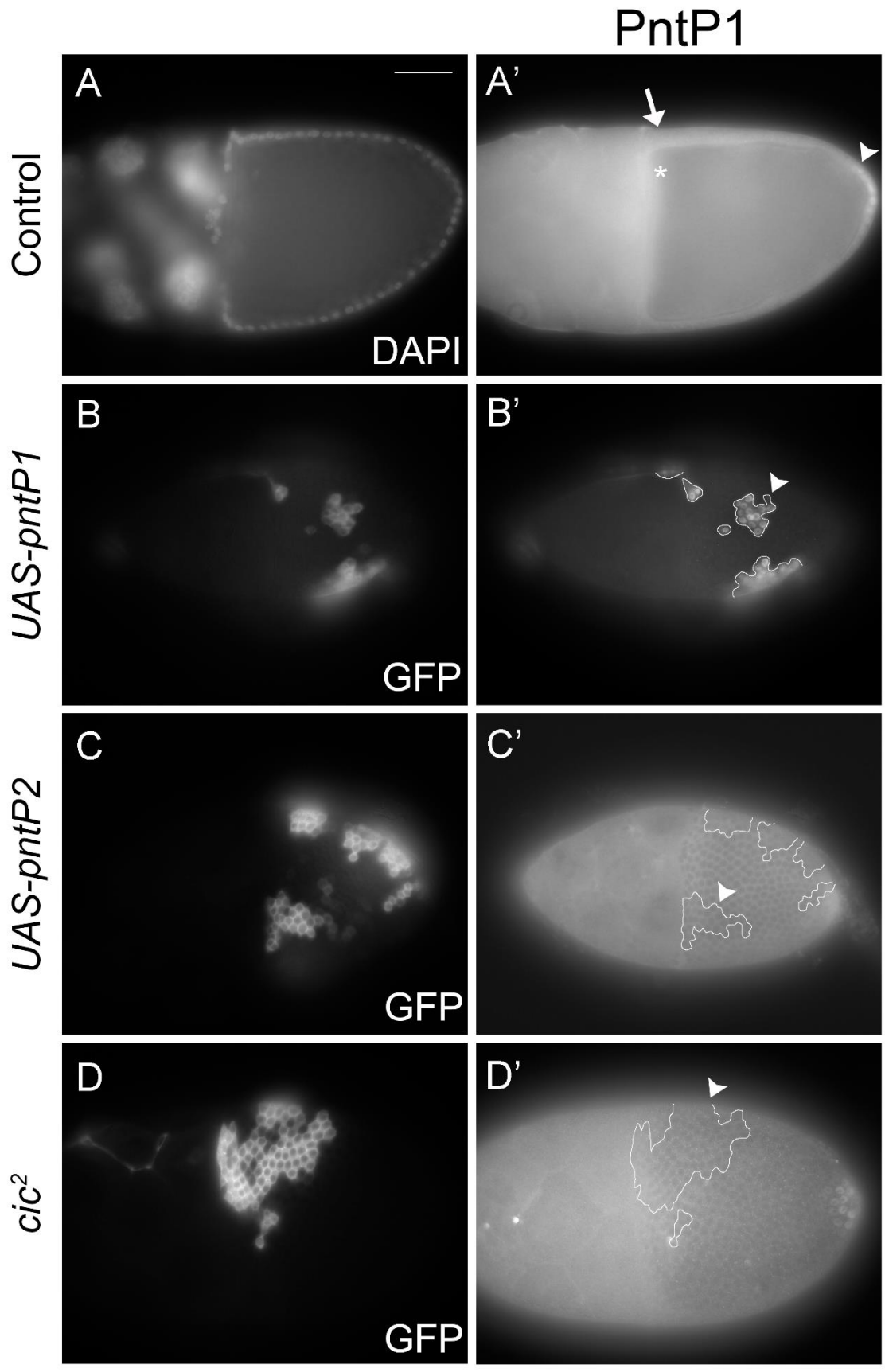
PntP1
Control
UAS-pntP1
UAS-pntP2
cic2
A
A'
B
B'
C
C'
D
D'
DAPI
GFP
GFP
GFP

Figure 3.6 – PntP1 is not induced in clones lacking *cic*

(A-A') Endogenous expression of PntP1. Expression is detected in the posterior (arrowhead) but not in the dorsal-anterior (arrow). (B-B') PntP1 staining is detected in clones expressing UAS-*pntP1*. (C-C') PntP1 staining is not detected in clones expressing UAS-*pntP2*. (D-D') PntP1 expression is not detected in loss-of-function clones cic^{2}. Pnt is expressed everywhere in the clones. All clones are positively marked with GFP. Dorsal side is indicated by an asterisk. Scale bar = 50μm for all panels.

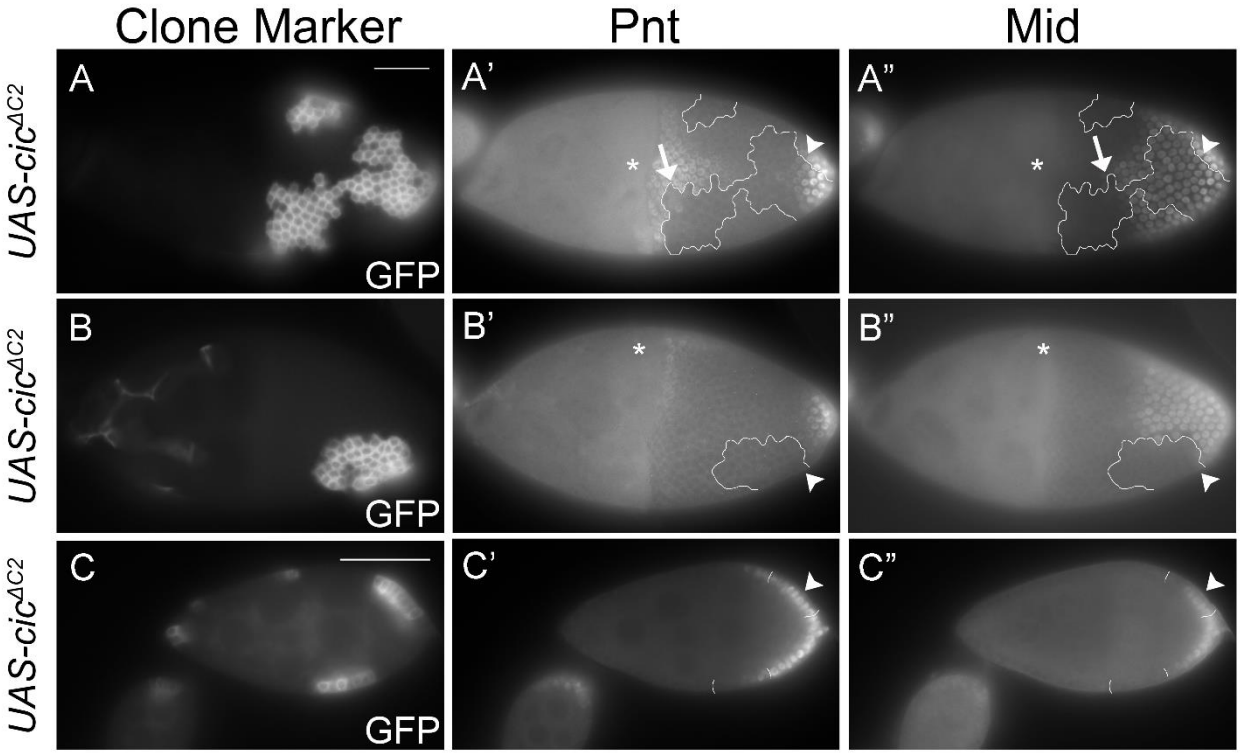
Clone Marker
Pnt
Mid
UAS-cic^ΔC2
UAS-cic^ΔC2
UAS-cic^ΔC2
A
A'
A"
B
B'
B"
C
C'
C"
GFP
GFP
GFP

