



Bases moleculares de la especificación del patrón dorso-ventral en *Drosophila*

María José Andreu Sauqué

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TESIS DOCTORAL

**BASES MOLECULARES DE LA ESPECIFICACIÓN
DEL PATRÓN DORSO-VENTRAL
EN *DROSOPHILA***

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Departamento de Genética
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Memoria presentada por

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Para optar al grado de

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Dr. Florenci Serras

A mi abuelo Buenaventura

“The secrets that engage me -that sweep me away- are generally secrets of inheritance: how the pear seed becomes a pear tree, for instance, rather than a polar bear”.

Cynthia Ozick

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INDEX

Abbreviations	1
Introduction.....	3
1. Cell signaling during development	3
2. Receptor tyrosine kinase signaling pathways	4
3. Determination of the body axes during oogenesis	7
3. 1. Specification of the AP axis during oogenesis	9
3. 2. Specification of the DV axis during oogenesis	9
3. 2. 1. Patterning of the dorsal structures of the eggshell	10
3. 2. 2. Patterning of the DV axis of the future embryo	11
4. Establishment of the body axes in the embryo	13
4. 1. Subdivision of the AP axis and the terminal system in the embryo	13
4. 2. Subdivision of the DV axis of the embryo	15
5. Function of Cic during development	17
5. 1. Cic downstream of EGFR signaling in the establishment of the DV axis	18
5. 2. Cic downstream of Torso signaling in the embryo	22
5. 3. Other possible roles of Cic downstream of EGFR signaling	23
5. 4. Role of Cic in human disease	24
Objectives	27
Publications	29
Informe sobre la contribución de la doctoranda	29
Informe sobre el factor de impacto de las publicaciones	33
Article 1. Mirr represses <i>pipe</i> expression in follicle cells to initiate dorso-ventral axis formation in <i>Drosophila</i>	35
Article 2. EGFR-dependent downregulation of Capicua and the establishment of <i>Drosophila</i> dorso-ventral polarity.....	43
Article 3. Capicua DNA-binding sites are general response elements for RTK signaling in <i>Drosophila</i>	51
Article 4. Gene Regulation by MAPK Substrate Competition	67
Discussion	83
1. Mechanism of DV axis polarization by EGFR signaling	84
1. 1. Mirr mediates cell-autonomous repression of <i>pipe</i> in dorsal follicle cells	85
1. 2. Cic supports pipe expression by repressing <i>mirr</i>	86

Index

1. 3. Significance of EGFR-dependent downregulation of Cic	88
2. Cic as a general sensor downstream of RTK signaling	90
3. Gene regulation during DV axis formation by MAPK substrate competition	93
4. Role of Cic in Dorsal-mediated repression downstream of Torso signaling	94
Conclusions	97
Bibliography	99
Summary in Spanish	113

ABBREVIATIONS

Abbreviation	Full name
BMP	Bone morphogenetic protein
DNA	Deoxyribonucleic acid
ETS	E-Twenty Six
<i>fet</i>	<i>fettucine</i>
HMG	High mobility group
MAPK	Mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
RNA	Ribonucleic acid
TGF- α	Transforming Growth Factor α
TGF- β	Transforming Growth Factor β
Wnt	Wingless-type
<i>wt</i>	wild-type

INTRODUCTION

Animal embryogenesis is a precise and complex process by which a single fertilized egg cell multiplies and differentiates into a mature individual composed of a wide variety of specialised organs, tissues and cell types. In *Drosophila*, as in other metazoans with bilateral symmetry, this process involves the establishment of two body axes: the antero-posterior (AP) and the dorso-ventral (DV) axes. They are already partly set up in the *Drosophila* egg, and become fully established in the early embryo, when it is still at the syncytial blastoderm stage. Genetic networks and signaling pathways with maternal and zygotic functions mediate the patterning of both axes. The maternal effect genes expressed in the mother's ovaries produce messenger RNAs (mRNAs) that are localized in different regions of the egg providing a framework of positional information which, after fertilization, is interpreted by the embryo's own genetic program. Zygotic genes act hierarchically and in a strict temporal sequence to establish broad regional differences, which are then refined to produce smaller developmental domains with a unique profile of gene activity. Thus, initial asymmetries arisen during oogenesis together with the subdivision of the early embryo by zygotic genes permit the establishment of the basic body plan of the future animal.

1. Cell signaling during development

Only a small fraction of all genes in a given animal constitute the toolkit that is required for the formation and patterning of the body plan. Two main classes of gene products constitute the genetic toolkit for development: transcription factors, which regulate the expression of other genes, and members of signaling pathways, which mediate short- and long-range interactions between cells. These genes are shared across eukaryotes although some of them have been modified during evolution; for example, they have gone through gene duplication and acquired new roles in development. Therefore, it is the regulated expression of specific transcription factors and cell signaling events what controls the formation, identity and patterning of most major features of the animal design. Animals rely on a small number of signaling pathways but these are used repeatedly during metazoan

Introduction

development: TGF β /BMP, Notch, Wnt/ β catenin, Hedgehog and Receptor Tyrosine Kinase (RTK) signaling among others.

In this thesis, we are focusing on RTK signaling and how it controls gene expression. We study the specification of *Drosophila* DV axis as a model where RTK signaling regulates region-specific expression of genes, which leads to cell fate determination.

2. Receptor tyrosine kinase signaling pathways

The ability of cells to receive, process and respond to information is essential for all those already mentioned processes. Signals may come from the environment or from other cells. In order to trigger a response, these signals must be transmitted across the cell membrane. Sometimes the signal itself can cross the membrane and other times the signal works by interacting with receptor proteins that contact both the outside and inside of the cell. Once a receptor protein receives the signal and is activated, it triggers intracellular signaling cascades that will eventually elicit a cellular response.

RTK signaling pathways regulate many biological processes in all metazoans from embryonic development to adult homeostasis. They are important in the control of cell proliferation, differentiation, metabolism, migration and survival. In addition, abnormal RTK signaling has been linked to multiple human syndromes and diseases including cancer.

The RTK family shares the same basic structure. All the receptors possess an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmatic domain, which contains the kinase activity. RTKs are activated through ligand-induced oligomerization, typically dimerization, which triggers auto- or trans-phosphorylation of tyrosine residues in their intracellular domains. Phosphorylated tyrosines on the receptor are then recognized by adaptor proteins that induce intracellular signaling cascades. RTKs signal through the activation of the small GTPase Ras, followed by serial activation of the protein kinases Raf, MAPKK/Dsor and MAPK/Rolled; or through the phosphatidyl-inositol-3-kinase (PI3K) and phospholipase C γ (PLC γ) pathways (Ullrich and Schlessinger, 1990).

The most prominent RTK signaling pathway is the Ras/MAPK pathway, which is highly conserved in eukaryotes and controls multiple developmental processes (Figure 1). Ras/MAPK signaling can alter the activities of transcription factors by phosphorylation in many ways which include control of their cellular localization, expression and protein stability; but also modulating their ability to remodel chromatin structure or to bind to DNA or transcriptional co-regulators (Whitmarsh, 2007). In *Drosophila*, the best-characterized effectors of the Ras/MAPK pathway belong to ETS transcription factor superfamily: the activator Pointed and the repressor Yan (Brunner et al., 1994; Rebay and Rubin, 1995; Tootle and Rebay, 2005). However, additional transcription factors have emerged as mediators of RTK signaling. In particular, the HMG-box factor Capicua (Cic) has been shown to function downstream of two different RTK/Ras/MAPK pathways, the Torso and EGFR signaling pathways, in different contexts (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Astigarraga et al., 2007; Tseng et al., 2007).

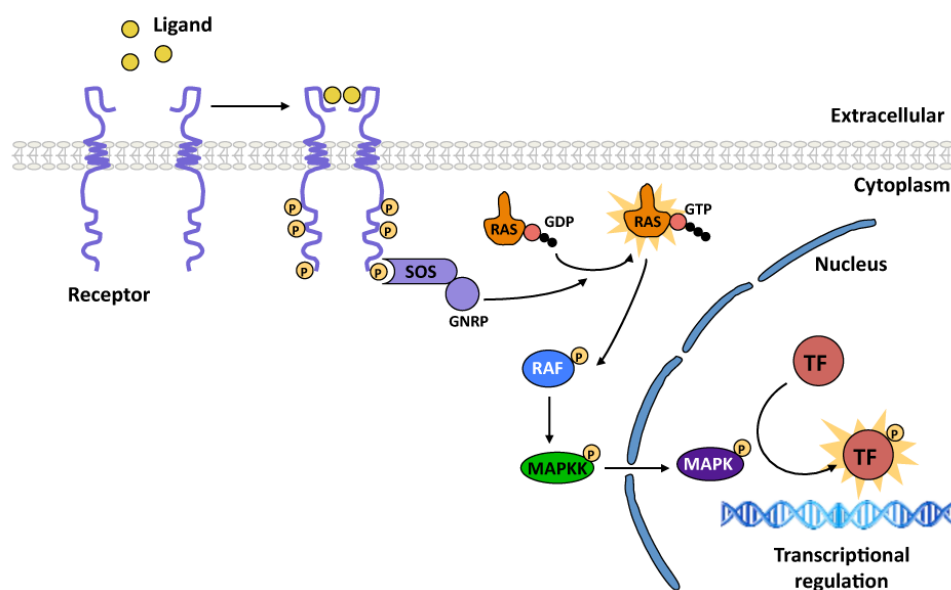


Figure 1. General scheme of RTK/Ras/MAPK signaling pathway. Ligand binding induces receptor dimerization and causes auto-phosphorylation of its tyrosine residues. The adaptor protein, SOS, recognizes the phosphorylated tyrosines on the RTK and activates an intermediate protein, GNRP, which promotes the exchange of GDP for GTP from the Ras G protein. Following Ras activation a serial phosphorylation of the protein kinases Raf, MAPKK and MAPK is triggered. Eventually, activated MAPK enters the nucleus and phosphorylates certain transcription factors which alter gene expression in the responding cell. (Adapted from Gilbert, 2003)

Introduction

Strikingly, although the same Ras/MAPK cassette is used in multiple developmental contexts, these signals are interpreted to produce a wide variety of cellular responses. The molecular mechanisms that provide signaling specificity to the RTK/Ras/MAPK signaling pathway have been a subject of study for years. One possible mechanism is based on the differential signaling kinetics: different cellular responses can be induced by altering the duration or strength of the activation of downstream signaling components (Traverse et al., 1994; Marshall, 1995; Sabbagh et al., 2001; Ebisuya et al., 2005). Different kinetics can be explained by intrinsic differences of distinct receptors or differences in the signaling triggered by distinct ligands of the same receptor (Figure 2A). A second aspect is the integration of multiple signaling pathways that converge to regulate a particular function in the cell. Different signaling pathways are able to combinatorially act to give specific cellular outcomes. This interaction can be at the level of the promoter of the target gene, at the level of signaling proteins or at the level of protein complexes (Szüts et al., 1998; Kretzschmar et al., 1997; Hill et al., 1995; Chuderland, 2005) (Figure 2B). Another mechanism that contributes to the specificity of the cascade is the formation of multiprotein complexes due to interaction with scaffold proteins. Different scaffolds can direct signaling components to regulate distinct processes by recruiting proteins belonging to a specific signal transduction event, controlling the localization of the cascade components or providing better stability to some of them to determine the threshold of signaling (Kolch, 2005; Jaeschke et al., 2004; Teis et al., 2002) (Figure 2C). Finally, specificity can also be conferred through the use of specific downstream effectors. Distinct tissues may express different proteins that respond to the same upstream signaling pathway to generate unique responses (Figure 2D). Likely, it is the combination of all different mechanisms what eventually induces the specific cellular phenotype.

In the next sections, we are focusing in the transcriptional responses mediated by EGFR and the Torso signaling pathways during *Drosophila* development. Specifically, we are interested in how localized EGFR signaling determines dorsal cell fate and leads to DV polarity in the ovary, which is crucial for embryonic DV patterning. In addition, we will study how activation of Torso signaling at the poles of the early embryo specifies both terminal regions.

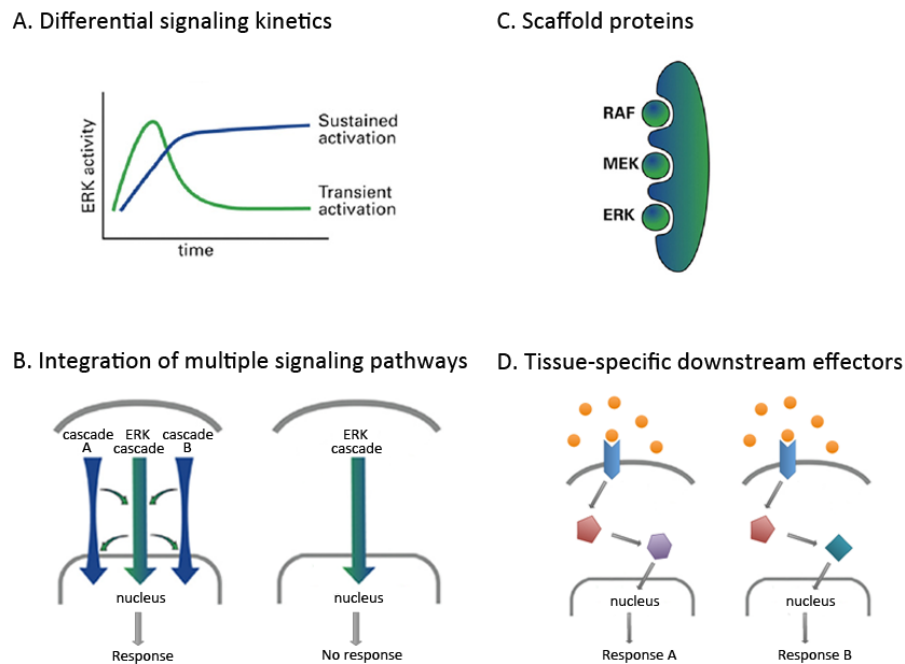


Figure 2. Schematic representation of mechanisms that determine the signaling specificity of the RTK signaling cascade. (A) Differences in the strength or the duration of the activation of a common signaling pathway might generate different cellular outcomes. **(B)** Different signaling pathways are able to combinatorially act to give specific responses. **(C)** Different scaffolds can direct signaling components to regulate distinct processes **(D)** Distinct tissues may express different proteins that generate unique cellular responses to the same upstream signaling pathway. (Adapted from Shaul and Seger, 2007)

3. Determination of the body axes during oogenesis

Specification of AP and DV axes in *Drosophila* begins in the female ovaries during oogenesis and involves localized activation of EGFR signaling. In both cases, axial polarization arises as a consequence of cell-to-cell signaling events between the oocyte and the somatic cells that surround it (Schüpbach, 1987). In order to understand these interactions, the main anatomic and functional features of the process of oogenesis are described below.

Introduction

A female *Drosophila* has two ovaries made up of approximately 18 ovarioles. Each ovariole is composed of 6-7 follicles or egg chambers at different stages of maturation, each one containing a single oocyte (Figure 3A). At the anterior end of the ovariole, there is the germarium that contains somatic and germline stem cells. Germline stem cells divide asymmetrically to produce another stem cell and a daughter cell, a cystoblast, which begins to differentiate. The cystoblast undergoes four mitotic divisions with incomplete cytokinesis to form a cyst of 16 cells interconnected to one another by cytoplasmic bridges known as ring canals. One of these cells moves to the posterior pole of the cluster and becomes the oocyte, while the other 15 cells adopt a nurse cell fate. Nurse cells synthesize nutrients and cytoplasmic components which are transported into the oocyte. On the other hand, somatic stem cells produce follicle cells that, coincident with the oocyte determination, migrate to form an epithelial monolayer that surrounds the germline cyst. This whole group of germline and somatic cells constitute the ovarian follicle or egg chamber which develops during 14 different stages. Egg chambers mature as they pass down the ovariole, reaching the posterior as mature eggs competent for fertilization (Figure 3B).

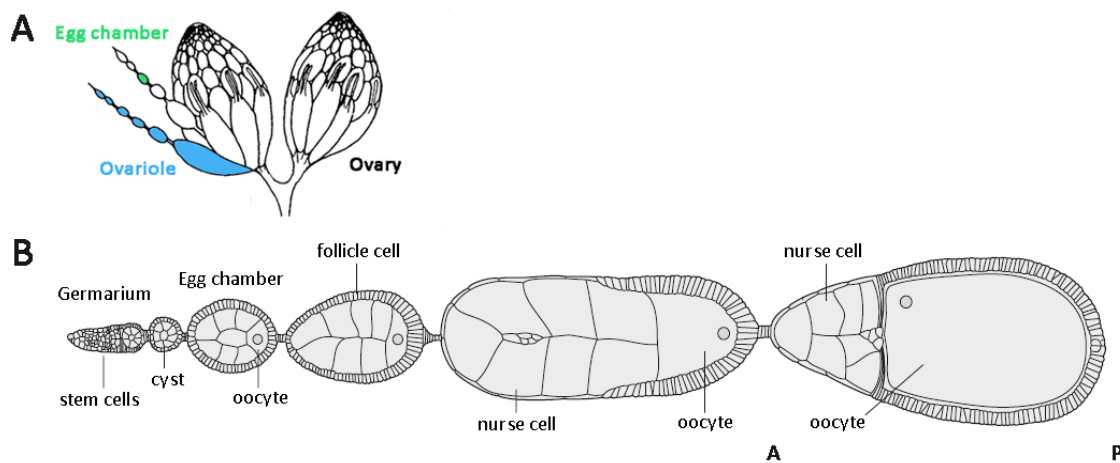


Figure 3. (A) Drawing of *Drosophila* ovaries and their organization. Females have two ovaries made up of 18 ovarioles each one. Each ovariole is composed of 6-7 egg chambers. **(B)** Drawing of the germanium and early stages of oogenesis. The youngest egg chambers are localized closer to the germanium.

3.1. Specification of the AP axis during oogenesis

During egg chamber formation, the oocyte-nurse cells cluster is polarized so that the oocyte is positioned posterior to the nurse cells. At stage 4 of oogenesis, the posteriorly localized oocyte produces a secreted TGF α -like growth factor, called Gurken (Grk). Grk accumulates surrounding the oocyte nucleus and, after secretion, acts as a ligand of the receptor tyrosine kinase EGFR (Epidermal Growth Factor Receptor) in the adjacent follicle cells membrane inducing them posterior cell fate (González-Reyes et al., 1995; Roth et al., 1995). Afterwards, during stage 6, posterior follicle cells generate a polarizing signal back to the oocyte that results in the reorganization of the cytoskeleton and the localization of essential determinants for AP polarity in the egg such as *bicoid* and *oskar* mRNAs in anterior and posterior regions, respectively. Likewise, polarization of the microtubules will lead to the movement of the nucleus of the oocyte to the future dorsal-anterior (DA) region. This is the initial step for the establishment of DV polarity in the egg chamber.

3.2. Specification of the DV axis during oogenesis

DV patterning starts during mid-oogenesis (stage 8) when the oocyte nucleus migrates from the posterior region to the DA corner, breaking its DV symmetry. This migration depends on the previous repolarization of the oocyte microtubules network after AP axis is specified. Local translation of *grk* mRNA associated with the nucleus leads to accumulation of the protein at the DA region of the oocyte (Neuman-Silberberg and Schüpbach, 1993). As a consequence, secreted Grk activates EGFR only on the overlying cells, as it happens in the establishment of the AP polarity (González-Reyes et al., 1995; Roth et al. 1995). EGFR activation initiates the intracellular Ras/Raf/MAPK cascade within follicle cells, which induces dorsal fate in that region of the follicular epithelium and leads to changes in gene expression necessary for regulation of two processes: formation of the dorsal structures of the chorion and establishment of DV polarity of the early embryo (Peri et al., 1999). Both branches of EGFR signaling in dorsal follicle cells are discussed below.