Figure 3.7 – Response of Pnt and Mid to clones expressing UAS-*cicS*$^{\Delta C2}$*-HA*

(A-A'") Dorsal view of an egg chamber expressing UAS-*cicS*$^{\Delta C2}$*-HA*. Clones in the anterior exhibit loss of Pnt (arrow in A') but not in the posterior (arrowhead). Mid expression at the posterior is not affected (arrowhead) but is slightly lost in the anterior domain of Mid expression (arrow in A"). (B-B") Ventral view of an egg chamber expressing UAS-*cicS*$^{\Delta C2}$*-HA* exhibiting lost of Mid expression (arrowhead). (C-C") Clones expressing UAS-*cicS*$^{\Delta C2}$*-HA* in early egg chambers do not exhibit loss of Pnt or Mid expression (arrowhead). All clones are positively marked with GFP. Dorsal side is indicated by an asterisk. Scale bar = 50μm for all panels.

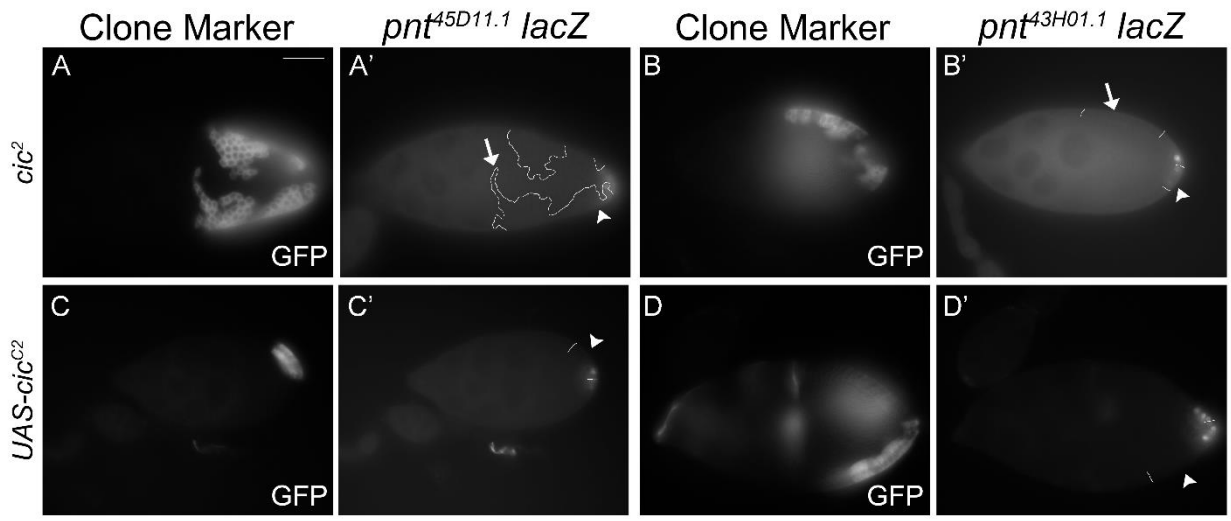
Clone Marker
pnt45D11.1 lacZ
Clone Marker
pnt43H01.1 lacZ
cic2
UAS-cicC2
A
A'
B
B'
C
C'
D
D'
GFP
GFP
GFP
GFP

Figure 3.8 – Response of the *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$ CRRs reporters to Cic

(A-A') Expression of the *pnt*$^{45D11.1}$ *lacZ* reporter is not affected in loss-of-function *cic*^{2} clones (arrowhead). (B-B') Expression of the *pnt*$^{43H01.1}$ *lacZ* reporter is not affected in loss-of-function *cic*^{2} clones (arrowhead). (C-C') Expression of the *pnt*$^{45D11.1}$ *lacZ* reporter is not affected in clones expressing UAS-*cicS*$^{\Delta C2}$*-HA* (arrowhead). (D-D') Expression of the *pnt*$^{43H01.1}$ *lacZ* reporter is not affected in clones expressing UAS-*cicS*$^{\Delta C2}$*-HA* (arrowhead). All clones are positively marked with GFP. Scale bar = 50μm for all panels.

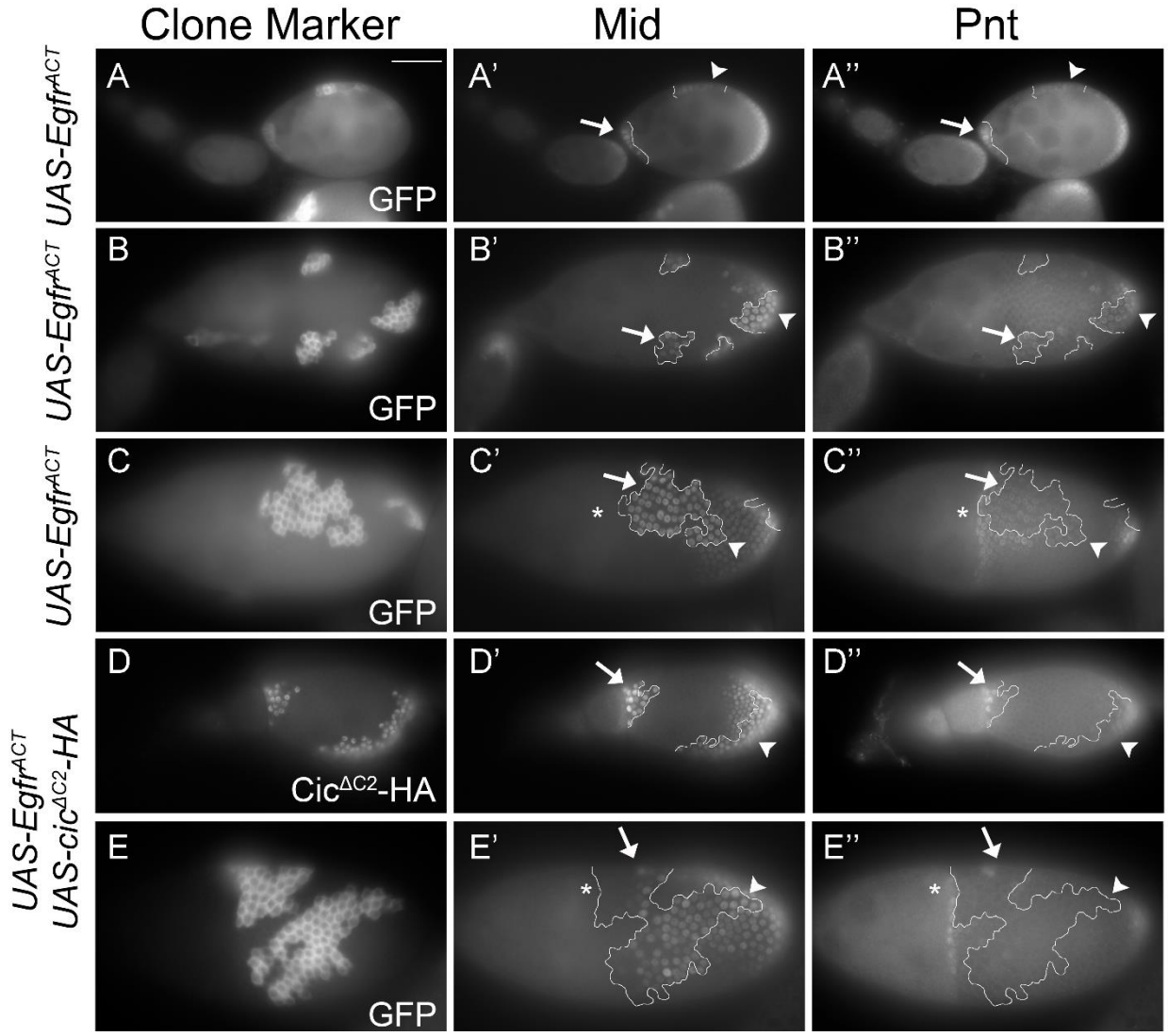
Clone Marker
Mid
Pnt
UAS-Egfr^ACT
UAS-Egfr^ACT
UAS-Egfr^ACT
UAS-Egfr^ACT
UAS-cic^ΔC2-HA
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''
GFP
GFP
GFP
Cic^ΔC2-HA
GFP

Figure 3.9 – Response of Mid and Pnt to UAS-*Egfr*^{ACT} and UAS-cicS$^{\Delta C2}$-HA

(A-A") Stage 8 egg chambers expressing UAS-*Egfr*^{ACT} exhibit gain of Mid and Pnt expression both in the anterior (arrow) and main body (arrowhead). Ectopic expression is higher in clones in the anterior. (B-B") Clones expressing UAS-*Egfr*^{ACT} in the ventral side of stage 10A egg chamber exhibit ectopic expression of Mid and Pnt, both in the posterior (arrowhead) and in the anterior (arrow). (C-C") Clones expressing UAS-*Egfr*^{ACT} in the dorsal side of a stage 10A egg chamber exhibit ectopic expression of Mid both in the posterior (arrowhead) and in the anterior (arrow). Pnt expression is induced in the posterior (arrowhead), but expression is reduced in the anterior (arrow). (D-D") Clones expressing UAS-*Egfr*^{ACT} and UAS-*cicS*$^{\Delta C2}$*-HA* exhibit gain of Mid expression, both in the anterior (arrow) and in the posterior (arrowhead). Pnt expression is induced in the anterior (arrow) but not in the posterior (arrowhead). Endogenous expression of Pnt is not affected. (E-E") Clones expressing UAS-*Egfr*^{ACT} and UAS-cicS$^{\Delta C2}$-HA in the dorsal side exhibit gain of Mid expression in the anterior (arrow) and the posterior (arrowhead). Pnt expression is not induced in the posterior (arrowhead) and is lost in the anterior (arrow). All clones are positively marked with GFP except for panel D which is marked with CicS$^{\Delta C2}$-HA. Dorsal side is indicated by an asterisk. Scale bar = 50μm for all panels.

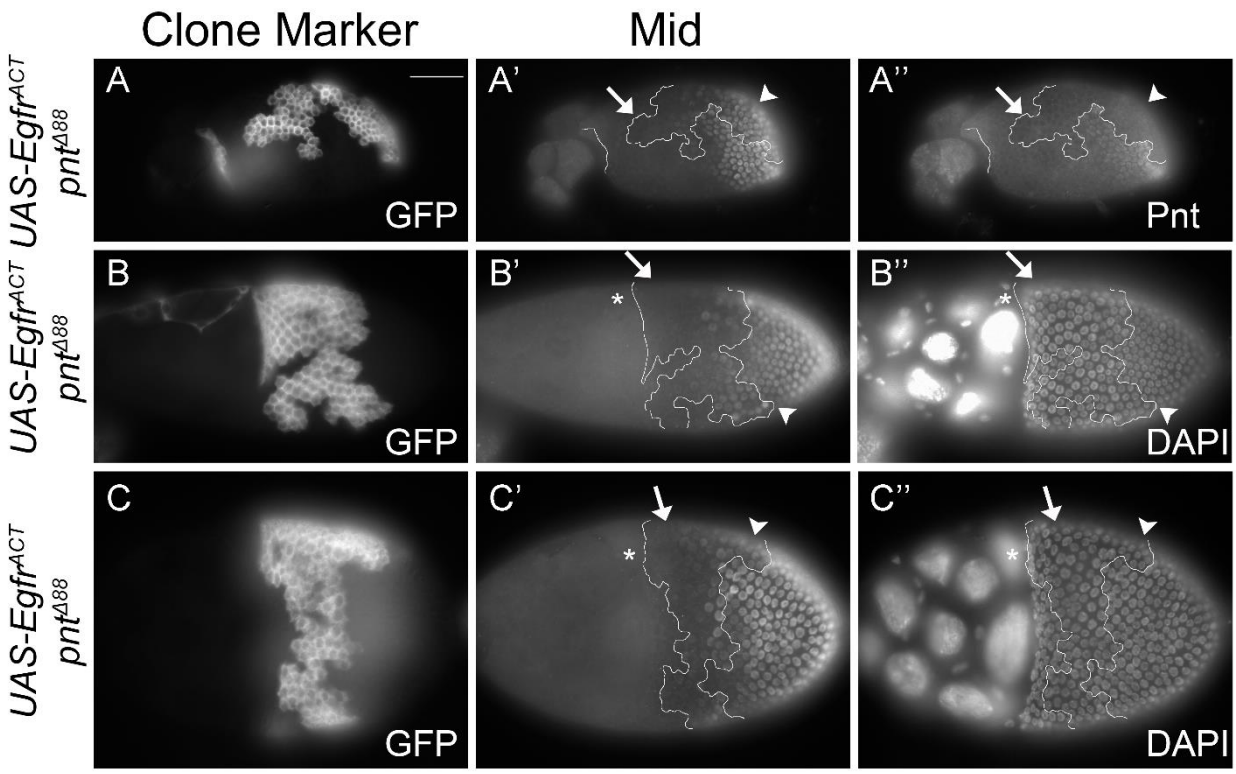
Clone Marker
Mid
UAS-Egfr^ACT pnt^Δ88
UAS-Egfr^ACT pnt^Δ88
UAS-Egfr^ACT pnt^Δ88
A
A'
A''
GFP
Pnt
B
B'
B''
GFP
DAPI
C
C'
C''
GFP
DAPI