3.2.1. Patterning of the dorsal structures of the eggshell

The patterning of the follicular epithelium is reflected in the eggshell that it produces, which exhibits pronounced axial asymmetries in its shape and in the presence of specialized structures, such as two dorsal respiratory appendages. Two main signaling pathways are responsible for eggshell patterning, EGFR and BMP (Deng and Bownes, 1997; Peri and Roth, 2000; Dobens and Raftery, 2000; Berg, 2005). On the one hand, localized activation of EGFR by Grk is essential for the determination of two distinct primordia that will produce the two eggshell appendages and also for their proper positioning along the DV axis (Schüpbach, 1987; Queenan et al. 1997; Peri et al., 1999). On the other hand, Decapentaplegic (Dpp), a BMP/TGF- β ligand, is produced by anterior-most follicle cells and regulates patterning along the AP axis (Twombly et al., 1996; Deng and Bownes, 1997; Peri and Roth, 2000). Initially, EGFR signaling reaches a broad field of DA cells and ultimately leads to the establishment within this field of two dorsolateral appendage primordia separated by a midline region. This pattern has been proposed to arise through a positive feedback loop in which Grk/EGFR signal leads to the activation of *rhomboid* (*rho*), an intracellular protease that acts activating Spitz (Spi). Spi is a diffusible TGF α -like ligand of EGFR, which amplifies EGFR initial signal in a broad DA zone. High levels of EGFR activity at the dorsal midline, in turn, induce the expression of an inhibitor of Spi, Argos (Aos). As a result of these positive and negative feedback loops, two lateral peaks of EGFR signaling are generated defining the regions where the respiratory appendages will form (Wasserman and Freeman, 1998; Klein et al., 2004).

More recently, a new model has been proposed after further studying the follicular epithelium changes during eggshell patterning. According to the new model, DV patterning of follicle cells is generated by spatial information derived directly from Grk signaling without the requirement of the Spi/Aos feedback loop. By contrast, it is postulated that Mirror (Mirr) and Pointed (Pnt), two transcription factors downstream of EGFR signaling, and Sprouty (Sty), an inhibitor of RTK signaling, play distinct roles in follicle cell patterning. Mirr is required for determination of DA cell fate, whereas Pnt is required to generate the dorsal midline region. The width of the midline region is restricted by Sty (Boisclar et al., 2009).

3.2.2. Patterning of DV axis of the future embryo

Regarding DV axis specification in the early embryo, the EGFR dorsalizing signal leads to restriction of *pipe* expression to ventral-most region by a yet unclear mechanism. Expression of *pipe* in ventral follicle cells is the cue that initiates the establishment of polarity in the resultant embryo (Nilson and Schüpbach, 1998; Sen et al., 1998). Pipe acts as a sulfotransferase enzyme involved in the modification of yet uncharacterized extracellular components that are secreted by the follicle cells into the perivitelline space of the forming egg (Zhu et al., 2007; Zhang et al., 2009a). After fertilization, ventrally localized Pipe activity initiates an extracellular proteolytic cascade of four serine proteases: Nudel, Gastrulation-defective, Snake and Easter (Smith and DeLotto, 1994; LeMosy et al., 2001). This serine-protease cascade finally culminates in the processing of the diffusible protein Spätzle, whose carboxyl terminus acts as a ligand of the embryo transmembrane receptor Toll (Hashimoto et al., 1991; DeLotto et al., 1998; Weber et al., 2003). Spatial control of the cascade is regulated by positive and negative intrinsic feedback loops and also by negative regulators as serpins (Morisato, 2001; Dissing et al., 2001; LeMosy et al., 2001; Misra et al., 1998; Ligoixigakis et al., 2003; Hashimoto, 2003). Activated Toll relays the extracellular signal to the embryonic nucleus by regulating the nuclear import of NF- κ B transcriptional factor Dorsal, otherwise sequestered in the cytoplasm by Cactus protein. Therefore, Pipe activity defines the width of an extraembryonic gradient of active Spätzle, which in turn leads to the generation of the intracellular gradient of Dorsal protein along the DV axis of the blastocyst (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Finally, it is the gradient of translocated Dorsal which allows the specification of the different regions of the embryo through activation or repression of zygotic genes (Rusch and Levine, 1996) (Figure 4).

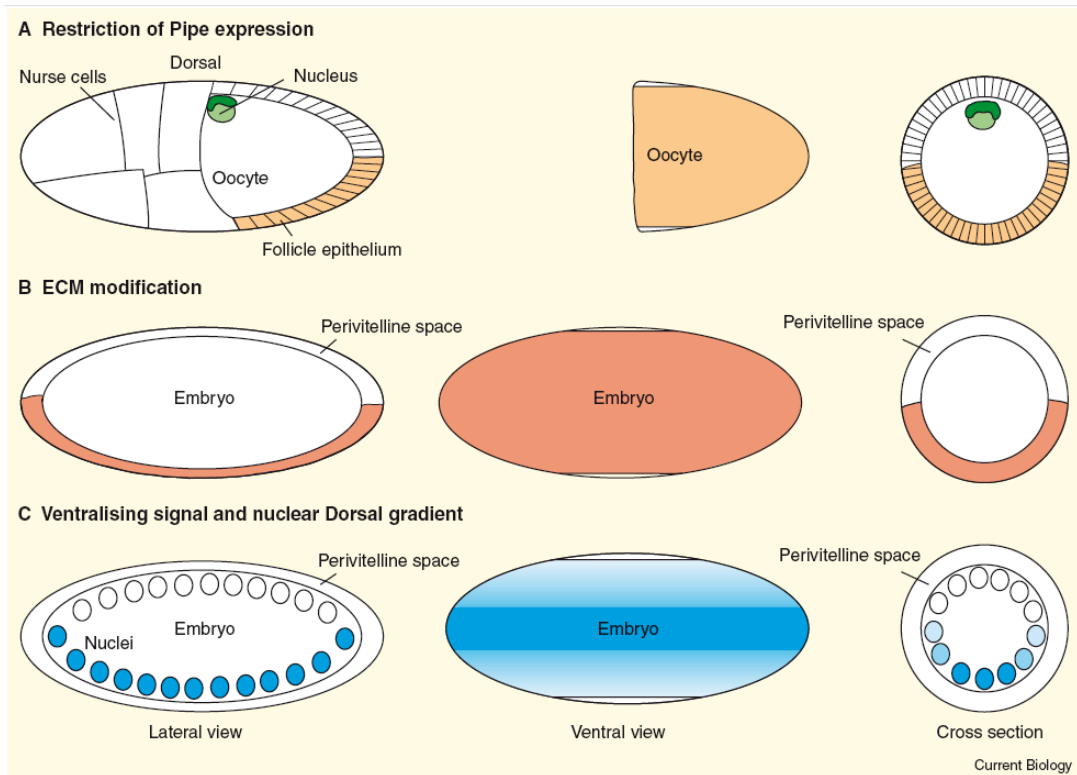


Figure 4. Major events during DV axis formation in *Drosophila*. (A) Grk (green) localized in the DA region of the oocyte is secreted and activates EGFR in the adjacent cells of the follicular epithelium. EGFR signaling restricts pipe expression (orange) to the ventral region. (B) Pipe activity leads to the modification of an unknown ECM component (red) which is secreted by the follicle cells into the perivitelline space of the forming egg. (C) After fertilization, an extracellular signal is generated within the region defined by the modified ECM component. This signal induces the nuclear transport of the transcriptional factor Dorsal. The signal has peak levels close to the ventral midline (dark blue). In the area between the dark blue stripe and the border of the red stripe in B, a gradient is established which accounts for the different ventro-lateral cell fates of the embryo. (From Moussian and Roth, 2005)

How *pipe* transcription is initially restricted to ventral follicle cells has been a long-standing issue. *pipe* gene is the only known gene involved in the induction of the embryonic DV axis which is asymmetrically expressed in the follicular epithelium, it is the key component responsible for transferring DV polarity from the egg chamber to the embryo. It has been shown that activated EGFR represses *pipe* expression cell-autonomously in DA and lateral follicle cells, indicating that it occurs in direct response to EGFR activity and does not require other secondary diffusible signals (Pai et al., 2000; James et al., 2002; Peri et al., 2002). In addition, some studies have reported that Grk forms a long range morphogen gradient from the dorsal side of

the egg chamber to the ventral side, whereas *pipe* exhibits a rather sharp border of expression, suggesting that *pipe* repression probably represents a switch-like response above a certain threshold of EGFR activity (Pai et al., 2000; Goentoro et al., 2006; Chang et al., 2008). In following sections, other factors implicated in the regulation of *pipe* expression will be discussed.

4. Establishment of the body axis in the embryo

Positional information generated during oogenesis is maintained during the early embryo development and leads to the full establishment of the body axis. After fertilization, the zygote nucleus undergoes a series of mitotic divisions without cytokinesis resulting in a syncytial blastomere, where the cytoplasm is already polarized by the localized maternal mRNAs. Along the AP axis the embryo becomes divided into different regions that later give rise to the head, thorax and abdomen of the larva. By contrast, the DV axis is divided into four regions in early embryogenesis from ventral to dorsal: the mesoderm, neuroectoderm, dorsal ectoderm and amnioserosa. The mesoderm will form muscles and other internal connective tissues. The neuroectoderm gives rise to the larval nervous system and the dorsal ectoderm to the larval epidermis. Finally, the amnioserosa is an extra-embryonic membrane on the dorsal side of the embryo which is required for dorsal closure.

In the subsequent two sections, we continue further into AP and DV axis specification in the early embryo.

4.1. Subdivision of the AP axis and the terminal system in the embryo

Three classes of maternal genes specify the AP axis: those that affect the anterior regions, such as *bicoid*; those that affect posterior regions, such as *oskar*; and those that affect both of the terminal regions (St. Johnston and Nüsslein-Volhard, 1992). Localized activity of maternal genes, which form gradients of morphogenetic proteins, generates a specific spatial pattern of zygotic genes expression. There are four main classes of zygotic genes acting along the AP axis in a hierarchy fashion.

Introduction

The gap genes, which are directly regulated by the maternal genes, define broad regional differences that will be refined by the action of the pair rule genes resulting in a periodic pattern of gene expression. The segment polarity genes elaborate the pattern within the segments. Together, the actions of these three classes of genes determine the spatial domain of homeotic selector genes that will define the identities of each segment (Figure 5).

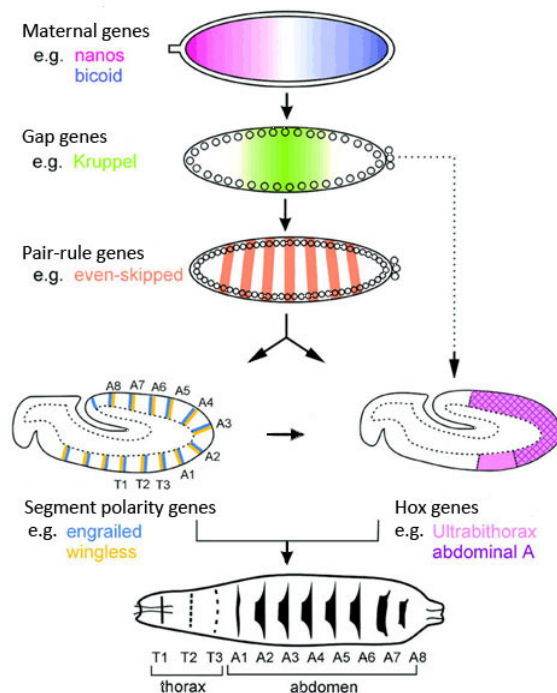
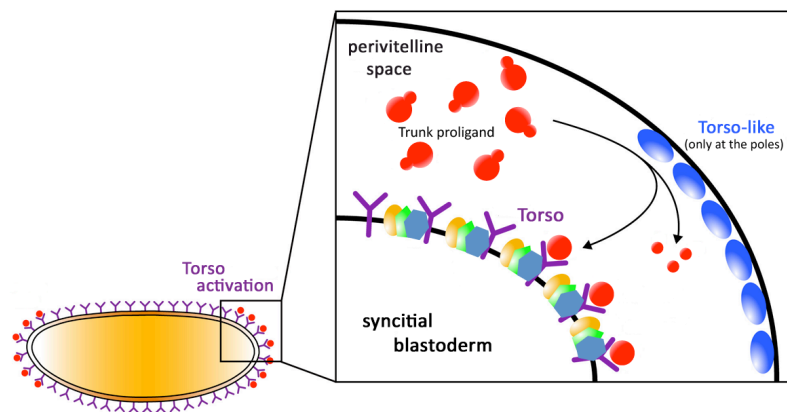


Figure 5. Patterning along the AP axis of the *Drosophila* embryo. The pattern is established by maternal effect genes that form gradients and regions of morphogenetic proteins. These morphogenetic determinants activate the gap genes, which define broad territories in the embryo. The gap genes regulate the expression of the pair-rule genes, each of which divides the embryo into regions about two segments wide. The segment polarity genes then divide the embryo into segment-sized units along the AP axis. Together, the actions of these genes define the spatial domains of the Hox genes that define the identities of each of the segments of the future larva. (From Sanson, 2001)

The terminal gene group is composed by the maternal genes that specify the structures at the extremities of the embryo: the acron and the head region at the anterior end, and the telson and most posterior abdominal segments at the posterior end. Specification of both terminal regions depends on the localized expression of the gap genes *tailless* (*tll*) and *huckebein* (*hkb*) in response to Torso signaling pathway at the poles of the embryo (Pignoni et al. 1990; Brönner and Jäckle, 1996; Duffy and Perrimon, 1994). Both *tll* and *hkb* expression patterns are also regulated by other factors such as Bicoid, which is expressed at the anterior region, and Dorsal, which forms a gradient along the DV axis (Liaw and Lengyel, 1993; Pignoni et al., 1992).

The *torso* mRNA is synthesized by the nurse cells, deposited in the oocyte and translated after fertilization (Sprenger and Nüsslein-Volhard, 1992). Torso is a receptor tyrosine kinase (RTK) evenly distributed throughout the plasma membrane but only activated by Trunk (Trk) at the terminal ends of the syncytial blastoderm (Schüpbach and Wieschaus, 1986; Casanova et al., 1995; Casali and Casanova, 2001). Trk is a factor synthesized by the early embryo and secreted into the perivitelline space. However, the fragment of Trk that acts as a ligand for Torso is generated only at the poles of the embryo, where the processing activity of Torso-like (Tsl) is localized (Savant-Bhonsale and Montell, 1993; Furriols et al., 1998; Casali and Casanova, 2001). The *tsl* gene is expressed only in follicle cells adjacent to the terminal regions of the egg. Tsl protein is secreted and anchored at the two poles of the perivitelline membrane enabling Torso localized activation (Martin et al., 1994; Furriols and Casanova, 2003; Stevens et al., 2003) (Figure 6). Tsl activity and stability at the poles requires the presence of Nasrat, Polehole and Closca proteins at the surface of the oocyte (Jiménez et al., 2002; Stevens et al., 2003; Ventura et al. 2010).

Figure 6. Localized activation of Torso signaling. In the early embryo, Trk is processed only at the poles by Tsl protein to activate the receptor Torso. Tsl localized activity requires the presence of Nasrat, Polehole and Closca proteins at the oocyte surface. (Adapted from LeMosy, 2003)



4.2. Subdivision of the DV axis in the embryo

A gradient of active gradient of Dorsal activity patterns the DV axis of the early embryo by controlling the expression of genes that will determine four regions: mesoderm, neuroectoderm, dorsal ectoderm and amnioserosa. Unlike Bcd, the maternally supplied *dorsal* transcript is distributed and translated uniformly

Introduction

throughout the fertilized egg. However, differential activation of Toll receptor along the DV axis results in a graded translocation of Dorsal protein to the nucleus. As previously exposed, an extracellular proteolytic cascade triggered by Pipe activity transmits the DV polarity of the egg chamber to the early embryo via activation of Toll pathway. This localized activation leads to an intracellular signaling which facilitates the degradation of Cactus, a cytoplasmic tethering protein, thereby releasing Dorsal from cytoplasmic retention (Belvin et al., 1995). On the other hand, recent *in vivo* imaging studies show that exclusion of Dorsal from the nucleus is not achieved by simply preventing Dorsal nuclear import, but by a balance between slow import and rapid export (DeLotto et al., 2007).

Nuclear-localized Dorsal regulates a number of genes in a concentration-dependent manner (Figure 7). High levels in ventral regions activate genes such as *twist* and *snail*, which are required for the specification of the mesoderm (Jiang et al., 1991; Pan et al., 1991; Ip et al., 1992a). Intermediate levels induce expression of genes such as *rhomboid*, *ventral neuroblast defective (vnd)* and *intermediate neuroblast defective (ind)*, important for the specification of the neurogenic ectoderm (Ip et al., 1992b; Stathopoulos et al., 2002). Low levels activate genes like *short-gastrulation* and *thisbe* which are required in lateral domains for patterning the dorsal ectoderm, amnioserosa and dorsal mesoderm (Markstein et al., 2002; Stathopoulos et al., 2002; François et al., 1994; Stathopoulos et al., 2004).

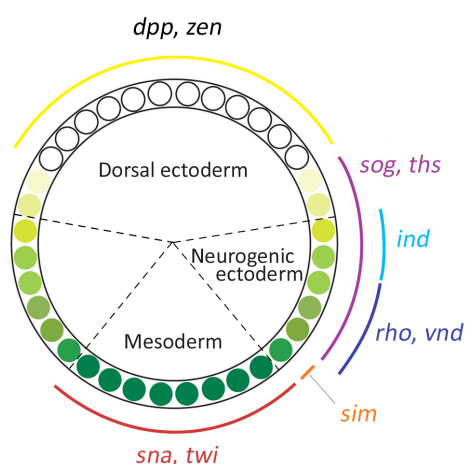


Figure 7. DV subdivision of the embryo. Schematic representation of the embryonic fate map of a cross section of the embryo. The nuclear gradient of Dorsal divides the embryo into three main subtissues: mesoderm, neurogenic ectoderm, and dorsal ectoderm. The neurogenic ectoderm can be further divided into ventral and dorsal halves. (Adapted from Reeves and Stathopoulos, 2009)

Dorsal functions as both an activator of transcription to induce gene expression and a repressor to keep genes silenced (Jiang et al., 1992; Pan and Courey, 1992). The same low levels of Dorsal that activate genes in lateral regions of the embryo also mediate repression of certain targets such as *decapentaplegic (dpp)* and *zerknüllt (zen)*, thereby limiting their expression to regions where nuclear Dorsal is absent (Doyle et al., 1989; Ip et al., 1991; Huang et al., 1993; Jiang et al., 1993; Kirov et al., 1993). These genes are required for patterning of the dorsal ectoderm and amnioserosa, which develops in the dorsal-most regions of the embryo.

5. Function of Cic during development

One mechanism by which RTK/Ras/MAPK signaling elicits its cellular responses is through changes in gene expression. Nuclear factors that are directly phosphorylated by components of the pathway have key roles in the interpretation of the specific response. In the last decade, the HMG-box transcription factor Cic has emerged as a new effector of RTK/Ras/MAPK signaling pathway, which is involved in the regulation of different processes during development (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Atkey et al., 2006; Astigarraga et al., 2007). Cic is conserved from cnidarians to vertebrates with single orthologs in *C. elegans*, mice and humans (Jiménez et al., 2000; Lee et al., 2002; Lam et al., 2006). Both *Drosophila* and mammals express at least two main isoforms (Cic-S and Cic-L), which differ in size and their N-terminal regions (Figure 8). At the present, the best-characterized activities of Cic correspond to the Cic-S isoform. The Cic-L isoform appears to have specific roles in oogenesis (Rittenhouse and Berg, 1995; Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Dorman et al., 2004; Astigarraga et al., 2007).

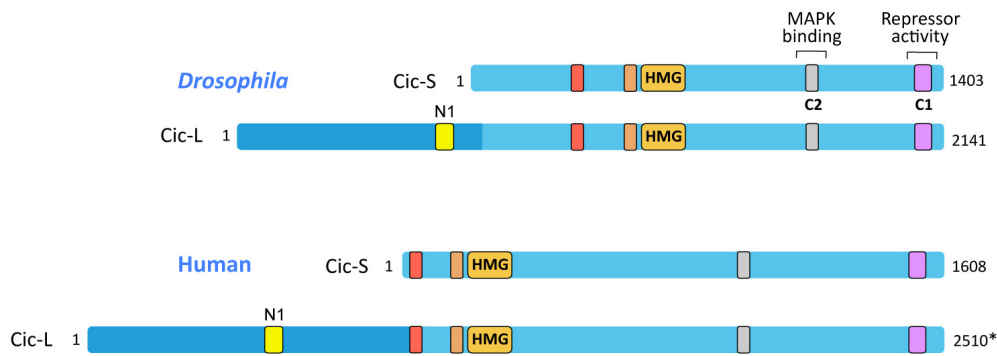


Figure 8. Structural features of Cic isoforms. Two main isoforms, short (Cic-S) and long (Cic-L), are present in both *Drosophila* and humans. In *Drosophila*, Cic-S performs most of known Cic functions. No differential functions have been assigned to short versus long isoforms in mammals. Functional domains are indicated. (Adapted from Jiménez et al., 2012)

Cic proteins share two well-conserved regions: the HMG-box, which is a DNA-binding domain, and a C-terminal motif (C1) of unknown molecular function, although it has been shown that is important for transcriptional repression (Jiménez et al., 2000; Astigarraga et al., 2007; Kamamura-Saito et al., 2006). In addition, there is a third motif (C2), which is less conserved in evolution, that functions as a MAPK-docking site and leads to Cic downregulation when it is phosphorylated (Astigarraga et al., 2007). The mechanisms mediating downregulation of Cic are not fully understood and it has been shown that differ among tissues. Torso signaling mediates Cic degradation at the poles of the embryo but, in the case of the ovary or the wing, EGFR signal induces a subcellular distribution of the protein (Astigarraga et al., 2007; Roch et al., 2002).