Figure 3.10 – Response of Mid to loss of *pnt* and gain of ectopic EGFR signalling

(A-A") Loss of function $pnt^{\Delta 88}$ expressing UAS-$Egfr^{ACT}$ exhibit weak induced of Mid in the anterior of the endogenous expression domain of Mid. There is no induction of Mid in the anterior (arrow). There is no effect on Mid in the posterior (arrowhead). (B-C") Loss of function $pnt^{\Delta 88}$ expressing UAS-$Egfr^{ACT}$ in the dorsal side exhibit weak ectopic expression of Mid in the anterior of the endogenous expression domain of Mid. There is no induction of Mid in the anterior (arrow). There is no effect on Mid in the posterior (arrowhead). All clones are positively marked with GFP. Dorsal side is indicated by an asterisk. Scale bar = 50µm for all panels.

Chapter 4 – Discussion and Future directions

4.1 Summary

4.1.1 JAK/STAT influences EGFR signalling by increasing levels of Pnt and by repressing Mirr

In this thesis, I have described how the outcome of a signalling pathway can be determined by other localized signals. The follicular epithelium of the Drosophila ovary is patterned along the anterior-posterior and dorsal-ventral axes by the EGFR signalling pathway. Early signalling occurring in the posterior follicle cells leads to expression of Mid and late signalling in the dorsal-anterior leads to expression of Mirr. Which EGFR target is expressed is determined by opposite gradients of JAK/STAT and BMP activity in the posterior and anterior respectively. I asked how inputs from the JAK/STAT signalling pathway determine EGFR outcome to promote posterior fate and Mid expression.

By characterizing a putative CRR controlling expression of Mid, mid^{up}, we were unable to find a direct regulation of Mid through binding of STAT92E to this sequence. Instead, we found that the EGFR effector Pnt is regulated by JAK/STAT signalling in posterior follicle cells and that Pnt controls Mid expression. Expressing Pnt in clones unable to respond to JAK/STAT signalling rescues loss of Mid, indicating that Mid is regulated by the JAK/STAT signalling pathway through Pnt.

In addition, De Vito, 2021 showed that a CRR controlling expression of Mirr is directly repressed through a STAT92E binding site, suggesting that *mirr* is a direct target of JAK/STAT signalling (De Vito, 2021). Together, this described how the JAK/STAT signalling pathway

influences the outcome of EGFR signalling; by increasing the levels of its effector Pnt which regulates Mid expression and by repressing the alternative target Mirr.

4.1.2 Pnt represents a point of crosstalk between the EGFR and JAK/STAT signalling pathways

We have described the role of Pnt as a major determinant of posterior fate in the follicular epithelium. This role has recently been described in another study (Stevens *et al.*, 2020). We also found that Pnt is jointly regulated by the EGFR and JAK/STAT signalling pathway in the posterior domain. In addition, we observe that neither JAK/STAT nor EGFR is sufficient to induce posterior expression of Pnt individually indicating that both inputs are needed for expression.

We have identified and characterized two putative CRRs recapitulating expression of Pnt in the posterior. Despite having similar expression pattern in the posterior, I found that the overlapping sequence shared between the two putative CRRs does not contribute to the expression pattern. This is indicating that the JAK/STAT response elements are scattered around the two CRRs. However, it is still unknown at this point if the regulation of Pnt by JAK/STAT is directly captured through binding of STAT92E to one or both of these putative CRRs.

4.1.3 Cic regulates Pnt in the dorsal-anterior but not in the posterior

Finally, I asked how the inputs from EGFR signalling are transduced in the posterior domain to induce expression of Pnt. Because of the well-known role of Cic as an effector of EGFR signalling in the dorsal-anterior and the fact Cic is regulating expression of Pnt in other tissues, I asked whether Cic was also controlling expression of Pnt in the follicular epithelium, and more specifically in the posterior. I found that although Cic regulates Pnt expression in the dorsal-anterior, it does not regulate expression of Pnt and Mid in the posterior domain. I found however, that loss of Cic shifted the anterior limit of Mid expression toward the anterior and gain of Cic

function shifted the limit toward the posterior, suggesting that if Cic does not directly regulate Mid and Pnt expression in the posterior, it is playing a role in setting up the boundary between the anterior and posterior domains.

Finally, I found that the dorsal-anterior and posterior expressions of Pnt are regulated by EGFR through two different mechanisms. In the dorsal-anterior, Cic repression leads to Pnt expression in a JAK/STAT independent manner, whereas in the posterior, Pnt expression requires JAK/STAT and EGFR signalling but is not regulated by Cic. How the signals from EGFR are transduced and captured in the posterior is still unknown.

4.1.4 Regulatory inputs on Mid are captured by two putative CRRs

In this thesis, I have characterized the response of mid^{up}, a putative CRR recapitulating Mid expression in the posterior and capturing inputs from Grk and Upd. Other lab members have also found that this CRR is capturing inputs from Mirr but not from Dpp (De Vito, 2021). As explained in chapter 2, we have characterized a derivative of the mid^{up} reporter exhibiting expression in the dorsal-anterior indicating that it is missing a response element required for dorsal-anterior repression. This allows us to map a region containing this response element which we hypothesized to be a Mirr binding site.