5.1. Cic downstream of EGFR signaling in the establishment of the DV axis

Cic protein accumulates in the nucleus of follicle cells except in DA region where it is downregulated by EGFR signaling (Goff et al., 2001; Astigarraga et al., 2007) (Figure 9). As mentioned previously, the EGFR pathway patterns the DV axis of both the embryo and the eggshell (Ray and Schüpbach, 1996). Mutations that prevent EGFR signaling lead to ventralization of the eggshell and the embryo, whereas ectopic EGFR activation dorsalizes the egg (Schüpbach, 1987; Queenan et al., 1997). Interestingly, maternal *cic* loss-of-function alleles produce dorsalization phenotypes

(Goff et al., 2001; Atkey et al., 2006). Dorsalized embryos produced by *cic* mutant females do not have ventral structures such as the ventral denticles and are composed exclusively of dorsal epidermis. Eggs laid by *cic* mutant females have broad dorsal appendages that are positioned more laterally and the dorsal gap between appendages is larger. Moreover, clonal analyses show that *cic* is required cell-autonomously in ventral follicle cells for normal DV pattern of the embryo and the eggshell (Goff et al., 2001; Atkey et al., 2006). In addition, double mutant females for *cic* and *Egfr* lay eggs with ventralized eggshell and embryo, indicating that Cic acts downstream of EGFR signaling (Goff et al., 2001). Moreover, loss of Cic does not affect EGFR signal as *grk* mRNA is properly localized in the DA region and the pattern of expression of known targets of EGFR, as *kekkon1*, does not change (Goff et al., 2001).

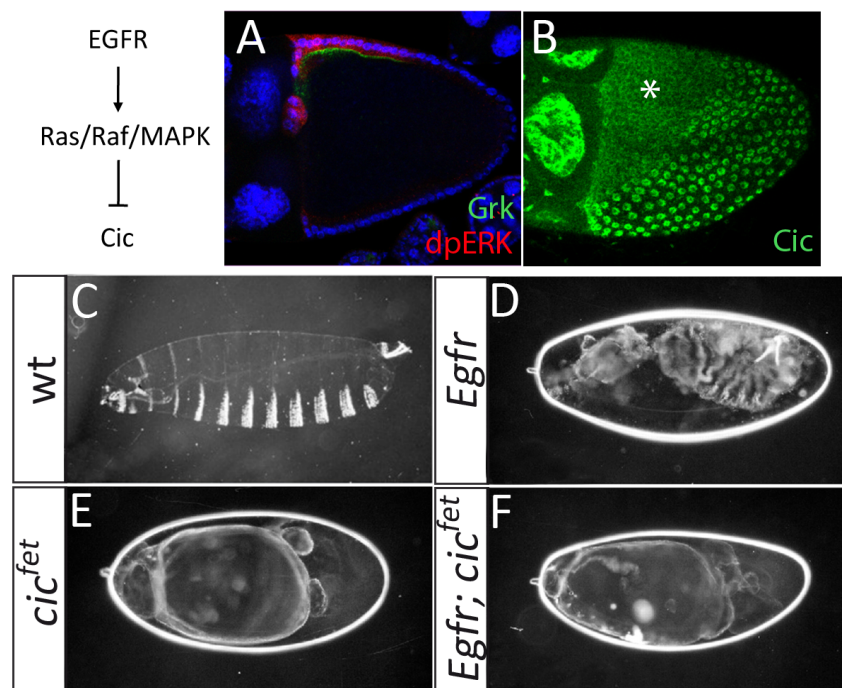


Figure 9. Cic downstream EGFR signaling pathway. (A) Grk ligand activates EGFR signaling at the DA region of the egg chamber. Activated MAPK staining in red and DAPI in blue. (B) Cic protein is downregulated in the DA region by EGFR signaling (asterisk). Cuticles of embryos produced by wild-type (C), *Egfr*⁻¹ (D), *cic*^{fet} and double mutant *Egfr*⁻¹; *cic*^{fet} (F) females (C-F). (Adapted from Goff et al., 2001)

DV patterning in the embryo is ultimately determined during oogenesis by expression of *pipe* in ventral follicle cells. This localized expression of *pipe* depends on EGFR activation in the DA region (Sen et al., 1998). *pipe* mRNA is first detected at

Introduction

stage 9 in egg chambers and continues through stage 10B. In *cic* mutant ovaries, *pipe* gene is no longer expressed in the follicular epithelium (Figure 10A'), only in a few posterior cells, indicating that Cic is required for *pipe* expression in ventral follicle cells for DV axis pattern of the embryo (Goff et al., 2001). Two models can be proposed: one in which Cic represses the activity of an unknown negative regulator of *pipe* expression and another in which Cic is required for positive activation of *pipe* gene in the ventral region.

Mirr, a homeodomain transcription factor, is required in DA follicle cells for determination of appendage-producing cell fate (Atkey et al., 2006). It is expressed in a dynamic pattern during oogenesis, but at stages 9 and 10 *mirr* is expressed in DA follicle cells where it is induced by EGFR signaling pathway (Jordan et al., 2000). In *cic* mutant egg chambers, expression of *mirr* is expanded ventrally only in the anterior region (Figure 10B'), indicating that *mirr* is also regulated by anterior positional cues (Goff et al., 2001; Atkey et al., 2006). On the other hand, reduction of *mirr* function in *cic* mutant egg chambers suppresses the *cic* phenotype (Atkey et al., 2006). Thus, Cic restricts appendage-producing fate through repression of *mirr* in the lateral and ventral follicle cells.

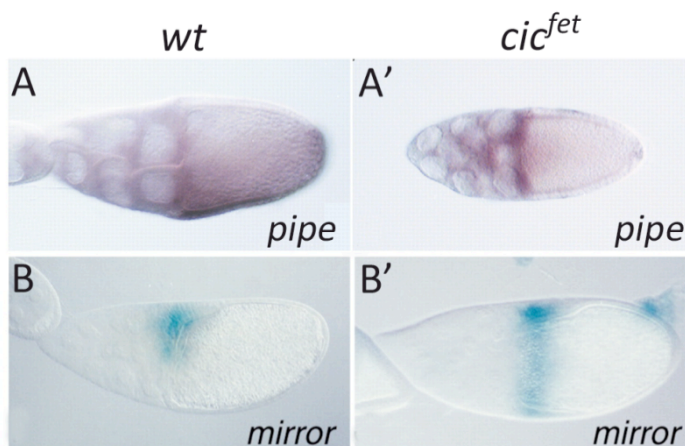


Figure 10. (A, A') Cic is required for *pipe* expression in ventral follicle cells, as *cic^{fet}* mutant egg chambers lack RNA expression pattern of *pipe*. **(B, B')** During stage 10, *mirr* expression expands to ventral regions in *cic^{fet}* mutant ovaries. (Adapted from Goff et al., 2001)

While an initial study of *mirr* function in oogenesis suggested that Mirr might act to repress *pipe* (Jordan et al., 2000), subsequent work indicated that *mirr* loss of function does not affect *pipe* expression (Peri et al. 2002). Moreover, while *pipe* expression spans the ventral-most third of the follicular epithelium, *mirr* is expressed in the dorsal-most third. In addition, ectopic *mirr* expression in *cic* mutant ovaries is only observed in the anterior half of the ventral follicle cells, whereas *pipe*

expression is repressed in all follicle cells (Goff et al., 2001). Therefore, *Mirr* is not a likely candidate for *pipe* repression even though it responds to EGFR signaling and is repressed by *Cic*. However, although *Mirr* does not seem to regulate *pipe* expression normally, ectopic expression of *Mirr* has been reported to repress *pipe* expression and to produce the dorsalization of the embryo (Jordan et al., 2000; Zhao et al., 2000a).

Apart from the maternal effect in the DV patterning of the embryo and the eggshell, *Cic* is then required during embryogenesis in the germline. As described before, DV patterning in the embryo depends on the gradient of Dorsal morphogen that accumulates in ventral nuclei of early embryos and acts as both an activator and repressor of transcription. Repression by Dorsal requires its association with the co-repressor Groucho (Gro) and other postulated co-repressors that bind next to Dorsal in the *zen* promoter (Jiang et al., 1993; Kirov et al., 1993; Dubnicoff et al., 1997; Valentine et al., 1998). This repressor complex is under negative regulation by Torso signaling at the poles of the embryo (Casanova, 1991; Rusch and Levine, 1994) and some results show that *Cic* can be one of the cofactors required for regulation of *zen* expression (Jiménez et al., 2000; Astigarraga et al., 2007) (Figure 11B). On the contrary, *Cic* is not required in the germline for the proper expression of genes as *twi* in the ventral region (Jiménez et al., 2000) (Figure 11D).

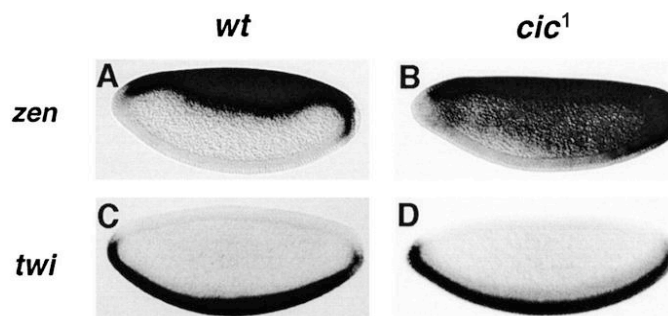


Figure 11. *Cic* is required for repression of *zen* but not for activation of *twi*. RNA expression patterns of *zen* (A, B) and *twi* (C, D) in embryos derived from wild-type (A, C) and homozygous *cic¹* (B, D) females. Derepression of *zen* is observed in *cic¹* embryos. In contrast, the similar pattern of *twi* expression in wild-type and *cic¹* embryos indicates that activation of *twi* by Dorsal is independent of *Cic*. (From Jiménez et al., 2000)

5.2. Cic downstream of Torso signaling in the embryo

Localized activation of the Torso receptor at each pole of the early blastoderm controls the specification of terminal body structures by inducing the expression of the gap genes *tll* and *hkb* (Pignoni et al., 1990; Brönner and Jäckle, 1991). However, this activation is indirect and involves a mechanism of derepression. Repression of *tll* and *hkb* requires several nuclear factors, including Cic and Gro, which are both downregulated by the Torso signal at the poles (Paroush et al., 1997; Jiménez et al., 2000; Goff et al., 2001; Astigarraga et al., 2007; Cinnamon et al., 2008). The mechanism of Cic downregulation by Torso signal is not fully understood but recently it has been suggested that MAPK phosphorylates Cic and, as a consequence, its subcellular localization changes leading to its degradation in the cytoplasm (Astigarraga et al., 2007; Grimm et al., 2012) (Figure 12).

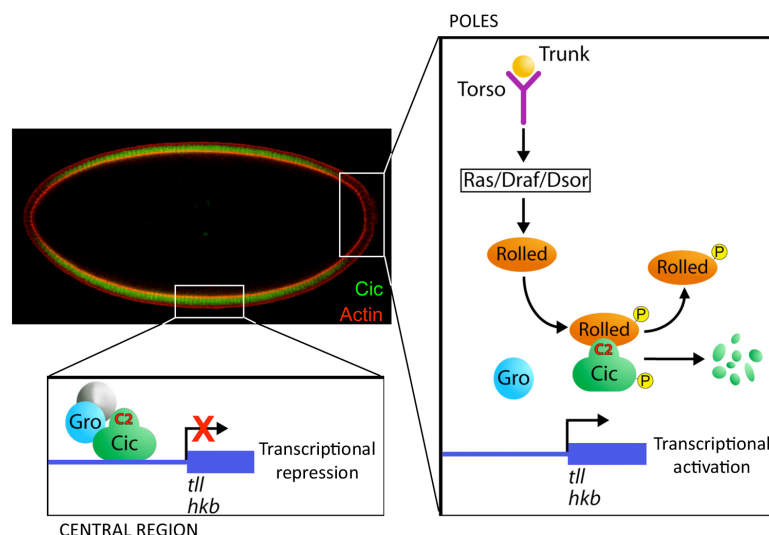


Figure 12. Cic downstream of Torso signaling pathway. Torso signaling pathway directly downregulates Cic protein at the poles of the embryo. MAPK/Rolled interacts with the C2 motif of Cic and phosphorylates it, leading to its degradation. Cic is part of a repressor complex, which probably involves other factors like Gro, that functions in the center of the embryo to inhibit gene expression.

Loss of Cic function causes derepression of *tll* and *hkb* in the center of the embryo (Jiménez et al., 2000; Goff et al., 2001) and, on the contrary, mutations of Cic insensitive to MAPK-downregulation cause reduced or absent posterior expression of both genes in the posterior poles (Astigarraga et al., 2007) (Figure 13). Nevertheless, direct binding of Cic to cis-regulatory regions of *tll* or *hkb* has not yet been demonstrated. In humans, a DNA-binding motif for Cic that resembles the *torso*

response element (torso-RE), a short regulatory element in *tll* upstream region responsible for its restriction to the poles of the embryo, has been identified (Kawamura-Saito et al., 2006; Liaw et al., 1995; Löhr et al., 2009). Therefore, Cic may bind to *tll* and *hkb* enhancers to directly regulate their expression.

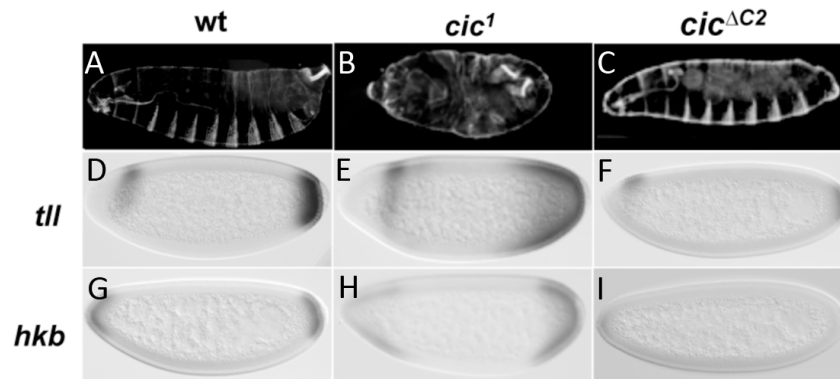


Figure 13. Cic is required for repression of *tll* and *hkb* during terminal development. Cuticles of wild-type (A), *cic*¹ with strongly suppressed trunk and abdomen (B) and *cic*^{ΔC2} with lack of posterior terminal structures (C) embryos. RNA expression patterns of *tll* and *hkb* in wild-type (D, G), *cic*¹ (E, H) and *cic*^{ΔC2} (F, I) embryos. Derepression of *tll* and *hkb* is observed in *cic*¹ mutant embryos. On the contrary, the *cic*^{ΔC2} mutant embryos exhibit repression of *tll* and *hkb* especially at the posterior pole. (Adapted from Astigarraga et al., 2007)

5.3. Other possible roles of Cic downstream of EGFR signaling

In addition to its role in DV patterning in the ovary, Cic is required for interpretation of the EGFR signal in other developmental contexts regulating gene expression and proliferation.

In the early embryo, EGFR signaling is essential for determining the formation and the specification of neuroblasts along the DV axis (Skeath, 1998; von Ohlen and Doe, 2000; Hong et al., 2008). One target of EGFR pathway in the neurogenic ectoderm is *ind*, which specifies the intermediate column cell identity (Weiss et al., 1998). Low levels of the Dorsal gradient work coordinately with EGFR signaling to define a broad domain where *ind* can be expressed. Spatially localized Vnd repressor keeps *ind* off in ventral regions of the neurogenic ectoderm. However, it remains unclear how the dorsal border of *ind* is established. Previous studies have identified a silencer element (*A-box* sequence) located downstream of the *ind* gene that controls

Introduction

its limit of expression through an unknown factor (Stathopoulos and Levine, 2005). A possible role of Cic in the dorsal repression of *ind* downstream of EGFR signaling has not been analyzed yet.

In the developing wing, Cic is necessary for proper specification of wing veins (Goff et al., 2001; Roch et al., 2002). The wing veins differentiate in regions of high EGFR signaling, whereas intervein regions maintain high levels of Cic throughout larval and pupal development. Again, RTK signaling (EGFR) downregulates Cic protein inducing the expression of vein-specific genes such as *argos* (*aos*). In *cic* mutant flies, *aos* is expressed ectopically and their wings exhibit ectopic vein tissue. Therefore, Cic mediates intervein specification by restricting vein formation to the proper regions. Nevertheless, several data suggest that EGFR signaling can mediate activation of some targets in the wing disc by other mechanisms independent of Cic inhibition underlining the complexity of the process (Ciapponi et al., 2001; Roch et al., 2002).

In addition to its important roles in tissue patterning, Cic also acts downstream of the EGFR pathway to regulate cell proliferation of intestinal stem cells (ISC) and larval imaginal discs (Tseng et al., 2007; Jiang et al., 2011; Krivy et al., 2013). In eye and wing imaginal discs, mutations in *cic* increase the rate of cell proliferation without affecting cell size and are able to bypass the requirement of EGFR signaling in promoting growth. Differently, mutations of other negative regulators of growth still depend on EGFR signaling to proliferate (Tseng et al., 2007). Likewise, inactivation of Cic induces ectopic ISCs proliferation in the adult midgut in the same way ectopic activation of EGFR signaling does (Jiang et al., 2011).

5.4. Role of CIC in human disease

Cic is an important factor in the interpretation of several RTK responses in *Drosophila* and increasing evidence suggests that its function as a transcriptional repressor is conserved in humans.

In mammals, CIC is expressed in the immature granule cells of the cerebellum, hippocampus and olfactory bulb during development of the central nervous system

(CNS) and is involved in the alveolarization of the developing lung (Lee et al., 2002; Lee et al., 2011). CIC has a role in the transcriptional regulation of PEA3 group of metalloproteases implicated in extracellular matrix (ECM) remodeling (Kawamura-Saito et al., 2006; Lee et al., 2011; Dissanayake et al., 2011). Studies with cultured cells and in mouse models have demonstrated that RTKs also regulate gene expression via CIC in vertebrates but the mechanisms are still not fully understood (Dissanayake et al., 2011; Fryer et al., 2011). Importantly, CIC dysregulation has been associated to several human diseases.

CIC has been shown to be involved in Spinocerebellar ataxia type 1 (SCA1), a neurodegenerative disease. In SCA1, polyglutamine-expanded Ataxin-1 (ATXN1) associates with CIC into aberrant complexes that mediate neurotoxicity by interfering with CIC normal repression activity (Lam et al., 2006, Lim et al., 2008).

In addition, the role of Cic in restricting cell growth in *Drosophila* appears to be conserved in humans. Missense mutations in the human *CIC* have been reported in tumors of breast and lung cancers (Sjöblom et al., 2006). In oligodendroglioma brain tumors, inactivating mutations in *CIC* gene are frequently found (Bettegowda et al., 2011, Yip et al., 2012). Moreover, CIC has been shown to be translocated in four cases of Ewing's sarcoma-like tumors producing chimeric CIC proteins fused to the C-terminal region of DUX4 with oncogenic properties (Kawamura-Saito et al., 2006; Yoshimoto et al., 2009).

All this evidence underlines the biomedical importance of further studying the mechanisms by which Cic exerts its functions and how it is regulated by RTK signaling, in order to advance in the understanding of the pathogenesis of the human diseases in which it is involved.

OBJECTIVES

RTK/Ras/MAPK signaling is one of the most common pathways for intercellular communication during development and in the adult organism. In addition, abnormal RTK signaling is associated with many pathological conditions, including cancer.

Taking advantage of the available genetic tools of *Drosophila*, we use the DV axis specification as a model to study the molecular mechanisms by which RTK signaling regulates gene expression.

At the time we started our work it was fully established that the initial asymmetries of the DV axis depend on the localized activation of EGFR at the dorsal follicle cells during oogenesis. However, it was an incognita how EGFR signal restricts *pipe* expression along the axis. Interestingly, it was also described that Cic, which has recently arisen as an important factor in the interpretation of the RTK/Ras/MAPK signaling in different contexts, is essential for *pipe* expression at the ventral follicle cells in the ovary.

Later during DV axis subdivision of the embryo, Cic is necessary for dorsal restriction of *zen*. The expression pattern of *zen* in the early embryo depends on the joined action of the terminal and the DV patterning systems. Torso-dependent downregulation of Cic is likely to play a role in *zen* expression at the poles.

Therefore, the general aim of this thesis is the characterization of the mechanisms of gene regulation of RTK signaling in the context of the DV patterning in *Drosophila*. Specifically, the objectives of this work are the following:

1. Characterize the mechanism by which EGFR signal controls *pipe* gene ventral expression in the ovary.
2. Analyse the role of Cic in *pipe* gene regulation during oogenesis.
3. Study Cic function in response to Torso and EGFR signaling during subdivision of the embryonic DV axis.

PUBLICATIONS

INFORME SOBRE LA CONTRIBUCIÓN DE LA DOCTORANDA A LAS PUBLICACIONES
DE ESTA TESIS DOCTORAL

La memoria de la tesis doctoral de **María José Andreu**, titulada **Bases moleculares de la especificación del patrón dorso-ventral en *Drosophila*** se presenta como un compendio de las cuatro siguientes publicaciones:

ARTÍCULO 1

Título: Mirror represses *pipe* expression in follicle cells to initiate dorsoventral axis formation in *Drosophila*

Autores: María José Andreu, Esther González-Pérez, Leiore Ajuria, Núria Samper, Sergio González-Crespo, Sonsoles Campuzano and Gerardo Jiménez

Referencia: *Development* **139**, 1110-1114 (2012)

En este artículo se describe el mecanismo de regulación transcripcional de *pipe*, un gen esencial en el establecimiento de la polaridad dorso-ventral en *Drosophila*, en respuesta a la señal de EGFR. María José ha llevado a cabo la gran mayoría de los experimentos genéticos, moleculares y bioquímicos presentados en este artículo, incluidos todos los análisis clonales en el ovario presentados en el trabajo (Figuras 1-3 y S1-2), así como la generación de diversas construcciones *reporter* del gen *pipe* y su análisis en distintos fondos genéticos (Figuras 2 y 3). Además, también ha realizado los ensayos de retardo en gel (EMSA) con la proteína Mirror recombinante (Figura 2I).

Otros autores han contribuido en diferentes aspectos del trabajo. E. González-Pérez y S. Campuzano generaron y caracterizaron las distintas deficiencias del complejo *iroquois* y realizaron hibridaciones *in situ* para caracterizar la expresión de los tres miembros del complejo en el ovario (Figura 1); L. Ajuria generó las líneas transgénicas que expresan las construcciones *M2-lacZ* y *M2^{r1mut.}-lacZ* (Figura 2C y

Publications

2G); N. Samper proporcionó soporte técnico al trabajo y S. González- Crespo ha participado en la concepción e interpretación de los experimentos del trabajo.

Este trabajo constituye parte de la tesis doctoral de L. Ajuria presentada en forma de compendio de publicaciones (“Función del represor Capicua en la interpretación de señales RTK-Ras-MAPK en *Drosophila*”; Universitat de Barcelona).

ARTÍCULO 2

Título: EGFR-dependent downregulation of Capicua and the establishment of *Drosophila* dorsoventral polarity

Autores: María José Andreu, Leiore Ajuria, Núria Samper, Esther González-Pérez, Sonsoles Campuzano, Sergio González-Crespo and Gerardo Jiménez

Referencia: *Fly* **6:4**, 234-239 (2012)

Este trabajo presenta una perspectiva general sobre la formación del patrón dorsoventral en *Drosophila*, así como nuevos resultados acerca de la regulación de la proteína Capicua (Cic) por la vía de EGFR en el ovario, complementando así los resultados presentados en el Artículo 1. Los nuevos experimentos fueron llevados a cabo en su totalidad por María José.

ARTÍCULO 3

Título: Capicua DNA-binding sites are general response elements for RTK signaling in *Drosophila*

Autores: Leiore Ajuria, Claudia Nieva, Clint Winkler, Dennis Kuo, Núria Samper, María José Andreu, Aharon Helman, Sergio González-Crespo, Ze’ev Paroush, Albert J. Courey and Gerardo Jiménez

Referencia: *Development* **138**, 915-924 (2011)

Este artículo describe las múltiples funciones de Cic como represor directo de genes regulados por vías RTK, mediando así las respuestas transcripcionales a dichas vías

a través de un mecanismo de desrepresión. La contribución de María José ha consistido en generar líneas transgénicas que expresan la construcción *CUASC-lacZ*, la cual ha sido esencial para demostrar la suficiencia de lugares octaméricos de unión de Cic para la interpretación de señales RTK *in vivo*. Además, María José ha participado en la generación de algunas de las cepas recombinantes necesarias para los experimentos de la Figura 5, habiendo colaborado también en el montaje de las alas y la elaboración de la Figura S4.

Este trabajo constituye parte de la tesis doctoral de L. Ajuria (“Función del represor Capicua en la interpretación de señales RTK-Ras-MAPK en *Drosophila*”; Universitat de Barcelona). Además, los experimentos en la Figura 3 del artículo han servido como material suplementario y de discusión en la tesis doctoral de C. Winkler (“A functional analysis of the corepressor Groucho in the development of *Drosophila melanogaster*”; University of California)

ARTÍCULO 4

Título: Gene Regulation by MAPK Substrate Competition

Autores: Yoosik Kim, María José Andreu, Bomyi Lim, Kwanghun Chung, Mark Terayama, Gerardo Jiménez, Celeste A. Berg, Hang Lu, and Stanislav Y. Shvartsman

Referencia: *Developmental Cell* **20**, 880–887 (2011)

Este artículo muestra cómo la competición molecular entre sustratos fosforilados por MAPK afecta la expresión de genes diana como *zerknüllt* (*zen*), revelando así un nuevo mecanismo de integración de los sistemas anterior, dorsoventral y terminal. La contribución de María José a este trabajo ha consistido en generar embriones mutantes *cic*, *trk* y dobles mutantes *trk cic* y analizar la expresión de *zen* en estos fondos mutantes. Estos experimentos mostraron que la regulación negativa de Cic dependiente de la vía de Torso contribuye a la desrepresión de *zen* en los polos del embrión. Estos resultados, junto con otros datos previos, han resultado esenciales para poder formular el nuevo modelo de regulación basado en la competición entre sustratos de MAPK.

Publications

Este trabajo constituye parte de la tesis doctoral de Y. Kim (“Quantitative analysis of signaling pathways: Imaging and modeling of the terminal patterning system of the *Drosophila* embryo”; Princeton University).

A handwritten signature in blue ink, appearing to read 'Gerardo Jimenez'.

Fdo. Gerardo Jiménez
Profesor de Investigación ICREA
IBMB-CSIC

INFORME SOBRE EL FACTOR DE IMPACTO DE LAS PUBLICACIONES

Los artículos presentados en esta tesis presentan resultados de gran relevancia y que suponen un avance de interés general para la comunidad científica. Los cuatro artículos listados se han publicado en las revistas *Development*, *Fly* y *Developmental Cell*, siendo las tres de ámbito internacional y ampliamente reconocidas en los campos de la Biología del Desarrollo, Molecular y Celular. Los índices de impacto para estas revistas en 2012 son: 6.208 para *Development*, 1.105 para *Fly* y 12.861 para *Developmental Cell*. Estos índices de impacto sitúan a las revistas *Development* y *Developmental Cell* en el primer cuartil de publicaciones en sus categorías.

ARTICLE 1: Mirror represses *pipe* expression in follicle cells to initiate dorsoventral axis formation in *Drosophila*

Authors

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Reference

Development **139**, 1110-1114 (2012)

Summary (in Spanish)

La formación del eje dorso-ventral (DV) en *Drosophila* se inicia con la activación localizada de EGFR, un receptor tirosina quinasa (RTK), en las células foliculares dorso-anteriores (DA) del ovario. Un evento crítico regulado por la vía de EGFR es la represión del gen *pipe* en las células foliculares dorsales, aunque el mecanismo de dicha represión sigue siendo una incógnita. El gen *pipe* codifica para una enzima sulfotransferasa esencial para la transmisión de la información posicional generada durante la oogénesis al embrión temprano. En este artículo mostramos cómo Mirror (Mirr), un factor de transcripción con homeodominio inducido por la vía de EGFR en las células foliculares DA, directamente reprime la expresión de *pipe* uniéndose a un elemento conservado de su región reguladora. Además, señalamos que el factor HMG-box Capicua (Cic) mantiene la expresión de *pipe* en las células foliculares ventrales mediante la represión de *mirr* en esta región. Cabe destacar que esta función de Cic se asemeja a su papel en la regulación del patrón del eje anteroposterior (AP), donde Cic permite la expresión de los genes *gap* de la región central del embrión mediante la represión de Tailless, un represor inducido por una señal RTK en los polos del embrión. Por lo tanto, circuitos similares RTK-Cic regulan los estadios tempranos de la formación del patrón de los ejes DV y AP en *Drosophila*.

Mirror represses *pipe* expression in follicle cells to initiate dorsoventral axis formation in *Drosophila*

María José Andreu¹, Esther González-Pérez², Leïore Ajuria¹, Núria Samper¹, Sergio González-Crespo¹, Sonsoles Campuzano² and Gerardo Jiménez^{1,3,*}

SUMMARY

Dorsoventral (DV) axis formation in *Drosophila* begins with selective activation of EGFR, a receptor tyrosine kinase (RTK), in dorsal-anterior (DA) ovarian follicle cells. A critical event regulated by EGFR signaling is the repression of the sulfotransferase-encoding gene *pipe* in dorsal follicle cells, but how this occurs remains unclear. Here we show that Mirror (Mirr), a homeodomain transcription factor induced by EGFR signaling in DA follicle cells, directly represses *pipe* expression by binding to a conserved element in the *pipe* regulatory region. In addition, we find that the HMG-box protein Capicua (Cic) supports *pipe* expression in ventral follicle cells by repressing Mirr in this region. Interestingly, this role of Cic resembles its function in regulating anteroposterior (AP) body patterning, where Cic supports gap gene expression in central regions of the embryo by repressing Tailless, a repressor induced by RTK signaling at the embryonic poles. Thus, related RTK-Cic repressor circuits regulate the early stages of *Drosophila* DV and AP body axis formation.

KEY WORDS: Dorsoventral patterning, EGFR signaling, *pipe*, Mirror, Capicua, *Drosophila*

INTRODUCTION

Dorsoventral (DV) embryonic patterning in *Drosophila* depends on inductive signals generated during oogenesis that are transmitted to the fertilized embryo (Nilson and Schüpbach, 1999; Moussian and Roth, 2005). During mid-oogenesis, localization of the oocyte nucleus in a dorsal-anterior (DA) position produces a local source of the Gurken (Grk)/TGF α -like secreted factor, which activates EGFR signaling in the overlying DA follicle cells. EGFR then signals via the Ras/Raf/MAPK cassette and represses transcription of the *pipe* gene, thereby restricting its expression to ventral follicle cells (Sen et al., 1998). *pipe* encodes a sulfotransferase that modifies structural components of the eggshell, a process required for subsequent signaling events that activate the Toll receptor on the ventral surface of the embryo (Sen et al., 1998; Moussian and Roth, 2005; Zhang et al., 2009).

The mechanism by which EGFR signaling represses *pipe* expression remains unclear. An important feature is that detectable EGFR signaling activity and *pipe* expression are not precisely complementary: at stage 10A of oogenesis, active phospho-MAPK and known EGFR targets are restricted to the dorsal ~30% of the follicular epithelium, whereas *pipe* expression spans the ventral ~40% region (Sen et al., 1998; Ghiglione et al., 1999; Peri et al., 1999; Reich et al., 1999; Jordan et al., 2000; Zhao et al., 2000). Accordingly, initial analyses suggested that *pipe* repression is not a direct consequence of EGFR signaling, but depends on long-range signals mediated by the homeodomain factor Mirror (Mirr) and the Notch-Fringe pathway (Jordan et al., 2000). However, subsequent studies indicated that Grk/EGFR signaling forms a

gradient that reaches ventral positions to directly control *pipe* expression (Pai et al., 2000; Goentoro et al., 2006). In agreement with this, loss of EGFR/Ras/MAPK activity causes cell-autonomous derepression of *pipe* in all lateral and dorsal positions (James et al., 2002; Peri et al., 2002). Furthermore, both Mirr and Notch-Fringe signals were found to be dispensable for *pipe* repression, arguing that this repression might depend on unknown factors (Peri et al., 2002).

Here, we report that Mirror, which is induced by EGFR signaling in DA cells (Jordan et al., 2000; Zhao et al., 2000), directly represses *pipe* expression by binding to a conserved element in its regulatory region. In addition, we find that the HMG-box protein Capicua (Cic) supports *pipe* expression in ventral follicle cells by repressing *mirr* in this region. Together, our results delineate a repressor circuit downstream of EGFR signaling that is essential for asymmetric *pipe* transcription in the follicle cell layer.

MATERIALS AND METHODS

Drosophila strains and genetic analyses

The following stocks were used: *mirr*^{J825} (Collins and Cohen, 2005), *fs(1)K10*¹ (Wieschaus et al., 1978), *pipe-lacZ* (Sen et al., 1998), *UAS-mirr* (McNeill et al., 1997), *cic*^{fe111}, *cic*^{fe116} (Goff et al., 2001), *FRT82B cic*^{Q474X} (Tseng et al., 2007) and *FRT82B Ras*^{ΔC40b} (James et al., 2002). *iro*^{EGP} deficiencies were generated using FRT-bearing transposon insertions from Exelixis (Thibault et al., 2004; Parks et al., 2004). Mosaic analyses were performed by FLP-FRT-mediated mitotic recombination (Xu and Rubin, 1993). Ectopic *mirr*-expressing clones were generated using the GAL4/UAS flip-out technique and an *hs-flp*; *UAS-CD8-GFP*; *tub>y*⁺ *Gal80>Gal4* line, resulting in GFP-labeled clones. Transgenic lines were established by standard P-element transformation.

Immunohistochemistry and in situ hybridization

The following primary antibodies were used: mouse anti- β -galactosidase (40-1a, 1:200 dilution) and mouse anti-Grk (1D12, 1:50) (Developmental Studies Hybridoma Bank). Mirr expression was visualized using a rat polyclonal antibody that recognizes all three Iro proteins (our unpublished data). Signals were detected using fluorochrome-conjugated secondary antibodies and DAPI. Whole-mount in situ hybridization was performed using digoxigenin-labeled RNA probes.

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DNA constructs

pipe enhancer fragments were amplified by PCR using the following primers: *m2-f* (5'-AGCTACTCACTTTCTTGGTAC-3') and *m2-r* (5'-AGCCAAACTTCGGGATCTAAG-3') to amplify module *M2*; and *m3-f* (5'-CTACTAAAAGTATTCACTCTAG-3') and *m3-r* (5'-ATTTCAGC-ATTGGTACCAAG-3') for *M3*. *M1* was generated by ligating *M2* and *M3* fragments through their common *KpnI* site. Reporter constructs were assembled in *pC4PLZ* (Wharton and Crews, 1993). *M1^{r1mut}* and *M2^{r1mut}* were made by replacing the 39 bp *r1* sequence with a *pC4PLZ* polylinker fragment (*BamHI*-*NotI*) of the same size.

In vitro DNA binding assays

Recombinant Mirr protein was synthesized by coupled transcription-translation (TNT, Promega). EMSA experiments were performed by incubating 0.5–2 µl TNT reactions with ~50 ng ³²P-end-labeled probes, 1 µg BSA and 1 µg poly(dI-dC) in a final volume of 20 µl supplemented with binding buffer (60 mM Hepes pH 7.9, 20 mM Tris-HCl pH 7.9, 300 mM KCl, 5 mM EDTA, 5 mM DTT, 12% glycerol). Complexes were resolved on 5% non-denaturing polyacrylamide gels run in 0.5× TBE at 4°C, and visualized by autoradiography. The control (c) probe in Fig. 2I is 5'-GAGTTTGGAGAAAAACACGTGTTAAGCT-3' (Mirr binding site is underlined) (Bilioni et al., 2005).

RESULTS AND DISCUSSION

Mirr mediates cell-autonomous repression of *pipe* in dorsal follicle cells

Although previous clonal analyses indicated that Mirr is not involved in *pipe* regulation (Peri et al., 2002), we reasoned that Mirr function in this context could be masked by redundant activities of the two other components of the *iroquois* complex (*Iro-C*), *araucan* (*ara*) and *caupolican* (*caup*), which encode homeodomain proteins highly related to Mirr (Gómez-Skarmeta et al., 1996; McNeill et al., 1997). Therefore, we generated follicle cell clones mutant for a deficiency, *iro^{EGP7}*, that removes the whole of *Iro-C* (Fig. 1A). Strikingly, *iro^{EGP7}* clones showed full, cell-autonomous derepression of *pipe-lacZ* expression at all positions where *pipe* is normally off (Fig. 1B). We then used two additional deficiencies, *iro^{EGP5}* and *iro^{EGP6}*, that specifically abolish *mirr* and *ara/caup* function, respectively (Fig. 1A). These analyses revealed a requirement for Mirr, but not Ara or Caup, in *pipe* repression: only *iro^{EGP5}* caused ectopic *pipe* expression equivalent to that seen with *iro^{EGP7}* (Fig. 1C,D and supplementary material Fig. S1). In addition, we found that *mirr* is the only member of *Iro-C* that is detectably expressed in follicle cells (Fig. 1E–G).

Thus, Mirr is essential for repression of *pipe* in dorsal follicle cells. Based on further analyses (supplementary material Fig. S1), it is possible that previous negative results were caused by residual Mirr activity of the *mirr^{ed8}* allele (Peri et al., 2002). In fact, *pipe* is highly sensitive to Mirr repression, as this repression occurs even in cells abutting the normal *pipe* domain where Mirr levels are very low or undetectable (Jordan et al., 2000; Zhao et al., 2000) (Fig. 1B and supplementary material Fig. S1). Furthermore, this is consistent with evidence that low EGFR/Ras/MAPK levels mediate *pipe* repression in lateral and dorsal-posterior cells close to its border of expression (Pai et al., 2000; Peri et al., 2002; James et al., 2002) (Fig. 3B).

Mirr binds to a conserved element in the *pipe* regulatory region

To further study *pipe* regulation, we identified two highly conserved sequence motifs in its 5' flanking region (designated *r1* and *r2*; Fig. 2A). Using different *lacZ* reporters, we defined a minimal *pipe* enhancer module, *M2*, which includes the *r1* element and recapitulates endogenous *pipe* expression (Fig. 2A–D). As

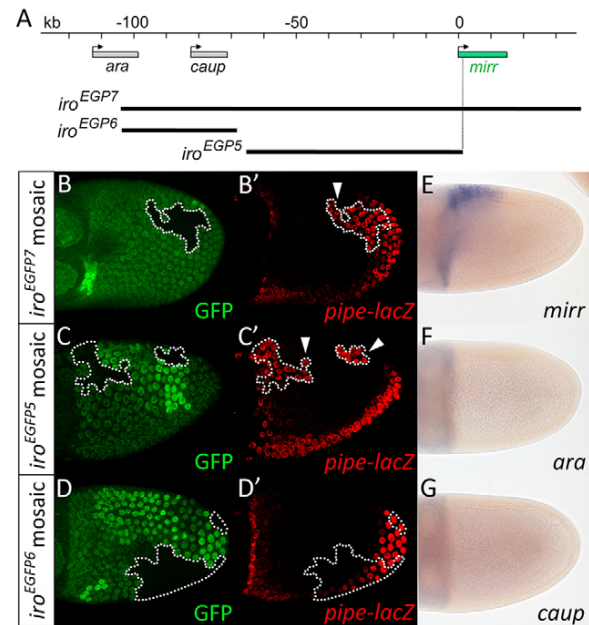


Fig. 1. Mirr represses *pipe* expression in dorsal follicle cells.