In addition, another putative CRR recapitulating expression of Mid in the posterior, mid^{intron} was identified. This putative CRR is responsive to Grk and Dpp but not to Upd and Mirr (De Vito, 2021). Therefore, response elements are scattered around the mid^{up} and mid^{intron} sequences, this is suggesting that the two putative CRRs are interacting with each other to integrate all the regulatory inputs controlling expression of Mid.

Interestingly, the *mid*^{intron} CRR is responsive to Pnt but not to Upd (De Vito, 2021). This is surprising because we have shown here that Pnt expression in the posterior requires JAK/STAT signalling. This could suggest that there is a JAK/STAT independent regulation of *pnt* in the posterior. However, it is unknown whether this CRR is directly regulated by Pnt. Future work should aim to find whether Mid is directly regulated by binding of Pnt to these CRRs.

4.2 How are inputs regulating Pnt integrated ?

4.2.1 Posterior regulation by JAK/STAT and EGFR signalling

In Chapter 3, we have characterized two putative CRRs recapitulating expression of Pnt in posterior follicle cells, *pnt*^{45D11} and *pnt*^{43H01}. Reporters of these putative CRRs are responsive to JAK/STAT signalling which suggest that inputs from Upd could be captured through STAT92E binding sites present in the genomic sequences they contain. A good candidate region to find such sites was the overlap sequence shared between the two CRRs. However, when isolated, this sequence does not drive expression of a reporter gene in the follicle cells indicating that there are no STAT92E binding site in this region required for expression. We have identified other putative binding sites in the derivatives missing the overlap regions (Figure 3.1F). Future studies should aim to characterize these sites by introducing mutations that would disrupt binding of STAT92E. If these sites are necessary for posterior expression of Pnt, we will observe loss of reporter expression. In addition, if we find that these reporters are activated through binding of STAT92E, it will be interesting to understand how in this context, binding of STAT92E activates gene expression but represses in the context of *mirr* regulation.

Pnt expression in the posterior also requires EGFR signalling (Morimoto *et al.*, 1996) and we have not yet characterized how EGFR signalling regulates Pnt expression in the posterior. Furthermore, whether the *pnt*^{45D11} and *pnt*^{43H01} reporters are responsive to EGFR signalling is

unknown. Searching for putative binding sites of other known regulators of EGFR signalling in the sequences could help identify other candidate effectors of EGFR signalling regulating Pnt expression in the posterior. However, we also observe that both CRRs are active in regions where Grk is absent: pnt^{45D11} is active in the border cells and pnt^{43H01} is active in anterior follicle cells. Expression in these regions is consistent with their regulation by JAK/STAT signalling (McGregor *et al.*, 2002; Silver and Montell, 2001; Xi *et al.*, 2003) but is not detectable by immunostaining for the endogenous Pnt. This suggest that these putative CRRs do not require EGFR signalling to be active, unlike endogenous Pnt. However, expression of the pnt^{45D11} and pnt^{43H01} reporters in the border cells and the anterior is only detected with Gal4:UAS-GFP reporter, not with the *lacZ* reporter and could suggest that endogenous Pnt expression is also not detected in these regions. Nonetheless, response to EGFR signalling of the pnt^{45D11} and pnt^{43H01} reporter to EGFR should be tested before looking for putative binding sites of EGFR effectors in these sequences.

4.2.2 Pnt regulation in the dorsal-anterior domain

4.2.2.1 What is/are the CRR(s) responsible for dorsal-anterior expression of Pnt

In our screen of the collection of reporters (Pfeiffer *et al.*, 2008), we have identified two putative CRRs recapitulating expression of Pnt in the posterior but none reproducing the dorsal-anterior expression. Another screen of reporter controlling expression of Pnt has also not detected dorsal-anterior CRRs (Revaitis *et al.*, 2017). Interestingly, a Pnt-GFP fusion protein made from a BAC containing 90kb around the *pnt* locus does have dorsal-anterior expression. In fact, this fusion protein faithfully reproduces endogenous Pnt expression, including both the posterior and dorsal anterior domains, and can rescue loss of endogenous Pnt (Boisclair Lachance *et al.*, 2014). This is indicating, that in the 90kb contained in the BAC, the CRR(s) controlling expression of Pnt in the dorsal-anterior must be present. All the reporters from the collection are located in the introns of

the *pnt* genes and the 10kb upstream and 20kb downstream regions of the *pnt* locus have not been screened (Figure 4.1), therefore, these sequences represent good candidates to look for putative CRRs controlling expression of *pnt* in the dorsal-anterior domain.

We can observe that the region upstream of the *pntP2* promoter is highly conserved but contain the *DNA polymerase epsilon subunit 1* gene. The sequence may therefore be conserved because of the presence of the gene rather than the CRR. The 10kb sequence downstream of the *pnt* gene is also highly conserved but contain the *cdc16, CG6763, wam* and *bb8* genes. The sequence more downstream is less conserved but seems to show similar levels of conservation compared to pnt^{45D11} and pnt^{43H01} but does span the *orb* gene. Because of the presence of these genes upstream and downstream of the *pnt* locus, it is difficult to rely on conservation to design reporter constructs of putative CRRs. Nevertheless, the relatively small size of the sequence to screen (around 30kb) could allow to make a series of derivative constructs of around 3-4kb to span the regions of interested to look for putative CRRs controlling expression of *pnt* in the dorsal-anterior domain.

Finally, an important caveat about this approach is the risk for false-negative, where a reporter containing a real CRR does not produce expression. A reason for this to occur is that some CRRs only interact with specific promoters (Juven-Gershon and Kadonaga, 2010; Suryamohan and Halfon, 2015), which in the assay that we are using is not taken into account. It would therefore be possible that some of CRR reporter lines tested contain a CRR controlling expression of Pnt in the dorsal-anterior that we have not detected using the promoter in our reporters.

4.2.2.2 Is Pnt a direct target of Cic ?

In the previous chapter, we have shown that Pnt expression is regulated by Cic. We observed that losing *cic* is sufficient to induce expression of Pnt which suggest there are no other

regulators of *pnt* transcription in the dorsal-anterior. In addition, *pnt* has been shown to be a direct target of Cic in the intestinal stem cells (Jin *et al.*, 2015) and in the wing disc (Pascual *et al.*, 2017). I have presented evidence toward that the hypothesis that the Pnt isoform regulated by Cic is PntP2. Therefore, an interesting region to look for a CRRs controlling expression of Pnt in the dorsal-anterior would be in the 10kb sequence upstream of the *pntP2* promoter.