(A) The *Iro* complex. Horizontal lines indicate the regions deleted in three *iro^{EGP}* deficiencies. *iro^{EGP5}* removes the first *mirr* exon and adjacent upstream sequences. (B–D') Stage 10 mosaic egg chambers carrying *iro^{EGP7}* (B), *iro^{EGP5}* (C) and *iro^{EGP6}* (D) clones marked by absence of GFP (green, outlined); B'–D' show *pipe-lacZ* expression (red). Dorsolateral (B,D) or lateral (C) views are shown. Arrowheads indicate ectopic *pipe-lacZ* expression. (E–G) Stage 10 egg chambers stained for *mirr* (E), *ara* (F) and *caup* (G) mRNA expression.

expected, *M2-lacZ* responded to EGFR regulation, as indicated by the loss of ventral-anterior expression in *fs(1)K10* mutant ovaries, which display ectopic EGFR activity (Wieschaus et al., 1978; Neuman-Silberberg and Schüpbach, 1993) (Fig. 2E). In addition, we found that clonal ectopic expression of Mirr repressed *M2-lacZ* in a cell-autonomous manner (Fig. 2H), consistent with EGFR signaling mediating *pipe* repression via Mirr.

We then found that *pipe* regulation crucially depends on the *r1* sequence, as mutation of this element resulted in dorsal derepression of reporter constructs (*M1^{r1mut}-lacZ* and *M2^{r1mut}-lacZ*, Fig. 2F,G). These mutant constructs, particularly *M2^{r1mut}-lacZ*, exhibit weaker and more variable expression than *M2-lacZ* throughout the epithelium, raising the possibility that the *r1* element includes sequences required for both repression in DA cells and full activation in ventral cells. Based on these results, we postulated that Mirr might repress *pipe* expression by binding to the *r1* element. EMSA experiments (Fig. 2I) showed that recombinant Mirr protein binds strongly to the *r1* sequence, but not to the mutated *r1mut* sequence that caused dorsal derepression in vivo. Using a set of mutations along the *r1* sequence (*mut A–D*), we then found that Mirr preferentially binds to a site containing the sequence ACACGA (Fig. 2I), which resembles the ACAnnTGT motif defined as a minimal Mirr binding site in vitro (Bilioni et al., 2005).

Cic supports *pipe* expression by repressing Mirr

Previous studies showed that Cic is required for *pipe* expression in ventral follicle cells; accordingly, embryos derived from *cic* mutant females are severely dorsalized (Goff et al., 2001). However, how

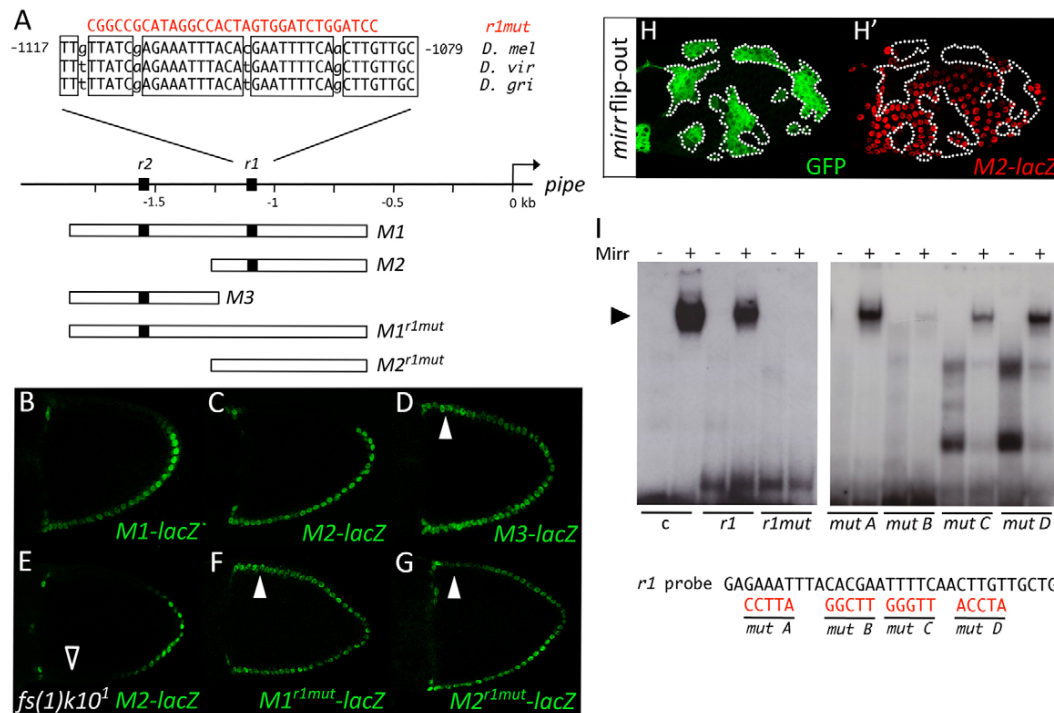


Fig. 2. Mirr binds to a conserved regulatory element in *pipe*. (A) The *pipe* upstream region showing *r1* sequences from *D. melanogaster*, *D. virilis* and *D. grimshawi* above; the *r1mut* sequence is also indicated. DNA fragments analyzed for enhancer activity are depicted beneath. (B-G) Lateral views of transgenic stage 10 egg chambers stained for *lacZ* reporter expression. Solid arrowheads indicate ectopic expression of constructs lacking the *r1* element. The open arrowhead in E indicates loss of ventral expression in the *fs(1)K10¹* background. In all cases, dorsal is up as confirmed by the dorsal position of the oocyte nucleus under Nomarski optics (not shown). (H, H') Ventrolateral views of a stage 10 mosaic egg chamber carrying *mirr* overexpression clones marked by expression of GFP (H, green, outlined); H' shows *M2-lacZ* expression (red). (I) EMSA analysis of in vitro synthesized Mirr protein binding to different oligonucleotide probes. Lanes labeled (-) contain TNT extract without *mirr* plasmid. The control probe (c) contains the ACACgTGT sequence (Bilion et al., 2005). The *r1mut* sequence is indicated in Fig. 2A; *mut A-D* probes contain the changes indicated in red.

Cic affects *pipe* expression is not understood (Goff et al., 2001; Atkey et al., 2006). Since Cic often represses genes induced by RTK signaling pathways (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Atkey et al., 2006; Löhr et al., 2009; Ajuria et al., 2011), we hypothesized that Cic might support *pipe*

transcription indirectly by repressing Mirr in ventral cells. Consistent with this idea, loss of *cic* function causes ectopic *mirr* expression, although only in anterior regions of the egg chamber (Goff et al., 2001; Atkey et al., 2006) (supplementary material Fig. S2).

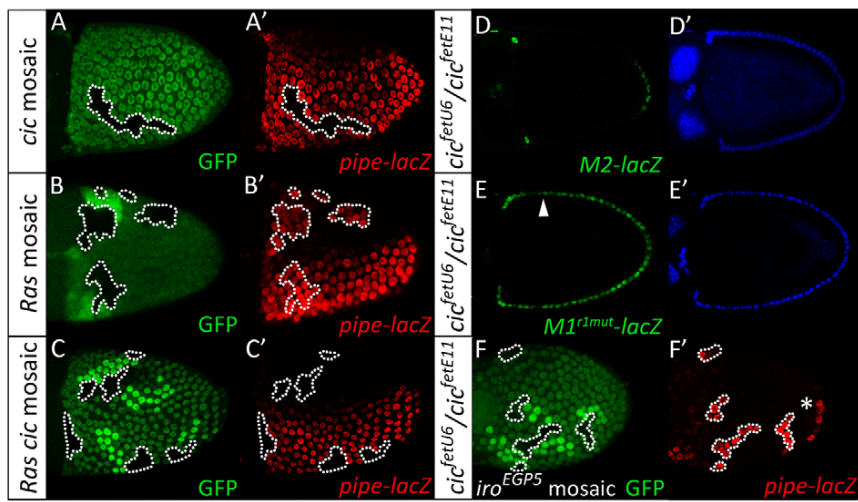


Fig. 3. Cic regulates *pipe* indirectly through the *r1* element. (A-C*) Stage 10 mosaic egg chambers carrying *cic^{Q474X}* (A), *Ras^{4C40b}* (B) and *Ras^{4C40b}/*cic^{Q474X}** (C) mutant clones marked by absence of GFP (green, outlined). A'-C' show *pipe-lacZ* expression (red). Ventrolateral (A) or lateral (B, C) views are shown. (D-E') Lateral views of stage 10 *cic^{fetU6}/*cic^{fetE11}** egg chambers stained for *lacZ* reporter expression; D' and E' show DAPI staining (blue). Dorsal is up (see Fig. 2 legend). (F, F') Lateral view of a stage 10 *cic^{fetU6}/*cic^{fetE11}** egg chamber carrying *iro^{EGP5}* mutant clones marked by absence of GFP (F, green); F' shows *pipe-lacZ* expression (red). The asterisk indicates residual *pipe-lacZ* expression in the *cic^{fetU6}/*cic^{fetE11}** background.

To further investigate Cic function, we performed mosaic analyses and found that Cic is required cell-autonomously for the expression of *pipe* (Fig. 3A). Since mutations in negative regulators of the EGFR pathway, such as Cbl, cause ventral *pipe* repression by upregulating EGFR signaling (Pai et al., 2000), we tested whether Cic might act similarly. To this end, we performed epistasis analyses using *cic* and *Ras* mutations. Whereas *Ras* clones cause ectopic *pipe* expression in dorsal cells and do not affect *pipe* expression in ventral cells (Fig. 3B) (James et al., 2002), *Ras cic* clones lacked *pipe* expression in all positions (Fig. 3C). This indicates that loss of *pipe* expression in *cic* mutant cells does not result from increased EGFR signaling in those cells.

We also analyzed the effect of *cic Ras* clones on *Mirr* expression and found that, like *cic* clones, they cause ectopic *Mirr* expression only in anterior regions (supplementary material Fig. S2). This is consistent with the observation that both *grk cic* and *cic* mutant ovaries exhibit expanded *mirr* expression (Atkey et al., 2006) and supports a model in which *mirr* is normally activated by EGFR signaling and by an independent anterior signal that may depend on Decapentaplegic (Twombly et al., 1996; Deng and Bowles, 1997; Peri and Roth, 2000), with Cic being required to counteract this latter input and repress *mirr* in ventral-anterior follicle cells (Atkey et al., 2006) (see below).

Although these observations are partly consistent with our hypothesis that Cic supports *pipe* expression by repressing *Mirr*, they raise a paradox: although Cic is required for *pipe* expression in both anterior and posterior follicle cells, loss of *cic* function derepresses *mirr* only in anterior cells. Since *Mirr* normally represses *pipe* in lateral and dorsal-posterior follicle cells where *Mirr* is undetectable, we reasoned that loss of Cic function in ventral-posterior cells might induce similar low-level expression of *Mirr* that is nevertheless sufficient to repress *pipe*. If this is correct, Cic should be dispensable for the expression of mutant *pipe* enhancers that lack the *r1* motif and are insensitive to *Mirr*. Indeed, whereas *M1-lacZ* and *M2-lacZ* require Cic activity for expression in ventral follicle cells, *M1^{r1mut}-lacZ* and *M2^{r1mut}-lacZ* do not (Fig. 3D,E; data not shown). This indicates that Cic regulates *pipe* expression in ventral cells by acting indirectly through the *Mirr*-responsive *r1* element. Furthermore, we have confirmed that *Mirr* represses *pipe* in the absence of Cic by generating *iro^{EGP5}* clones in *cic^{fatU6}/cic^{fatE11}* mutant ovaries that normally lack *pipe* expression (Goff et al., 2001) (Fig. 3D). We find that *pipe* expression is depressed in all such mutant clones, irrespective of their position (Fig. 3F; data not shown), indicating that ectopic, low-level activity of *Mirr* accounts for the loss of *pipe* expression in *cic* ovaries (see also Fig. 4B).

Conclusions

Mirr had previously been implicated in the specification of dorsal cell fates during eggshell patterning (Atkey et al., 2006), but it was assumed to be dispensable for establishing embryonic polarity (Peri et al., 2002; Moussian and Roth, 2005; Atkey et al., 2006). Accordingly, it is well accepted that EGFR signaling in the follicle cell layer bifurcates into one branch that regulates DV axis formation and another that controls eggshell patterning, with *pipe* being required only in the first branch and *Mirr* only in the second. However, our results clearly identify *Mirr* as a common mediator of EGFR signaling responses in both developmental processes (Fig. 4A).

Our data also show that Cic affects *pipe* expression by repressing *mirr* throughout the *pipe* domain. It should be noted that, although Cic is normally downregulated by EGFR signaling in DA cells (Astigarraga et al., 2007), this downregulation does

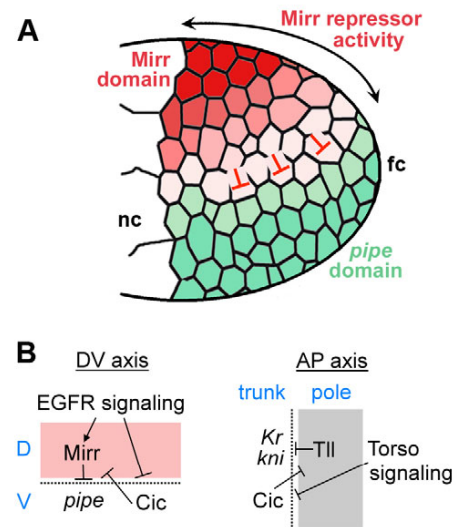


Fig. 4. Roles of *Mirr* and *Cic* in *pipe* regulation. (A) *Mirr*-dependent regulation of *pipe* in follicle cells. The *Mirr* gradient peaks at the dorsal-anterior region (red). Lateral and dorsal-posterior follicle cells (pale red) contain very low levels of *Mirr* protein that are nevertheless sufficient to repress *pipe*, thereby restricting its expression to ventral cells (green). nc, nurse cells; fc, follicle cells. (B) Model of regulatory networks involved in dorsoventral (DV) and anteroposterior (AP) embryonic patterning. Both processes require RTK signals that, together with other inputs, induce expression of repressor factors (*Mirr* and *Tll*). Note that Torso signaling induces *tll* expression simply by relieving *Cic* repression (Astigarraga et al., 2007; Cinnamon et al., 2008). In regions where RTK signaling is low or absent, *Cic* represses *mirr* and *tll*, thereby supporting the expression of key patterning genes such as *pipe*, *Kr* and *kni*.

not play a major role in modulating the effect of Cic on *Mirr*, nor in inducing *Mirr* expression in response to EGFR signaling. Indeed, *Cic* derivatives that are insensitive to EGFR-mediated downregulation do not repress *Mirr* in DA cells, implying that EGFR signaling induces *Mirr* by mechanisms that do not require downregulation of *Cic* (Atkey et al., 2006; Astigarraga et al., 2007) (see above).

Finally, the role of Cic in DV patterning is analogous to its function in the anteroposterior (AP) axis, where it supports the expression of gap genes such as *Kruppel* (*Kr*) and *knirps* (*kni*) in the segmented trunk of the embryo. In this case, Cic supports gap gene expression by repressing *Tailless* (*Tll*), a repressor of *Kr* and *kni* induced by Torso RTK signaling at the posterior embryonic pole (Fig. 4B) (Paroush et al., 1997; Jiménez et al., 2000; Goff et al., 2001; Morán and Jiménez, 2006; Löhr et al., 2009; Ajuria et al., 2011). Therefore, similar interactions between Cic and RTK-induced repressors are essential for the early subdivision of both DV and AP embryonic axes.

Note added in proof

Two related papers on *pipe* regulation have appeared while this article was in revision (Technau et al., 2011; Fuchs et al., 2012).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at

<http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076562/-/DC1>

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Supplemental Information

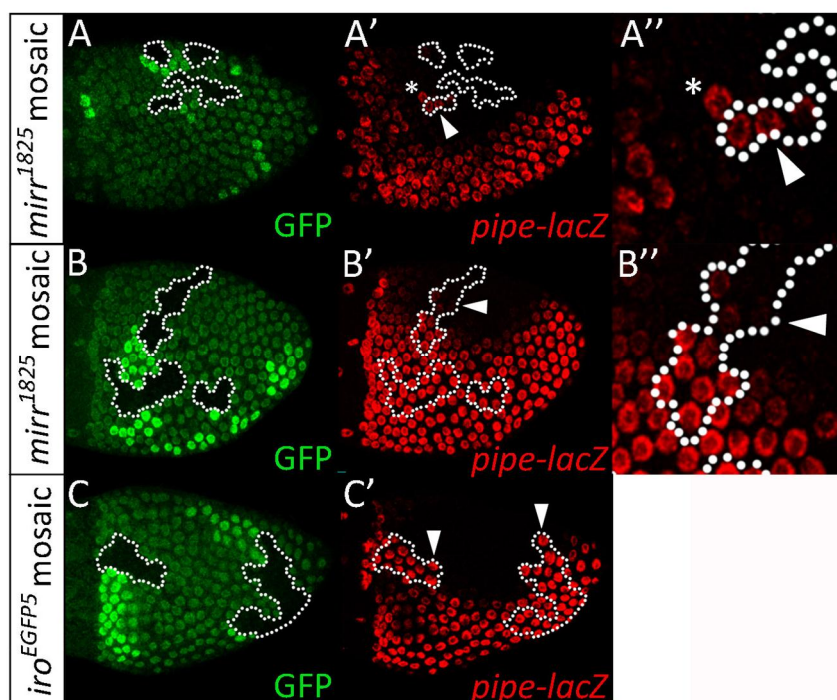


Figure S1. Effects of *mirr*¹⁸²⁵ and *iro*^{EGP5} mutations on *pipe* expression. (A-C) Stage 10 mosaic egg chambers carrying *mirr*¹⁸²⁵ (A, B) and *iro*^{EGP5} (C) loss-of-function clones marked by absence of GFP (green). (A'-C') Anti- β -galactosidase staining of *pipe-lacZ* expression (red) in these egg chambers. (A'', B'') Magnifications of A' and B', respectively. Lateral (A, C) or ventro-lateral (B) views are shown. The *mirr*¹⁸²⁵ examples exhibit partial derepression of *pipe-lacZ* in only a fraction of clones. Note the ectopic reporter expression in one of the clones shown in A' (arrowhead); one rare case of non-cell-autonomy is also seen in this clone (stained nucleus marked with asterisk). By contrast, complete loss of *mirr* function in the *iro*^{EGP5} background causes full derepression of *pipe* (C). This suggests that *mirr*¹⁸²⁵ represents a hypomorphic allele retaining residual Mirr function. This is consistent with the regional effects of *mirr*¹⁸²⁵ clones, which predominantly show ectopic *pipe-lacZ* expression nearby the normal *pipe* domain (see B''); if these clones retain residual Mirr activity, their effects should be weaker in DA cells where Mirr levels are higher, and more pronounced in lateral cells where Mirr levels are low. Accordingly, derepression of *pipe* in DA cells requires the complete loss of Mirr activity as in *iro*^{EGP5} mutant cells.

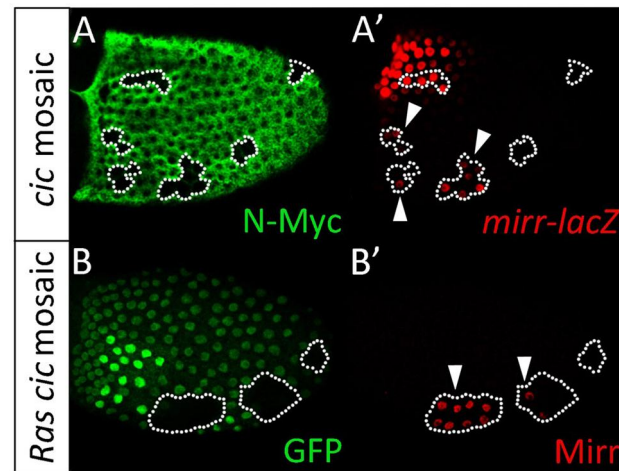


Figure S2. Cic represses *mirr* expression. (A, B) Stage 10 mosaic egg chambers carrying *cic^{fetU6}* (A) and *ras^{ΔC40b} cic^{Q474X}* (B) loss-of-function clones marked by absence of N-Myc (A) or GFP (B) (green). (A') *mirr* expression visualized using the *mirr^{F7}* (*mirr-lacZ*) enhancer trap line and anti-β-galactosidase staining (red). (B') *Mirr* expression visualized by anti-Iro staining (red). Lateral (A) or ventral (B) views are shown. In both cases, the mutant clones exhibit ectopic *mirr* expression, but only in the anterior half of the follicular epithelium (arrowheads)

ARTICLE 2: EGFR-dependent downregulation of Capicua and the establishment of *Drosophila* dorsoventral polarity

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Reference

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Summary (in Spanish)

La formación del eje dorso-ventral (DV) en *Drosophila* se inicia durante la oogénesis mediante la activación en forma de gradiente de la vía de señalización EGFR/Ras/MAPK en el epitelio folicular de la cámara ovárica. La señal de EGFR, que es más elevada en las células foliculares dorsales, reprime la expresión del gen *pipe* delimitando de esta forma un dominio ventral de actividad de la proteína que es crítico para la posterior inducción de destino ventral en el embrión. Hemos caracterizado el circuito transcripcional que une la vía de EGFR con la represión de *pipe*: en las células foliculares dorsales, el factor de transcripción con homeodominio Mirror (Mirr), el cual es inducido por la vía de EGFR, reprime directamente la transcripción de *pipe*; mientras en las células foliculares ventrales, la proteína HMG-box Capicua (Cic) sostiene la expresión de *pipe* mediante la represión de *mirr*. A pesar de que Cic es regulado negativamente a nivel post-transcripcional por la vía de Ras/MAPK en distintos contextos, la relevancia de este mecanismo para la interpretación de la señal de EGFR durante la formación del patrón DV es desconocida. En este artículo, proponemos un modelo en el que la regulación negativa de Cic mediada por la vía de EGFR, modula la distribución espacial de la proteína Mirr en las células foliculares laterales contribuyendo así a definir la posición en la que se forma el borde de expresión de *pipe*.

EGFR-dependent downregulation of Capicua and the establishment of *Drosophila* dorsoventral polarity

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Dorsoventral (DV) axis formation in *Drosophila* begins during oogenesis through the graded activation of the EGF receptor (EGFR)-Ras-MAPK signaling pathway in the follicle cell layer of the egg chamber. EGFR signaling, which is higher in dorsal follicle cells, represses expression of the sulfotransferase-encoding gene *pipe*, thereby delimiting a ventral domain of Pipe activity that is critical for the subsequent induction of ventral embryonic fates. We have characterized the transcriptional circuit that links EGFR signaling to *pipe* repression: in dorsal follicle cells, the homeodomain transcription factor Mirror (Mirr), which is induced by EGFR signaling, directly represses *pipe* transcription, whereas in ventral follicle cells, the HMG-box protein Capicua (Cic) supports *pipe* expression by repressing *mirr*. Although Cic is under negative post-transcriptional regulation by Ras-MAPK signaling in different contexts, the relevance of this mechanism for the interpretation of the EGFR signal during DV pattern formation remains unclear. Here, we consider a model where EGFR-mediated downregulation of Cic modulates the spatial distribution of Mirr protein in lateral follicle cells, thereby contributing to define the position at which the *pipe* expression border is formed.

A remarkable feature of *Drosophila* development is the highly elaborate maternal control that is required for embryonic patterning. This is perhaps best illustrated by the establishment of embryonic

dorsoventral (DV) polarity, which requires multiple maternal functions that regulate the progressive transfer of DV positional information from the ovarian egg chamber to the embryo.^{1,2} This transfer occurs in two main steps. First, the ventral follicle cells around the oocyte secrete localized determinants that are incorporated in the ventral portion of the vitelline membrane, the innermost eggshell layer, where they remain stored until fertilization takes place. Then, following fertilization, the ventral determinants initiate a secondary ventralizing signal from the vitelline membrane to the embryo. This signal relies on a cascade of serine proteases that cleaves the Spätzle ligand so that it can activate the Toll receptor on the ventral surface of the embryo. When Toll is activated, the Rel-family protein Dorsal is translocated to the nucleus, where it acts as a morphogen to regulate the expression of zygotic genes along the embryonic DV axis.^{1,3}

While many aspects of this inductive process have been well established, the mechanisms regulating its early steps in the follicle cell layer have remained less understood. A key regulator in this context is Pipe, a sulfotransferase that is homologous to vertebrate glycosaminoglycan-modifying enzymes.⁴ Pipe, which is expressed in ventral, but not dorsal, follicle cells, mediates sulfation of structural proteins that are secreted by the follicle cells and become embedded in the ventral side of the vitelline membrane.^{4,5} These sulfated components function as the ventral determinants required to activate the proteolytic cascade that triggers Spätzle-Toll

Keywords: *Drosophila*, dorsoventral patterning, oogenesis, follicle cells, EGFR signaling, Capicua, Mirror, MAPK, *pipe*

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signaling, possibly by promoting functional interactions between the Snake and Easter proteases.⁶

A long-standing issue has been how *pipe* transcription is initially restricted to the ventral follicle cells. In fact, the first symmetry-breaking event that determines DV polarity in the egg chamber occurs in dorsal positions. By mid-oogenesis (stage 7–8), the oocyte nucleus migrates to a cortical, anterior location within the oocyte, which will become the future dorsal-anterior (DA) region of the egg. The asymmetric positioning of the oocyte nucleus directs the transport of *gurken* transcripts to the DA corner of the oocyte. By stage 9–10, the Gurken product, a TGF- α -related factor, is secreted from the oocyte and activates the EGF receptor (EGFR) in the overlying DA follicle cells (reviewed in ref. 7). Activated EGFR then signals through the Ras-MAPK pathway and regulates DV polarity by repressing *pipe* expression in DA and lateral follicle cells.^{8–10} Genetic analyses have shown that this repression is cell-autonomous, indicating that it occurs in direct response to EGFR activity and does not require other secondary, diffusible signals (see also below). Furthermore, since EGFR signaling forms a dorsal-to-ventral gradient (due to diffusion of Gurken from the dorsal side), whereas *pipe* exhibits a rather sharp border of expression, the repression of *pipe* probably represents a switch-like response above a certain threshold of EGFR activity.¹¹

Mechanism of *pipe* Repression

Recently, work from different laboratories, including ours, has characterized the molecular pathway by which EGFR signaling represses *pipe* transcription.^{12–14} The results show that Mirror (Mirr), a homeodomain transcription factor induced by EGFR signaling in DA follicle cells,^{15,16} represses *pipe* expression by binding to a cis-regulatory element in the *pipe* upstream region. This repression occurs cell-autonomously in all dorsal and lateral follicle cells where *pipe* is normally off, implying that Mirr is responsible for the repressive activity of EGFR signaling on *pipe* transcription.¹² Given that Mirr is distributed forming a gradient with high levels in DA cells and low levels in

lateral follicle cells^{15,16} (see below), the simplest model derived from these studies is that Mirr establishes a lateral repression threshold for *pipe* transcription that prevents its activation by broadly distributed factors. Ultimately, these opposing inputs would be integrated at the level of *pipe* cis-regulatory elements, thus generating sharp borders of *pipe* expression.

Previous studies had demonstrated a role of Mirr in the specification of DA follicle cells that direct the formation of dorsal respiratory appendages in the eggshell.^{17,18} This process is also initiated by EGFR signaling, but was considered to be independent of the gene circuit controlling embryonic patterning. However, the current evidence shows that Mirr is a key factor in both processes. Interestingly, Fuchs et al. (ref. 13) have identified related Mirr-responsive elements in *pipe* and the *broad* gene, an EGFR-regulated target involved in dorsal appendage morphogenesis. The authors found that Mirr represses both genes in dorsal follicle cells during stage 9–10A, whereas by stage 10B Mirr plays a role in activating a late *broad* enhancer. This appears consistent with a more complex regulation of eggshell morphogenesis compared with that of embryonic patterning.^{13,17,19}

Role of Capicua in DV Patterning

We have also addressed the role of Capicua (Cic) in DV axis formation.¹² Cic is an HMG-box repressor that functions as a sensor of Ras-MAPK signaling pathways.²⁰ In the ovary, Cic activity is essential to maintain *pipe* expression in all ventral follicle cells; consequently, loss of maternal Cic function leads to severe dorsalization of the embryo.²¹ However, the mechanism of Cic function in this context was unclear. It turns out that Cic represses *mirr* expression in ventral follicle cells, thereby supporting *pipe* transcription in this region.^{12,17} Cic-mediated repression of *mirr* is particularly evident in ventral anterior follicle cells: mosaic analyses using *cic* mutations cause clear derepression of *mirr* in anterior but not posterior regions of the follicular epithelium.¹⁷ The interpretation of this regional effect is that Cic blocks the ventral induction of *mirr* by anterior positional cues that may

include a Decapentaplegic (Dpp)/TGF β signal.¹⁷ These anterior inputs may function cooperatively with EGFR signaling to activate *mirr* expression in DA cells, whereas they must be counteracted via Cic repression in ventral cells.^{17,20} Importantly, we have shown that the same repressor circuit operates in ventral-posterior regions, where *pipe* expression also depends on Cic-mediated repression of *mirr*.¹² Thus, loss of Cic activity in ventral-posterior follicle cells induces low-level activity of Mirr that is nevertheless sufficient to repress *pipe*.

EGFR-Mediated Downregulation of Cic

In several contexts, Ras-MAPK activation leads to downregulation of Cic nuclear levels.²⁰ In DA follicle cells, MAPK-dependent phosphorylation of Cic causes partial re-localization of Cic protein to the cytoplasm, reducing its nuclear levels by ~50%.²² This may contribute to the induction of *mirr* transcription through derepression. However, EGFR signaling has a positive input on *mirr* expression that is independent of Cic: *cic* mutant egg chambers show high expression of *mirr* in DA cells and low ectopic expression in ventral cells, whereas *gurken; cic* double mutant ovaries show low *mirr* expression throughout the anterior circumference of the epithelium.¹⁷ Thus, although Cic is essential for DV axis formation, the contribution of EGFR-dependent downregulation of Cic in this process is unclear.^{12,17,22}

To re-examine this issue, we have tested if EGFR-mediated downregulation of Cic detectably reduces Cic repressor activity in dorsal follicle cells during stage 10. To this end, we analyzed a synthetic enhancer composed of Cic repressor and Gal4 activator binding sites (*CUASC*; Fig. 1A). This enhancer functions as a sensor of Cic-mediated repression in the presence of Gal4 protein.²³ We find that a *CUASC-lacZ* reporter shows preferential dorsal transcription in egg chambers with uniform Gal4 expression (Fig. 1B), and that this pattern expands ventrally in *f(1)K10* mutant ovaries (Fig. 1C), which display ectopic EGFR activity in ventral regions.²⁴ This indicates that Cic repressor activity is indeed downregulated by

EGFR signaling in dorsal follicle cells, either through the observed decrease in Cic nuclear concentration, or through some additional inhibition of Cic function in response to MAPK phosphorylation.

We have also analyzed a Cic mutant derivative, *Cic^{ΔC2}*, which lacks a MAPK docking site that is essential for EGFR-mediated downregulation of Cic.²² We previously reported that *Cic^{ΔC2}*, when expressed at physiological levels using genomic transgenes, does not cause overt repression of *mirr* in DA follicle cells²² (see also Fig. 2A and B). This is consistent with the notion that EGFR-induced activation of *mirr* is not just a consequence of Cic downregulation, but involves other unknown mechanism(s)^{17,22} (see above). However, *Cic^{ΔC2}* does cause moderate expansion of *pipe* mRNA expression toward the dorsal side of the egg chamber.²² Using a *pipe-lacZ* reporter, we also see modest expansion of *lacZ* expression in *cic^{ΔC2}* egg chambers (Fig. 2E and F). This suggests that EGFR-mediated downregulation of Cic plays a role in setting up the precise position at which the *pipe* expression border is formed. Below, we argue that this effect on *pipe* expression is a consequence of Cic acting as a repressor of *mirr*.

We have seen that Mirr directly controls the *pipe* expression boundary in lateral and dorsal-posterior follicle cells. In these cells, the levels of Mirr are very low or undetectable, albeit sufficient to mediate repression of *pipe*;¹² see also Figure 2A and E showing that detectable *mirr* expression does not reach the *pipe* domain. Therefore, we reasoned that downregulation of Cic might influence the pattern of Mirr distribution within this region of low Mirr expression, thereby affecting *pipe* transcription. To further test this idea, we have assayed the effects of expressing Cic and *Cic^{ΔC2}* proteins in follicle cell clones using the GAL4-UAS system. We found that overexpression of Cic does not cause detectable changes in *mirr* expression, irrespective of the position where the clone is formed (Fig. 2C). However, Cic overexpression does induce ectopic *pipe* transcription in lateral regions close to the endogenous *pipe* domain, although such ectopic expression did not occur in more dorsal positions

(Fig. 2G). In contrast, high-level expression of *Cic^{ΔC2}* produced full repression of *mirr*, and the corresponding derepression of *pipe*, in all lateral and dorsal clones (Fig. 2D and H). This stronger effect of *Cic^{ΔC2}* is likely attributable to its ability to escape EGFR-mediated downregulation in dorsal cells, so that high Cic activity in the *Cic^{ΔC2}*-expressing clone can effectively repress *mirr*. Thus, changes in Cic repressor activity can affect the expression of *mirr* throughout the entire follicular epithelium, causing either ectopic ventral expression when Cic is absent or complete dorsal repression when Cic activity is increased (refs. 12, 17 and this work). Altogether, the results suggest a competition mechanism between Cic-mediated repression and EGFR-dependent and -independent activation of *mirr*, which leads to graded expression of *mirr* in dorsal and lateral follicle cells. Additionally, this is consistent with a role of Cic downregulation in facilitating *mirr* expression in lateral and dorsal-posterior follicle cells, where low, but functional

Mirr activity defines the *pipe* expression border.

Conclusion

In summary, we propose a model where EGFR signaling regulates the size of the *pipe* expression domain through both Cic-dependent and -independent mechanisms (Fig. 3). In DA cells, EGFR signaling induces Mirr expression, which then represses *pipe*; in this position, downregulation of Cic may contribute to Mirr induction but other EGFR-dependent mechanisms are also involved.^{17,22} In lateral and dorsal-posterior cells, declining levels of EGFR signaling and Mirr reach a threshold where *pipe* is no longer repressed and its border is formed. The precise position of this border is influenced by EGFR-dependent downregulation of Cic, which potentiates *mirr* expression and places the *pipe* border more ventrally. Lastly, in ventral follicle cells, very low EGFR signaling activity and other positive anterior inputs that act on *mirr* (see above)

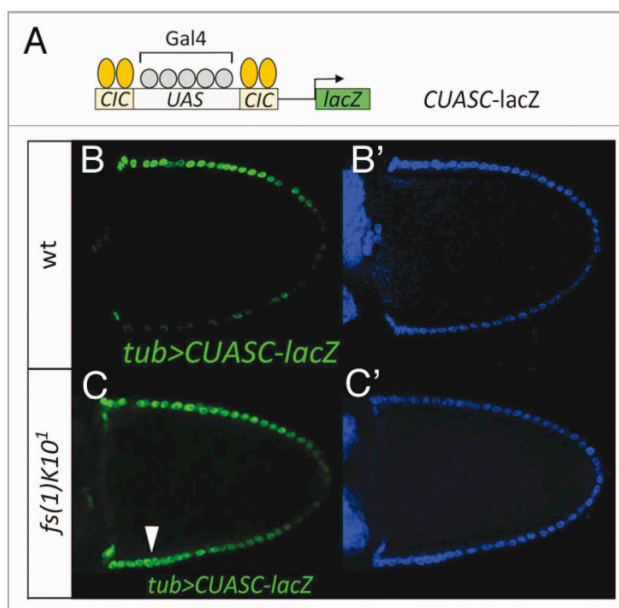


Figure 1. EGFR signaling downregulates Cic repressor activity in dorsal follicle cells. (A) Diagram of the *CUASC-lacZ* reporter construct containing five Gal4/UAS binding sites flanked by two Cic binding sites on either side. (B and C) Lateral views of stage-10 wild-type (B and B') and *fs(1)K101* (C and C') egg chambers carrying *tubulin-Gal4* and *CUASC-lacZ* insertions. Double stainings with anti- β -galactosidase antibody (B and C, green) and DAPI (B' and C', blue) are shown. Dorsal is up as indicated by the dorsal position of the oocyte nucleus under Nomarski optics (not shown). *CUASC-lacZ* expression shows preferential dorsal expression in a wild-type background, and is ectopically expressed in ventral regions of the *fs(1)K101* egg chamber (arrowhead, C).

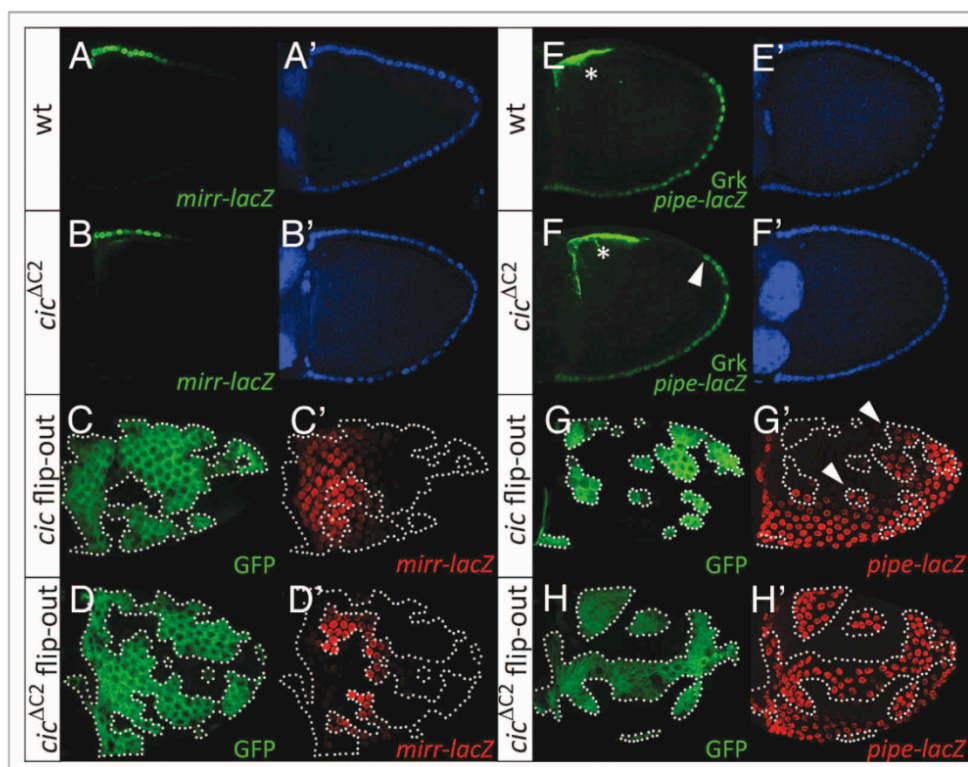


Figure 2. EGFR signaling modulates Cic-mediated repression of *mirr*. (A and B) Lateral views of stage-10 wild-type (A and A') and *cic*^{ΔC2} (B and B') egg chambers carrying the *mirr*^{lacZ} (*mirr-lacZ*; ref. 33) enhancer trap. Double stainings with anti-β-galactosidase (A and B, green) and DAPI (A' and B', blue) are shown. (C and D) Dorsal views of stage-10 mosaic egg chambers expressing Cic (C and C') and *Cic*^{ΔC2} (D and D') proteins in clones marked by expression of GFP (C and D, green). (C' and D') show anti-β-galactosidase stainings of *mirr-lacZ* expression (red). Only *Cic*^{ΔC2} causes full repression of *mirr-lacZ*. (E and F) Lateral views of stage-10 wild-type (E and E') and *cic*^{ΔC2} (F and F') egg chambers carrying the *M2-lacZ* (*pipe-lacZ*; ref. 12) reporter. Stainings with anti-β-galactosidase and anti-Gurken antibodies (E and F, green) and DAPI (E' and F', blue) are shown; the Gurken signals are indicated with asterisks. A single genomic *cic*^{ΔC2} transgene (see ref. 22) leads to expanded *pipe-lacZ* expression by an average of 1.3 cells (n = 20) in the dorsal-posterior region (arrowhead in panel F). (G and H) Lateral views of stage-10 mosaic egg chambers expressing Cic (G and G') and *Cic*^{ΔC2} (H and H') proteins in clones marked by expression of GFP (G and H, green). (G' and H') show anti-β-galactosidase stainings of *M1-lacZ* (*pipe-lacZ*; ref. 12) expression (red). Note that *Cic*-expressing clones show partial derepression of *pipe-lacZ* close to the endogenous *pipe* domain (arrowheads, G'), whereas *Cic*^{ΔC2} causes robust derepression of the reporter in all positions.

are counteracted by Cic, so that *mirr* is repressed and *pipe* can be transcribed.