However, the Cic binding sites identified in the *pnt* locus in the two studies mentioned above are all located within the introns and in the sequences included in the CRR reporters screened (De Vito, 2021; Revaitis *et al.*, 2017). This could favor the hypothesis of a false negative result in our screen and that the CRR(s) controlling expression of Pnt in the dorsal-anterior need to interact with the *pntP2* promoter.

4.2.2.3 Is Pnt regulating its own expression ?

Finally, if my data suggest that PntP2 is the isoform induced in the dorsal-anterior, it was reported that *pntP1* also is expressed in the dorsal-anterior (Morimoto *et al.*, 1996; Yakoby *et al.*, 2008). It is currently unknown how PntP1 expression is controlled in this domain. As mentioned above, my data suggest that only PntP2 expression is controlled by Cic, which would suggest that PntP1 is regulated by another factor. In the developing eye, it was shown that activation of PntP2 leads to transcription of PntP1 (Shwartz *et al.*, 2013). This mechanism could explain how PntP1 gets expressed in the dorsal-anterior. To test this hypothesis, one could take advantage of a Pnt-GFP fusion protein (Boisclair Lachance *et al.*, 2014) and test whether ectopic activation of PntP2 could induce expression of the Pnt-GFP. Because this Pnt-GFP rescues loss of Pnt, it is impossible to do a loss of *pnt* function experiment with the GFP fusion protein. Interestingly, if this PntP2 induces expression of PntP1 in the dorsal-anterior, it would not be able to sustain its own expression

because Pnt expression is lost in the dorsal-midline after stage 10B (Morimoto *et al.*, 1996; Yakoby *et al.*, 2008), potentially due to derepression of Cic (Astigarraga *et al.*, 2007).

It was proposed that the induction of PntP1 by PntP2 allows for sustained EGFR signalling after the ligand has been removed (Shwartz *et al.*, 2013). Therefore, because, in the posterior, Mid and Pnt stay expressed after the migration of Grk toward the dorsal-anterior, I hypothesized that posterior Pnt could regulates its own expression. However, preliminary data suggests that it is not the case. Indeed, clones homozygous for the null allele $pnt^{\Delta 88}$ (Scholz *et al.*, 1993), do not lose expression of the $pnt^{45D11.1}$ and $pnt^{43H01.1}$ reporters (37/48 for $pnt^{45D11.1}$, 32/35 for $pnt^{43H01.1}$, Figure 4.2A-B'). Conversely, clones gaining PntP2 do not exhibit expression of any of the two reporters (0/41 for $pnt^{45D11.1}$, 0/77 for $pnt^{43H01.1}$, Figure 4.2C-D'). This is suggesting that Pnt does not regulates it own expression in the posterior. However, I could not test the response of the CRRs to gain of PntP1.

4.3 How are signals from the EGFR signalling pathway transduced to establish posterior fate and regulate Pnt expression ?

4.3.1 Does the CicL isoform regulates Pnt expression and posterior fate ?

Previously, we have presented evidence suggesting that Pnt is not regulated in the posterior by Cic. However, the null allele used or the RNAi in the loss of function clones did not distinguish between the two Cic isoforms and the gain of function clones only expressed a non-repressible form of the short Cic isoform (CicS). Unlike the long Cic isoform (CicL), CicS contains a N2 domain which is necessary for Groucho (Gro) mediated repression of Cic target genes (Forés *et al.*, 2015; Jiménez *et al.*, 2000). Gro is a transcriptional corepressor whose activity is inhibited through EGFR mediated phosphorylation by MAPK (Cinnamon et al., 2008; Cinnamon and Paroush, 2008; Paroush et al., 1997).

It was shown that Cic function independently of Gro in the dorsal-anterior. Indeed, unlike clones lacking *cic*, clones lacking Gro do not exhibit ectopic expression of *mirr*, indicating that Cic does not require Gro to repress expression of *mirr* (Forés *et al.*, 2015). However, we do not know if this is also true in the posterior domain. If Gro is working with Cic to control expression of *pnt* in the posterior, then clones expressing CicS$^{\Delta C2}$-HA would not be able to repress *pnt* expression, because Gro would be repressed by MAPK (Cinnamon *et al.*, 2008). To test this hypothesis, we could generate clones expressing a non-repressible form of Gro. Cinnamon et al. have generated a non-repressible Gro transgene (Gro^{AA}) in which the residues phosphorylated by MAPK are replaced with alanine residues, preventing phosphorylation (Cinnamon *et al.*, 2008). If Cic requires Gro to repress *pnt* expression in the posterior, then expressing a non-repressible form of Gro will lead to loss of *pnt* expression.

Alternatively, the CicL isoform does not contain the N2 domain, suggesting that it represses targets without needing Gro. Expressing a non-repressible form of CicL (CicL$^{\Delta C2}$) in the dorsal-anterior leads to loss of Mirr expression (Gerardo Jimenez, personal communication). Therefore, if *pnt* is regulated in the posterior by Cic, generating clones expressing CicL$^{\Delta C2}$ will lead to repression of Pnt in the posterior. This would test whether Pnt is regulated by the CicL isoform and will also indicate if Pnt is repressed without input from Gro.

A potential caveat with the experiment with the Cic transgene lacking the C2 domain came from an observation made by Jin et al. that showed that despite keeping nuclear localization in presence of EGFR signalling, nuclear foci of Cic staining normally observed in the absence of EGFR signalling are no longer detected and staining of CicS$^{\Delta C2}$ is homogenous within the nuclei. This is suggesting that there would be other MAPK dependent mechanisms of Cic regulation that would not involve the C2 domain. Jin et al. hypothesized that Cic$^{\Delta C2}$ dissociates from the chromatin

but is not excluded from the nucleus (Jin *et al.*, 2015). A MAPK mediated repression of Cic that does not involve exclusion from the nuclei has recently been described in which Cic targets are derepressed before Cic is excluded from the nucleus (Keenan *et al.*, 2020). In fact, unlike the CicS isoform, CicL is not excluded from the nucleus in cells experiencing EGFR activity despite still being repressed, which can be observed by derepression of Cic target genes (Gerardo Jimenez, personal communication). This regulation that does not involve exclusion from the nucleus may explain why expressing $CicS^{\Delta C2}$ under the endogenous *cic* promoter does not affect Mirr expression (Astigarraga *et al.*, 2007) or Pnt (data not shown). The levels of expression may be too low to overcome the C2 independent inhibition, which is not the case with MARCM clones that drive much higher levels of expression (Lee and Luo, 2001).

4.3.2 Are there other factors regulating posterior fate and Pnt expression ?

Alternatively, it is possible that factors other than the canonical downstream effectors of EGFR signalling play a role in the establishment of posterior fate. Another determinant of posterior fate in the follicular epithelium is the Hippo (Hpo) signalling pathway (Edgar, 2006; Halder and Johnson, 2011). Posterior follicle cells lacking Hpo, a kinase that is a core component of the Hpo signalling pathway, do not adopt posterior fate (Meignin et al., 2007; Polesello and Tapon, 2007). These cells also do not express a *pnt* enhancer trap and expression is lost in a cell autonomous manner. This is suggesting that *pnt* expression in the posterior requires input from the Hpo signalling pathway.

Another phenotype observed in clones lacking Hpo is an over proliferation leading to multilayering of the epithelium in the posterior domain. This is thought to happen through the Notch signalling pathway, because expression of Hindsight, a downstream effector of Notch is lost in clones lacking Hpo (Polesello and Tapon, 2007). In addition, ectopic expression of Notch intra-

cellular domain (NICD), which causes activation of Notch signalling pathway, rescues the phenotypes observed in *hpo* mutant clones (Yu et al., 2008). Lack of Notch signalling could prevent posterior follicle cells from exiting the mitotic cycle (Sun and Deng, 2007). However, it is still unknown why only posterior follicle cells are affected in *hpo* mutant clones.

Because Upd requires Notch signalling to be expressed (Assa-Kunik et al., 2007), it is possible that cells cannot adopt posterior fate due to the absence of Upd. However, activity of the JAK/STAT signalling is not affected in posterior *hpo* mutant clones, expression of a reporter of STAT92E activity (Bach et al., 2007; Polesello and Tapon, 2007) and *dome-lacZ*, which is expressed in response to Upd (Xi *et al.*, 2003; Yu *et al.*, 2008) are still detected. Interestingly, it was reported than Upd expression and migration of border cells are affected in anterior clones unable to respond to the Hpo signalling pathway, suggesting unlike what was previously reported (Meignin *et al.*, 2007; Polesello and Tapon, 2007), the Hpo signalling pathway is active in anterior follicle cells and it affects Upd expression differently between the posterior and the anterior (Lin et al., 2014).

Finally, because the Hpo and EGFR signalling pathways are both important to establish posterior fate, Polesello and Tapon asked whether the Hpo signalling pathway is acting downstream of the EGFR pathway. They found that the Hpo and EGFR signalling pathways work in parallel by generating clones mutant for *warts* and *ras*, two components of the Hpo and EGFR signalling pathway respectively. In clones lacking *ras*, Dystroglycan (DG) is upregulated and FasIII expression is unaffected. In clones lacking *warts*, DG expression is unaffected and FasIII is upregulated. In clones lacking both *ras and warts*, both DG and FasIII are upregulated, suggesting that the Hpo and EGFR signalling pathways act in parallel to induce posterior fate. However, they have only looked at Dystroglycan and FASIII expression, which are only affected by the EGFR

and Hpo signalling pathways respectively but have not addressed the fact that *pnt* is regulated by both pathway (Polesello and Tapon, 2007; Poulton and Deng, 2006). The fact that Pnt seems to be a target of the EGFR, Hpo, and JAK/STAT signalling pathways in the posterior suggest that these pathways could work together to induce posterior fate, despite what the results from Dystroglycan and FASIII staining indicates. Pascual et al. have recently described that the Hpo signalling pathway influences outcome of EGFR signalling by controlling expression of Pnt and Cic in the wing disc (Pascual *et al.*, 2017). In this tissue, production of Upd is also controlled by the Hpo signalling pathway but it seems to not be the case in the posterior domain of the follicular epithelium (Polesello and Tapon, 2007; Yu *et al.*, 2008). Interestingly, Pnt is central in the crosstalk between the three signalling pathways (Pascual *et al.*, 2017), similarly to what we have described here. The role of the Hpo signalling pathway in the establishment of posterior fate and how it interacts with the EGFR and JAK/STAT signalling pathways remain poorly understood. Studying this potential interaction could give insight in the mechanisms patterning the follicular epithelium.

4.3.3 Is Pnt indirectly regulated by EGFR signalling in the posterior ?

One of the goals of this project was to characterize the mechanisms used by the EGFR signalling pathway to induce *mid* expression and posterior fate. We have found that the EGFR effector Pnt is regulating *mid* expression and is sufficient to induce posterior fate, but we have not identified how *pnt* expression is controlled by EGFR signalling in the posterior. A hypothesis that we have not explored here is the possibility that Pnt is indirectly regulated by EGFR signalling in the posterior.

Posterior clones lacking *egfr* adopt anterior fate and lose expression of a *pnt* enhancer trap (Gonzalez-Reyes and St Johnston, 1998). However, loss of Pnt does not lead to anterior fate (Stevens et al., 2020) and we observed that Mid is not completely lost in clones lacking Pnt.

Interestingly, we and others observe that clones unable to response to JAK/STAT or Hpo signalling also do not adopt anterior despite losing posterior identity (McGregor *et al.*, 2002; Meignin *et al.*, 2007; Polesello and Tapon, 2007; Xi *et al.*, 2003; Yu *et al.*, 2008). Clones unable to response to JAK/STAT signalling also only partially lose *mid* expression (Fregoso Lomas *et al.*, 2016). It is unknown whether *mid* expression is lost in clones unable to respond to Hpo signalling but the loss of Pnt and posterior markers observed suggest that Mid would most likely be lost in these clones.

Another characteristic of posterior follicle is the ability to signal back to the oocyte to initiate microtubules rearrangement and migration of the oocyte toward the dorsal anterior. This signal and rearrangement require EGFR signalling in the posterior (González-Reyes *et al.*, 1995). Egg chambers with posterior clones unable to respond to Hpo signalling also have absence of oocyte nucleus migrating (Meignin *et al.*, 2007; Polesello and Tapon, 2007; Yu *et al.*, 2008). Defects in migration are not seen in clones unable to respond to JAK/STAT signalling or lacking Pnt suggesting that the changes in fate with these different mutations are not equivalent. This would suggest that EGFR signalling induces posterior fate through another mechanism that does not include Pnt. It is therefore possible that expression of Pnt results from this change of fate, rather than being the cause of it. In this case, EGFR signalling would make the posterior follicle cells competent to induce expression of Pnt by an unknown mechanism.

4.4 Conclusion

The objective of my project was to describe how the JAK/STAT signalling pathway influences outcome of EGFR signalling in the posterior follicle cells to induce Mid expression. We found that Mid is regulated by JAK/STAT signalling through regulation of the EGFR effector Pnt. In addition, the alternative EGFR target Mirr is directly repressed by through a STAT92E binding

sites. Together, this describes how a mechanism by which a signalling pathway influences outcome of another; by increasing levels of its effector and by repressing alternatives targets.