In addition, we have noted that this patterning network resembles the mechanism by which the antagonistic activities of Torso-Ras-MAPK signaling and Cic coordinate the subdivision of the early embryo along the anteroposterior axis.^{12,20} In this case, Torso signaling at the embryonic poles downregulates Cic activity to induce gap repressors such as Tailless (Tll) and Hucklebein (Hkb), which are kept repressed by Cic in middle regions of the embryo. Cic-mediated repression of *tll* and *hkb* is required for the normal patterning of central body structures, just as Cic-mediated repression of *mirr* is essential for patterning of ventral embryonic fates.

However, downregulation of Cic plays a more critical role in inducing *tll* expression than it does during EGFR-dependent activation of *mirr*.²⁰ Understanding these differential responses will require further analyses of the effector mechanism(s) by which EGFR signaling induces *mirr* expression, and a detailed characterization of other transcriptional inputs that activate *mirr* and *tll* expression in each context.

Finally, there are other cases where positional signals are translated into opposing gradients of repressor factors, which in turn regulate spatially restricted expression of downstream targets. For example, Dpp signaling in the *Drosophila* wing disc generates a complementary gradient of Brinker

activity that represses Dpp targets such as *optomotor blind* and *spalt*.²⁵⁻²⁸ Moreover, activity of the Bicoid morphogen in the early embryo establishes opposing gradients of repressor factors (including Runt) that antagonize Bcd-mediated activation;²⁹ additionally, the Torso-regulated gradient of Cic activity at the anterior pole also represses Bcd targets.³⁰ Similarly, interpretation of the Sonic Hedgehog signaling gradient in the vertebrate neural tube depends on cross-repressive interactions among downstream transcription factors (for example see refs. 31 and 32). Thus, the establishment of antagonistic repressor gradients in response to morphogenetic signals may represent a general regulatory principle in development.

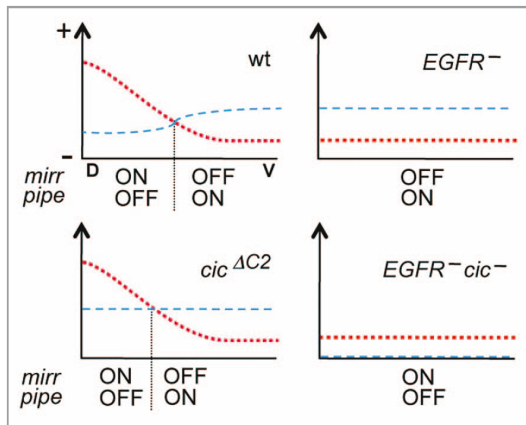


Figure 3. Model for the regulation of *mirr* and *pipe* expression in response to EGFR and Cic activities. Each diagram represents the regulatory inputs that control *mirr* expression in four different genetic backgrounds. For simplicity, only the anterior region of the egg chamber is represented along the DV axis (D, dorsal; V, ventral). The red dotted line represents the combined activation inputs from EGFR signaling and anterior positional cues that may depend on Dpp. The blue dashed line indicates the level of Cic-mediated repression. These inputs are proposed to define the position at which *Mirr* levels are sufficient to repress *pipe* (vertical dotted line), thereby establishing its border of expression. In *cic*^{ΔC2} egg chambers, this position is shifted dorsally as a result of uniform Cic activity throughout the follicular epithelium. Note also that EGFR mutant backgrounds retain a weak activation input from anterior positional cues; this input is insufficient to drive *mirr* expression unless Cic repressor activity is absent.

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ARTICLE 3: Capicua DNA-binding sites are general response elements for RTK signaling in *Drosophila*

Authors

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Reference

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Summary (in Spanish)

Las vías de señalización RTK/Ras/MAPK juegan un papel clave en el desarrollo de los metazoos; sin embargo, se desconoce el mecanismo por el que controlan la expresión de sus genes diana. En *Drosophila*, en general se ha asumido que la mayoría de respuestas transcripcionales a la activación de las vías RTKs dependen de la unión de proteínas de la familia ETS a elementos cis específicos contenidos en las regiones reguladoras de sus genes diana. En este artículo mostramos cómo múltiples vías RTK en *Drosophila* controlan la expresión de genes mediante elementos octaméricos comunes que actúan como lugares de unión del factor HMG-box Capicua (Cic), un represor transcripcional regulado negativamente por la señal de RTK en diferentes contextos. También mostramos cómo la regulación de la expresión de los genes *gap* terminales dependiente de la señal RTK de Torso en el embrión temprano, depende de octámeros de unión a Cic y que dicha unión es esencial para el reclutamiento del correpresor Groucho a la región reguladora de *huckebein* in vivo. Además, indicamos que la posterior activación de la vía de EGFR en la región del neuroectodermo del embrión controla la expresión de genes dorso-ventrales mediante la regulación negativa de Cic, y que dicho control depende de motivos octaméricos de unión a Cic. Por consiguiente, un mecanismo similar de regulación por vías RTKs actúa durante la subdivisión de los ejes antero-posterior y dorso-ventral del embrión. Asimismo, observamos que secuencias octaméricas

idénticas median la regulación dependiente de Capicua de otras dianas de EGFR, como *argos*, en el desarrollo del ala. Cabe remarcar que la simple combinación de lugares de unión a activadores y motivos de unión a Capicua es suficiente para el establecimiento de patrones complejos de expresión génica en respuesta a la activación de las vías de Torso y EGFR en diferentes tejidos. Nuestros resultados indican que los motivos octaméricos de unión a Capicua son elementos generales de respuesta a vías RTK en *Drosophila*.

Capicua DNA-binding sites are general response elements for RTK signaling in *Drosophila*

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SUMMARY

RTK/Ras/MAPK signaling pathways play key functions in metazoan development, but how they control expression of downstream genes is not well understood. In *Drosophila*, it is generally assumed that most transcriptional responses to RTK signal activation depend on binding of Ets-family proteins to specific cis-acting sites in target enhancers. Here, we show that several *Drosophila* RTK pathways control expression of downstream genes through common octameric elements that are binding sites for the HMG-box factor Capicua, a transcriptional repressor that is downregulated by RTK signaling in different contexts. We show that Torso RTK-dependent regulation of terminal gap gene expression in the early embryo critically depends on Capicua octameric sites, and that binding of Capicua to these sites is essential for recruitment of the Groucho co-repressor to the *huckebein* enhancer in vivo. We then show that subsequent activation of the EGFR RTK pathway in the neuroectodermal region of the embryo controls dorsal-ventral gene expression by downregulating the Capicua protein, and that this control also depends on Capicua octameric motifs. Thus, a similar mechanism of RTK regulation operates during subdivision of the anterior-posterior and dorsal-ventral embryonic axes. We also find that identical DNA octamers mediate Capicua-dependent regulation of another EGFR target in the developing wing. Remarkably, a simple combination of activator-binding sites and Capicua motifs is sufficient to establish complex patterns of gene expression in response to both Torso and EGFR activation in different tissues. We conclude that Capicua octamers are general response elements for RTK signaling in *Drosophila*.

KEY WORDS: Capicua, *Drosophila*, RTK signaling

INTRODUCTION

Receptor tyrosine kinase (RTK) signaling pathways control a broad spectrum of developmental decisions, including cell proliferation, differentiation, morphogenesis and survival (Schlessinger, 2000; Simon, 2000). Many RTK pathways signal through the conserved Ras/MAPK cassette, which then leads to phosphorylation of nuclear transcription factors and other cellular proteins. At the transcriptional level, RTK signals induce a wide variety of target gene responses in different contexts, but the molecular mechanisms underlying these responses are not well understood. In *Drosophila*, *in vivo* validated RTK effectors include the Ets factors Pointed and Yan (Simon, 2000; Tootle and Rebay, 2005), the HMG-box repressor Capicua (Cic) (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Astigarraga et al., 2007; Tseng et al., 2007) and the Groucho (Gro) co-repressor (Hasson et al., 2005; Cinnamon et al., 2008; Cinnamon and Paroush, 2008; Jennings and Ish-Horowicz, 2008). Consequently, the analysis of these effectors can provide general insights into the regulatory mechanisms by which RTK signals control gene expression and development.

The *Drosophila* Torso RTK pathway represents an excellent model of transcriptional regulation in response to RTK activation (Furriols and Casanova, 2003). In this system, localized activation of the Torso receptor at each pole (termini) of the early blastoderm embryo controls the specification of terminal body structures by inducing the expression of two zygotic gap genes: *tailless* (*tll*) and *huckebein* (*hkb*) (Pignoni et al., 1990; Brönnert and Jäckle, 1991). This induction involves a mechanism of derepression: both genes are normally repressed in medial regions of the embryo and the Torso signal relieves this repression at the poles (Liaw et al., 1995; Paroush et al., 1997; Jiménez et al., 2000). Repression of *tll* and *hkb* requires several nuclear factors, including Cic and Gro, which are both downregulated by the Torso signal (Paroush et al., 1997; Häder et al., 2000; Jiménez et al., 2000; Goff et al., 2001; Astigarraga et al., 2007; Cinnamon et al., 2008). Thus, loss of Cic or Gro function causes derepression of *tll* and *hkb* in medial regions of the embryo, which then leads to repression of central gap genes such as *knirps* (*kni*) and *Krüppel* (*Kr*) (Paroush et al., 1997; Jiménez et al., 2000; Goff et al., 2001; Löhr et al., 2009) (see Fig. S1 in the supplementary material). Conversely, mutations that render Cic or Gro insensitive to MAPK phosphorylation cause inappropriate repression of *tll* and *hkb* at the poles (Astigarraga et al., 2007; Cinnamon et al., 2008). Additionally, various studies have implicated other factors, such as GAGA/Trx-like, Dorsal, Retained (Retn; also known as Dead-ringer) or Tramtrack, in *tll* and/or *hkb* regulation (Liaw et al., 1995; Häder et al., 2000; Chen et al., 2002).

It is currently assumed that terminal gap genes contain complex enhancer regions that are bound by several, perhaps redundantly acting, transcription factors. However, how these activities converge to regulate Torso-dependent expression of *tll* or *hkb* is not

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understood. For example, analysis of a *hkb* enhancer indicated a role of Dorsal, Retn and Gro in Torso-mediated regulation of this enhancer (Häder et al., 2000). Cic is also required for *hkb* repression, but it has not yet been possible to demonstrate direct binding of Cic to *hkb* cis-regulatory regions (Jiménez et al., 2000). Recently, a DNA-binding motif for the Cic protein has been identified in humans (Kawamura-Saito et al., 2006), and it has been noted that this motif resembles a short regulatory element in the *tl* upstream region, the *torso response element* (*tor-RE*), which restricts *tl* expression to the posterior pole of the embryo (Liaw et al., 1995; Löhr et al., 2009). Consequently, it is possible that Cic represses *hkb* expression by binding to *tor-RE*-like elements, thus contributing to Torso-dependent regulation of this target.

Here, we report that *tor-RE*-like octameric sequences present in the *hkb* enhancer region function as binding sites for Cic and play a central role in the response of this target to Torso regulation. We also show that these Cic-binding motifs are essential for recruitment of the Gro co-repressor to *hkb* enhancer sequences in vivo. We then show that similar elements control the restricted expression of the *intermediate neuroblasts defective* (*ind*) gene in the neuroectodermal region of the embryo. This regulation occurs downstream of the EGFR RTK signaling pathway, indicating that Cic-binding sites function downstream of different RTK signals. Identical sites mediate Cic-dependent regulation of another EGFR target, *argos*, in the developing wing. Using synthetic enhancer constructs, we find that Cic octamers are sufficient to provide the regulatory information necessary to translate RTK signaling inputs into precise transcriptional responses in different tissues. We conclude that Cic octameric sites are general response elements for RTK signaling in *Drosophila*.

MATERIALS AND METHODS

DNA constructs

A GST-Cic^{HMG} expression construct was generated by amplifying a fragment encoding the *Drosophila* Cic HMG-box region (corresponding to residues 481–580) with primers *hmg1* (5' AAT GAA TTC CCG CAG CTG GGC AGC 3') and *hmg2* (5' TAT CCC GGG TCC GCT CGC CTT TCC 3'), and subcloning the resulting fragment into *pGEX-6P-2*. This construct (*pGEX-6P-2-Cic^{HMG}*) is structurally equivalent to the *pGEX6P-2-Cic-HMG* construct from human Cic made by Kawamura-Saito et al. (Kawamura-Saito et al., 2006).

To generate *hkb^{0.4}-lacZ*, the *hkb^{0.4}* fragment was amplified using primers *hkb1* (5' AAT GAA TTC ACG TTC GCT GGC CGA G 3') and *hkb2* (5' GAA GGA TCC ATA AAA CGC GGT CCG 3'), digested with *EcoRI* and *BamHI*, and subcloned in *EcoRI/BamHI*-digested *pCaSpeR-hs43-lacZ*. *hkb^{0.4mut}-lacZ* was made similarly but using a *pUC57-hkb^{0.4mut}* plasmid template in which the two TGAATGAA sites had been mutated to CACACGCA by recombinant PCR.

hb-lacZ was generated by amplifying a 270 bp *hb* enhancer with primers *hb1* (5' ATG AAT TCG CTA GCT GCC TAC TCC 3') and *hb2* (5' AAT GCG GCC GCA CGC GTC AAG GGA 3') and digesting the resulting product with *EcoRI* and *NcoI* for cloning into *pCaSpeR-hs43-lacZ*. *hbC-lacZ* was made by inserting two TGAATGAA sites as *NcoI-SpeI* and *SpeI-BamHI* adaptors downstream of the *hb* sequence.

Bcd-lacZ was made by amplifying a synthetic array of four Bcd-binding sites separated by scrambled spacers (Hanes et al., 1994), digesting the PCR product with *EcoRI* and *BamHI*, and subcloning the resulting fragment in *pCaSpeR-hs43-lacZ*. To generate *CBcdC-lacZ*, we first joined a 45 bp module from *hkb^{0.4}* containing two TGAATGAA sites with the above Bcd-binding site fragment using recombinant PCR. This fragment was subcloned upstream of a second copy of the above 45 bp element to create a *CBcdC* module, which was then inserted as an *EcoRI-BamHI* fragment into *pCaSpeR-hs43-lacZ*. *CBcdC^{TRE}-lacZ* and *CBcdC^{mut}-lacZ* were made similarly, using versions of the *hkb* 45 bp module mutated to TCAATGAA or CACACGCA, respectively.

ind^{0.5}-lacZ was created by amplifying the *ind^{0.5}* fragment with primers *ind1* (5' AAT GAA TTC AAA CGT TTT GTT ATA ATC 3') and *ind2* (5' GAA GGA TCC GGA AGA CAC TTC ATG 3'), and subcloning the resulting fragment in *pUC57*. The 0.5 kb *ind^{0.5}* fragment was then recovered by digesting the *pUC57-ind^{0.5}* plasmid with *BamHI* and (partially) with *EcoRI*, and ligated to *EcoRI/BamHI*-digested *pCaSpeR-hs43-lacZ*. *ind^{0.5mut}-lacZ* was made similarly using a *pUC57-ind^{0.5mut}* plasmid template in which the TGAATGAA sites had been mutated to CACACGCA by recombinant PCR.

argos^{1.0}-lacZ was generated using the *argos^{1.0}* enhancer fragment amplified with primers *argos1* (5' ATG AAT TCG AGA TGA AAG TTT ATA G 3') and *argos2* (5' CAT TTT CAC ACC TGA CTG CAG 3'), and subcloning the resulting fragment in T-overhang *pUC57*. *argos^{1.0}* was then recovered as an *EcoRI-BamHI* fragment and subcloned into *pCaPLZ*. *argos^{1.0mut}-lacZ* was made similarly using the corresponding *argos^{1.0mut}* fragment carrying mutated Cic sites (CACACGCA).

CUASC-lacZ was made by first joining five tandem Gal4-binding sites to the 45 bp module from *hkb^{0.4}* containing two Cic sites. This fragment was then inserted upstream of a second copy of the Cic-site module to create a *CUASC* enhancer, which was then subcloned as an *EcoRI-BamHI* fragment in *pCaPLZ*.

Protein expression and EMSA experiments

GST-HMG-box fusion proteins were expressed and purified as described previously (Paroush et al., 1994). In vitro binding assays were carried out as described by Kawamura-Saito et al. (Kawamura-Saito et al., 2006). Briefly, incubations were performed in a 15 µl volume containing 0.1–0.2 µg of GST-HMG-box protein, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 6% glycerol, 0.5% Triton-X100, 10 µg BSA, 1–2.5 µg poly(dI-dC) and 1 µg of single-stranded DNA. After 15 minutes of preincubation at 4°C, ~0.05 pmol of ³²P-labeled DNA probe was added and the incubation was continued for another 45 minutes at the same temperature. Reactions were resolved on 5% nondenaturing polyacrylamide gels at 4°C in 0.5× TBE.

Drosophila stocks

The *cic¹*, *cic²*, *cic^{fe1E11}* and *tor⁴⁰²¹* alleles have been described before (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Klinger et al., 1988). *cic^{AC2}* embryos were obtained from transheterozygous females carrying two different *cic^{AC2}* insertions (Astigarraga et al., 2007). Embryos devoid of maternal *gro* activity were obtained using the *gro^{34B36}* allele (Jennings et al., 2008) in combination with the *ovo^D-FLP-FRT* system (Chou et al., 1993). Embryos lacking maternal *Ras* function, alone or in combination with *cic*, were generated similarly using the *Ras^{AC40b}* and *cic^{Q474X}* alleles (Tseng et al., 2007). *dorsal* (*dl*) mutant embryos were derived from *dl¹/dl⁴* mothers (FlyBase). Other transgenic insertions and mutants used were *cic-HA* construct (Astigarraga et al., 2007), *argos^{w11}* (Freeman et al., 1992), the *rho^{ve} vn¹* combination (Diaz-Benjumea and García-Bellido, 1990), *UAS-cic* (Lam et al., 2006) and *UAS-λop* (Quecuan et al., 1997). Transgenic lines were obtained by standard P-element transformation and several independent lines were analyzed for each reporter construct.

Embryo and wing disc analyses

Embryos were fixed in 4% formaldehyde-PBS-heptane for 20 minutes. In situ hybridizations were carried out using digoxigenin-UTP labeled antisense RNA probes, and anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche). Immunostainings were performed using the following primary antibodies: anti-dpErk (Cell Signaling; 1:50 dilution), anti-HA (12CA5, Roche; 1:400 dilution) and anti-β-galactosidase (40-1a, Developmental Studies Hybridoma Bank; 1:250 dilution). Signals were detected using secondary fluorochrome-conjugated antibodies (Molecular Probes). Embryos were mounted in Permount (in situ hybridizations) or Fluoromount-G (immunostainings). Wing discs were fixed in 4% paraformaldehyde-PBS for 20 minutes, processed for immunostaining using anti-HA and anti-β-galactosidase (anti-β-Gal) antibodies, and mounted in Fluoromount-G.

Chromatin immunoprecipitation assays

ChIP assays were performed using staged embryo collections from homozygous lines containing the *hkb^{0.4}-lacZ* or *hkb^{0.4mut}-lacZ* transgenes. Embryos were dechorionated in 100% bleach and subsequently fixed for 20 minutes in 10 ml crosslinking buffer (3% formaldehyde, 50 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl) and 30 ml heptane. Crosslinking was stopped with 125 mM glycine. Crosslinked chromatin was sheared by sonication to an average size of 500 bp and immunoprecipitated using anti-Gro antibodies (two different rabbit polyclonal antisera raised against the N-terminal region of the protein). Control experiments using pre-immune serum or no antibody resulted in signals below 0.05% of input. Immunoprecipitated complexes were sequentially washed with low salt buffer [50 mM HEPES (pH 7.9), 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 140 mM NaCl, 0.1% deoxycholate], high salt buffer [50 mM HEPES (pH 7.9), 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, 0.1% deoxycholate], LiCl buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 0.5% deoxycholate, 0.5% NP-40] and TE. The chromatin was eluted with TE containing 1% SDS and 0.1 M NaHCO₃, and cross-linking was reversed by incubating at 65°C overnight. The resulting DNA was purified by chloroform extraction and ethanol precipitation, and quantified by qPCR using the FastStart SYBR Green Master Mix (Roche) on an Opticon Monitor 2 system (Bio-Rad). Three to five independent biological replicates (in which independent embryo collections were subjected to separate crosslinking and IP before separate qPCR) were analyzed for each amplicon. As reported in other Gro ChIP studies (Martinez and Amosti, 2008), these replicates produced some variability reflected in the standard deviation (s.d.) of the data. Nevertheless, the results were highly consistent over multiple experiments using the two anti-Gro antibodies. A *P*-value was calculated by comparing *hkb^{0.4}* with *hkb^{0.4mut}* for all data points from amplicons within *hkb^{0.4}* (amplicons C-F) using a two-tailed *t*-test.