I found that establishment of posterior fate does not require Cic. However, Cic plays a role in establishing the boundary between the posterior and dorsal-anterior domains, potentially through the regulation of Mirr and PntP2.

Together, my work provides insight in the role of CRRs to capture and integrate inputs from different signalling pathways to generate pattern of gene expression and in the mechanisms used by signalling pathways to determine outcome of signalling and generate bistable systems.

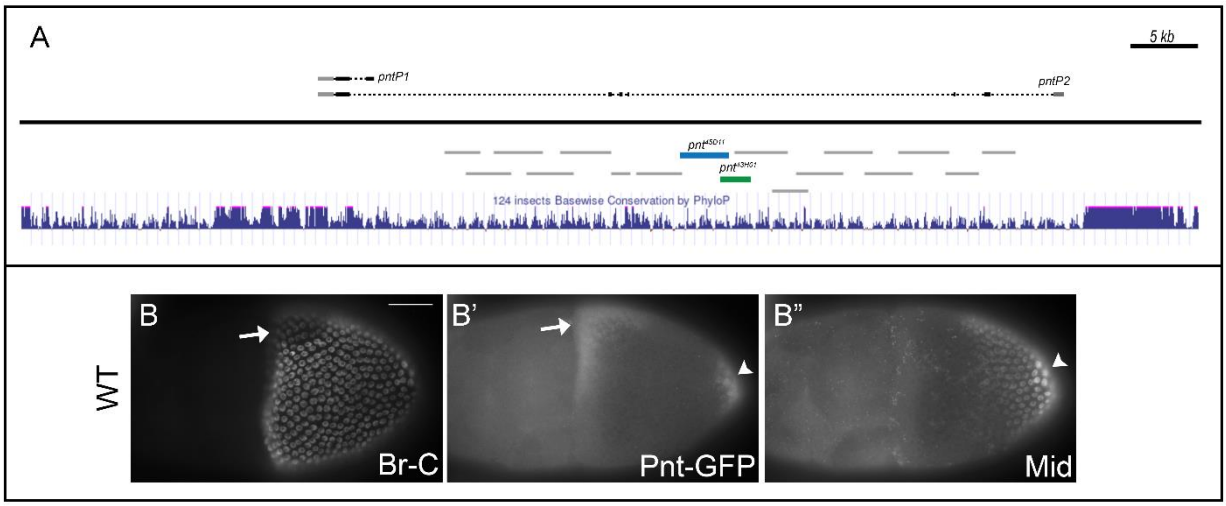
A
5 kb
pntP1
pntP2
124 insects Basewise Conservation by PhyloP
WT
B
Br-C
B'
Pnt-GFP
B''
Mid

Figure 4.1 – Genomic sequences included in the BAC containing Pnt-GFP fusion protein

(A) Map of the sequenced included in the BAC containing the Pnt-GFP fusion protein. Reporter containing putative CRRs with no expression in the follicle cells are shown in gray, pnt^{45D11} and pnt^{43H01} are shown in blue and green respectively. Conversation is shown below. (B) WT egg chamber expressing Pnt-GFP. Pnt-GFP is expressed in the posterior domain, where Mid is also expressed (arrowheads) and in the dorsal midline which correspond to the low Broad domain (arrows). Scale bar indicates 50μm.

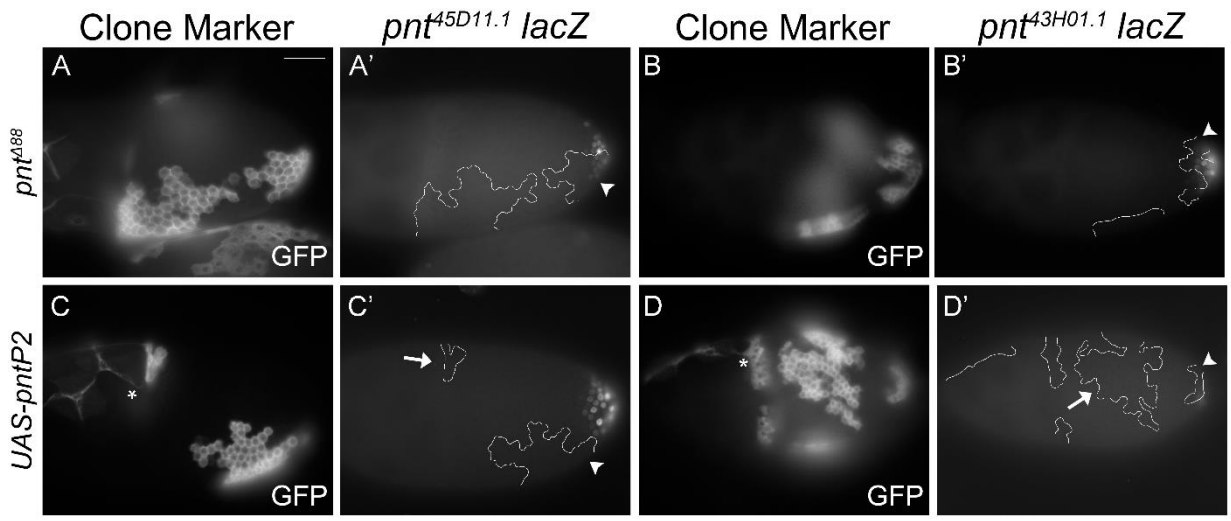
Clone Marker
pnt45D11.1 lacZ
Clone Marker
pnt43H01.1 lacZ
pntΔ88
UAS-pntP2
A
A'
B
B'
C
C'
D
D'
GFP
GFP
GFP
GFP

Figure 4.2 – Response of the *pnt*$^{45D11.1}$ and *pnt*$^{43H01.1}$ CRRs reporters to Pnt

(A-A') Expression of the *pnt*$^{45D11.1}$ *lacZ* reporter is not affected in loss-of-function *pnt*$^{\Delta 88}$ clones (arrowhead). (B-B') Expression of the *pnt*$^{43H01.1}$ *lacZ* reporter is not affected in loss-of-function *pnt*$^{\Delta 88}$ clones (arrowhead). (C-C') Expression of the *pnt*$^{45D11.1}$ *lacZ* reporter is not affected in clones expressing UAS-*pntP2* in the posterior (arrowhead) or in the anterior (arrow). (D-D') Expression of the *pnt*$^{43H01.1}$ *lacZ* reporter is not affected in clones expressing UAS-*pntP2* in the posterior (arrowhead) or in the anterior (arrow). All clones are positively marked with GFP. Scale bar = 50μm for all panels.

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