RESULTS

Cic represses *hkb* expression via TGAATGAA octamers

The human Cic protein binds the octameric sequence TGAATG(G/A)A (Kawamura-Saito et al., 2006). This element exhibits a single-nucleotide mismatch when compared with the core sequence of the *tor-RE*, TGCTCAATGAA (Liaw et al., 1995; Löhr et al., 2009). We assayed the ability of human and *Drosophila* Cic to bind to TGAATGAA and TCAATGAA sequences in gel-shift assays and observed similar interactions with both sites, indicating that T(G/C)AATGAA motifs are recognized by *Drosophila* Cic in vitro (see Fig. S2 in the supplementary material). We have also analyzed the role of Cic in repression of *tll* via the *tor-RE*. Using transgenes that contain *tll* enhancer sequences (Liaw et al., 1995), we provide evidence that Cic represses *tll* by binding to the *tor-RE*, and that this repression is inhibited by Torso signaling at the posterior pole (see Fig. S3 in the supplementary material).

We then searched for T(G/C)AATGAA motifs in the *hkb* upstream region and identified several TGAATGAA elements that are well conserved among *Drosophila* species (Fig. 1A; data not shown). Two such conserved sites are included in the *hkb* enhancer region identified by Häder et al. (Häder et al., 2000). To test whether Cic represses *hkb* through these motifs, we first defined a minimal *hkb* enhancer fragment that accurately reproduces the endogenous *hkb* pattern (Fig. 1A; data not shown). This 0.4 kb enhancer (designated *hkb^{0.4}*) directs highly restricted expression at both poles of the embryo (Fig. 1B). This pattern depends on Cic repression because it expands in *cic¹* embryos lacking maternal *cic* function (Fig. 1C) (Jiménez et al., 2000). Conversely, *cic^{ΔC2}* embryos expressing a Cic derivative insensitive to Torso-mediated downregulation (Astigarraga et al., 2007) show diminished *hkb^{0.4}-lacZ* expression at both poles (Fig. 1D). Mutagenesis of the two

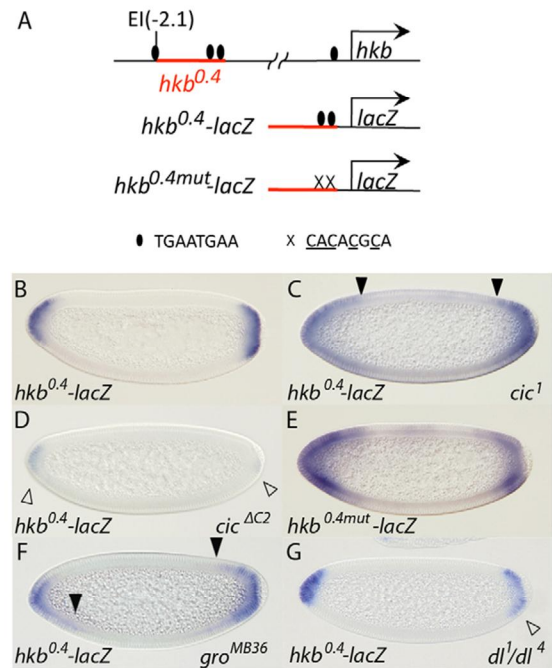


Fig. 1. Torso signaling regulates *hkb* expression via TGAATGAA repressor elements. (A) The *hkb* locus depicting the *hkb^{0.4}* enhancer (red line). E1, EcoRI restriction site located 2.1 kb upstream of the transcription start site. The structure of *lacZ* reporters is shown below. (B-G) mRNA expression patterns of *hkb^{0.4}-lacZ* (B-D, F, G) and *hkb^{0.4mut}-lacZ* (E) in otherwise wild-type (B, E), *cic¹* (C), *cic^{ΔC2}* (D), *gro^{MB36}* (F) and *dl¹/dl⁴* (G) embryos. Closed arrowheads in C and F indicate derepressed *hkb^{0.4}-lacZ* expression in *cic¹* and *gro^{MB36}* embryos. Open arrowheads in D and G indicate reduced *hkb^{0.4}-lacZ* expression in *cic^{ΔC2}* and *dl¹/dl⁴* embryos.

TGAATGAA sites in *hkb^{0.4}-lacZ* causes expanded reporter expression that resembles the pattern of *hkb^{0.4}-lacZ* in *cic¹* embryos (Fig. 1E). We conclude that regulation of *hkb* expression requires direct binding of Cic to conserved TGAATGAA cis-acting octamers.

For comparison, we also analyzed *hkb^{0.4}-lacZ* expression in embryos devoid of maternal Gro function. Gro activity is essential for restricting *tll* and *hkb* expression to the embryonic poles, although the mechanism of Gro action in this context remains uncertain (Paroush et al., 1997; Jiménez et al., 2000; Häder et al., 2000; Cinnamon et al., 2008) (see below). As shown in Fig. 1F, there is significant *hkb^{0.4}-lacZ* derepression in *gro^{MB36}* mutant embryos, similar to the effect seen in *cic¹* embryos. Thus, both Cic and Gro play similar roles in repressing the *hkb^{0.4}* enhancer. By contrast, embryos lacking Dorsal activity, another maternal regulator which functions as both an activator and repressor and is implicated in *hkb* regulation (Häder et al., 2000; Hong et al., 2008), displayed reduced *hkb^{0.4}-lacZ* expression at the posterior pole (Fig. 1G), indicating that Dorsal is required for activating *hkb* expression in posterior regions (see below).

Cic repressor sites are sufficient to mediate Torso-dependent regulation

Although binding of Cic to *hkb^{0.4}* is essential for repressing this enhancer, the response to Torso regulation might involve additional factors bound to the enhancer. To address this issue, we asked

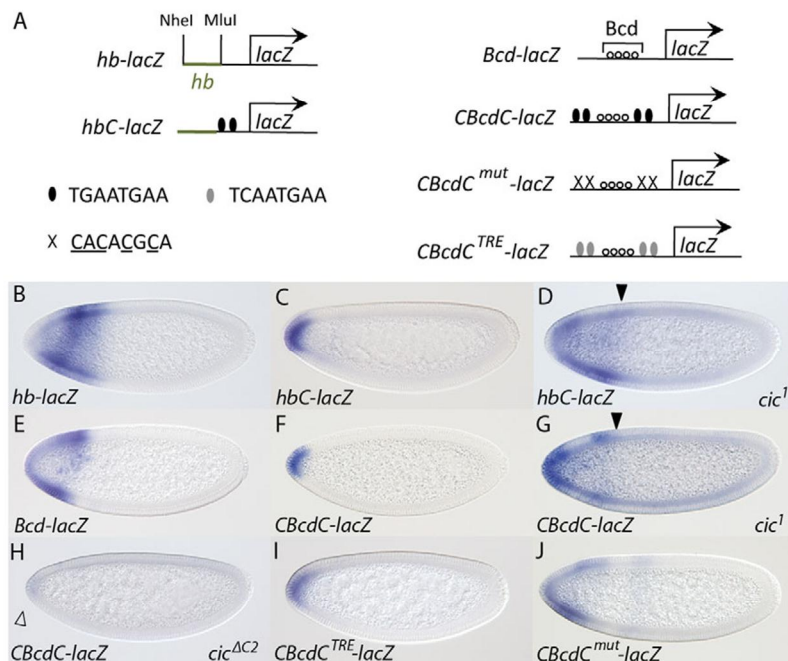


Fig. 2. Cic-binding motifs confer Torso-dependent regulation to synthetic enhancers.

(A) Diagram of *lacZ* reporters containing Bcd-activating sequences and T(G/C)AATGAA sites. The 270 bp *hb* enhancer (delimited by *NheI* and *MluI* restriction sites) is indicated in green. (B–J) mRNA expression patterns of *hb-lacZ* (B), *hbC-lacZ* (C,D), *Bcd-lacZ* (E), *CBcdC-lacZ* (F–H), *CBcdC^{TRE}-lacZ* (I) and *CBcdC^{mut}-lacZ* (J) in otherwise wild-type (B,C,E,F,I,J), *cic¹* (D,G) and *cic^{ΔC2}* (H) embryos. Closed arrowheads in D and G indicate expanded *hbC-lacZ* and *CBcdC-lacZ* expression in *cic¹* embryos. The open arrowhead in H indicates residual *CBcdC-lacZ* expression at the anterior pole.

whether Cic octamers are sufficient for Torso-dependent regulation of synthetic enhancers. We first tested whether Cic-binding sites (TGAATGAA) linked to a heterologous enhancer would make it responsive to Torso regulation. We selected a 270 bp promoter fragment from the *hunchback* (*hb*) gene, which normally drives intense staining in the anterior third of the embryo (construct *hb-lacZ*; Fig. 2A,B) (Struhl et al., 1989). Linking the same fragment to a single pair of Cic-binding motifs (construct *hbC-lacZ*) caused restricted expression from ~91 to 100% embryo length (EL; 0% being the posterior tip of the embryo; Fig. 2C). This pattern resembles the anterior domain of *hkb* expression and precisely corresponds to the area of Cic downregulation by the Torso pathway (Jiménez et al., 2000; Kim et al., 2010). Furthermore, this pattern depends on Cic because it expands posteriorly in *cic¹* embryos (Fig. 2D). Thus, the addition of Cic repressor sites confers Torso-dependent expression to the *hb* enhancer.

The *hb* enhancer is activated by the anteriorly expressed Bicoid (Bcd) factor (Struhl et al., 1989; Driever and Nüsslein-Volhard, 1989). Therefore, we tested whether a simple combination of Bcd and Cic sites would also respond to Torso regulation. A construct containing four multimerized Bcd sites drive anterior expression from ~73 to 100% EL (construct *Bcd-lacZ*; Fig. 2A,E). By contrast, a transgene in which the Bcd sites are flanked by two Cic sites on either side is expressed in a restricted pattern from 92 to 100% EL (construct *CBcdC-lacZ*; Fig. 2A,F). In *cic¹* embryos, *CBcdC-lacZ* expression expands posteriorly up to ~74% EL (Fig. 2G), whereas it almost disappears in *cic^{ΔC2}* embryos (Fig. 2H). A similar construct containing TCAATGAA sites corresponding to the *torRE* (*CBcdC^{TRE}-lacZ*) also showed highly restricted expression in the Torso signaling domain (Fig. 2I). Finally, mutation of the four Cic sites to CACACGCA caused derepressed reporter expression similar to the *Bcd-lacZ* pattern (construct *CBcdC^{mut}-lacZ*; Fig. 2J). These results indicate that Cic repressor sites combined with Bcd activator sequences are sufficient to provide a direct highly localized readout of Torso signaling activity at the anterior pole.

Cic-binding motifs are required for recruitment of Gro to the *hkb* enhancer

Because the Gro co-repressor does not bind DNA, it is believed to be recruited to terminal enhancers by one or more DNA-bound repressors (Paroush et al., 1997; Häder et al., 2000; Jiménez et al., 2000; Cinnamon et al., 2008; Jennings and Ish-Horowitz, 2008). We and others have proposed different mechanisms by which Gro could interact with terminal repressors such as Dorsal, Retn or Cic to silence *tll* and *hkb* expression (Häder et al., 2000; Jiménez et al., 2000). Given that *hkb^{0.4}-lacZ* expression depends on both Gro activity and intact Cic regulatory sites (Fig. 1), we analyzed whether such sites are required for recruitment of Gro to the *hkb^{0.4}-lacZ* transgene by chromatin immunoprecipitation (ChIP) assays using anti-Gro antibodies and qPCR. These experiments were performed using staged embryo collections (90–180 minutes after egg laying) carrying two copies of *hkb^{0.4}-lacZ*. We designed a set of amplicons that span the *hkb^{0.4}* enhancer and the flanking sequences present in the reporter construct (Fig. 3A). Some of these amplicons (A, B, G, H and I) are specific for the reporter and do not amplify endogenous genomic sequences, whereas amplicons C–F potentially amplify both the homozygous transgenic and endogenous *hkb^{0.4}* enhancers. As shown in Fig. 3B, we found association of Gro with most of the intact *hkb^{0.4}* enhancer (blue bars for amplicons C–F), but not with regions flanking the enhancer (amplicons A, B, G and I), although a small peak is observed at the transcriptional start site (amplicon H). Interestingly, within the enhancer, Gro levels were somewhat higher upstream of the Cic sites (amplicons D and E). This upstream region includes binding sites for Dorsal and Retn (Fig. 3A), two factors that have been implicated in *hkb* regulation and are known to bind Gro directly (Dubnicoff et al., 1997; Valentine et al., 1998; Häder et al., 2000).

We then used the same approach to assay binding of Gro to the *hkb^{0.4mut}* enhancer containing mutant Cic sites. In this case, Gro was detected at significantly lower levels compared with the wild-

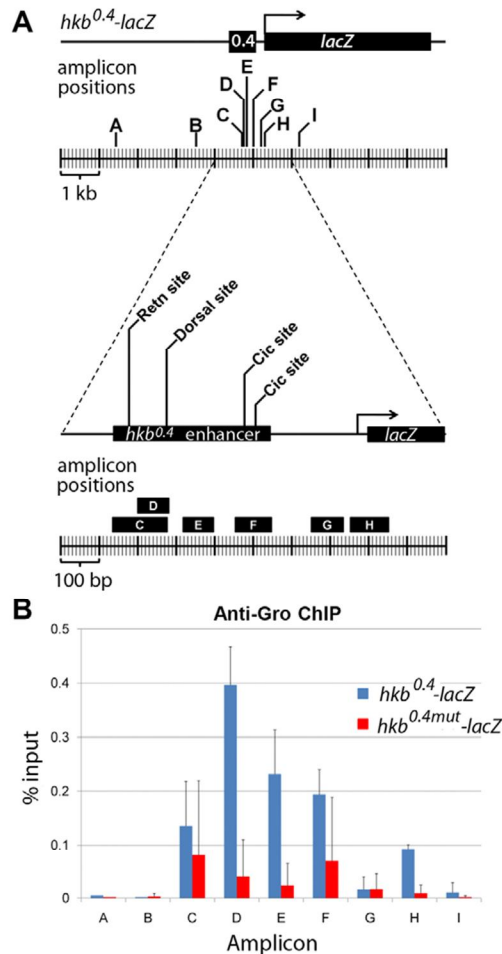


Fig. 3. Association of Gro with the *hkb^{0.4}* enhancer requires intact Cic regulatory sites. (A) The *hkb^{0.4}-lacZ* transgene contains the 0.4 kb *hkb* enhancer, which includes two Cic-binding sites, a Dorsal-binding site and a Retn-binding site upstream of the *lacZ* reporter. The positions of amplicons A-I are shown relative to *hkb^{0.4}-lacZ*. (B) Crosslinked chromatin was isolated from embryos carrying the *hkb^{0.4}-lacZ* (blue bars) or the *hkb^{0.4mut}-lacZ* (red bars) transgenes. Anti-Gro ChIP was assayed by qPCR using amplicons A-I. Each bar represents the average ($\pm$ s.d.) of three to five independent biological replicates. Background levels resulting from pre-immune ChIP controls were subtracted out of all signals.

type enhancer (red bars in Fig. 3B). This decrease is observed throughout the enhancer, including the region upstream of the mutant Cic sites. Averaging across the four amplicons within the enhancer, we find that mutagenesis of the Cic sites reduces Gro association with the enhancer by 4.5-fold ($P < 0.01$). As amplicons C-F should detect signals derived from both transgenic and endogenous enhancers, the larger than twofold decrease observed for the mutant sample suggest a higher efficiency of Gro immunoprecipitation from transgenic versus endogenous *hkb^{0.4}* sequences, perhaps owing to a more open conformation of the transgene. Similar data were obtained using two different anti-Gro antibodies (data not shown). Taken together, these results indicate that binding of Cic to specific sites in *hkb^{0.4}* is essential for recruitment of Gro to this enhancer.

Cic represses *ind* expression downstream of EGFR signaling

We searched for potential Cic-binding sites in genes that might be targets of other RTK signaling pathways. One gene identified in these analyses, *ind*, functions as a target of the EGFR RTK pathway in the neuroectodermal region of the embryo (Skeath, 1998; Weiss et al., 1998; von Ohlen and Doe, 2000; Hong et al., 2008). *ind* expression begins at mid stage 5, forming sharp four- or five-cell wide longitudinal stripes on either side of the embryo (Weiss et al., 1998) (Fig. 4C). This pattern requires activator inputs from the Dorsal morphogen, as well as from the EGFR pathway, which is active in lateral domains overlapping the *ind* stripes (Skeath, 1998; von Ohlen and Doe, 2000; Hong et al., 2008) (Fig. 4B,D). The *ind* stripes are limited ventrally by the Ventral neuroblasts defective (*Vnd*) repressor expressed in the ventral neuroectoderm, and dorsally by the dorsal limit of the EGFR signaling domain (Weiss et al., 1998; von Ohlen and Doe, 2000). Previous analyses of *ind* regulation have identified a repressor element (the *A-box* motif) that controls the dorsal limit of *ind* expression through an unknown factor (Stathopoulos and Levine, 2005). We noted that the *A-box* sequence (WTTTCATTCA) matches the complementary sequence of the Cic-binding motif. This, together with the requirement of EGFR signaling for *ind* expression, prompted us to study the role of Cic in *ind* regulation.

We reasoned that activation of *ind* expression by the EGFR pathway could involve downregulation of Cic protein in the lateral ectoderm. Indeed, EGFR activity in the neuroectoderm (as visualized by immunostaining against double-phosphorylated Erk/MAPK) precisely correlates with a sharp decline in Cic protein levels in this region (Fig. 4B). This suggests that EGFR signaling controls the dorsal limit of *ind* expression by defining a corresponding limit of Cic downregulation. We then monitored *ind* expression in *cic¹* embryos and observed altered *ind* stripes that appeared both dorsally expanded at the anterior and retracted from the posterior (Fig. 4E). In embryos derived from *cic¹/cic²* females, which contain even lower Cic activity (Roch et al., 2002), the *ind* stripes are shorter and further expanded towards the dorsal side, resulting in eight- to 10-cell wide staining at late stage 5 (Fig. 4F). Thus, Cic has two effects on *ind* expression: it defines the dorsal limit of the *ind* stripes and maintains their expression in the abdominal region. The latter effect is reminiscent of the indirect positive role of Cic on *kni* and *Kr* expression (see Fig. S1 in the supplementary material), and it could thus reflect repression of *ind* by the terminal gap genes extending from the posterior pole in the absence of Cic. Consistent with this idea, we fail to detect *ind* expression in *torso⁴⁰²¹* gain-of-function mutant embryos where the terminal gap genes are severely derepressed (Fig. 4G) (Klinger et al., 1988; Brönnner and Jäckle, 1991).

To test whether Cic represses *ind* through *A-box* elements, we generated a *lacZ* reporter driven by a 0.5 kb *ind* enhancer fragment encompassing two *A-box* motifs and a Dorsal-binding site (Fig. 4A) (Stathopoulos and Levine, 2005). This reporter (*ind^{0.5}-lacZ*) forms weak, discontinuous lateral stripes of expression in late blastoderm embryos (Fig. 4H). As in the case of endogenous *ind* stripes, the *ind^{0.5}-lacZ* stripes are abolished in embryos lacking EGFR/Ras signaling activity (derived from females carrying *Ras^{ΔC40b}* germ-line clones; Fig. 4D,I). In *cic¹* embryos, the *ind^{0.5}-lacZ* stripes are more uniform and expand by three to five cell diameters dorsally (Fig. 4J), whereas *cic¹/cic²* embryos show ectopic expression almost up to the dorsal midline (Fig. 4K). Additionally, this expanded *ind^{0.5}-lacZ* expression persists in embryos derived from *Ras^{ΔC40b} cic^{Q474X}* double mutant germline

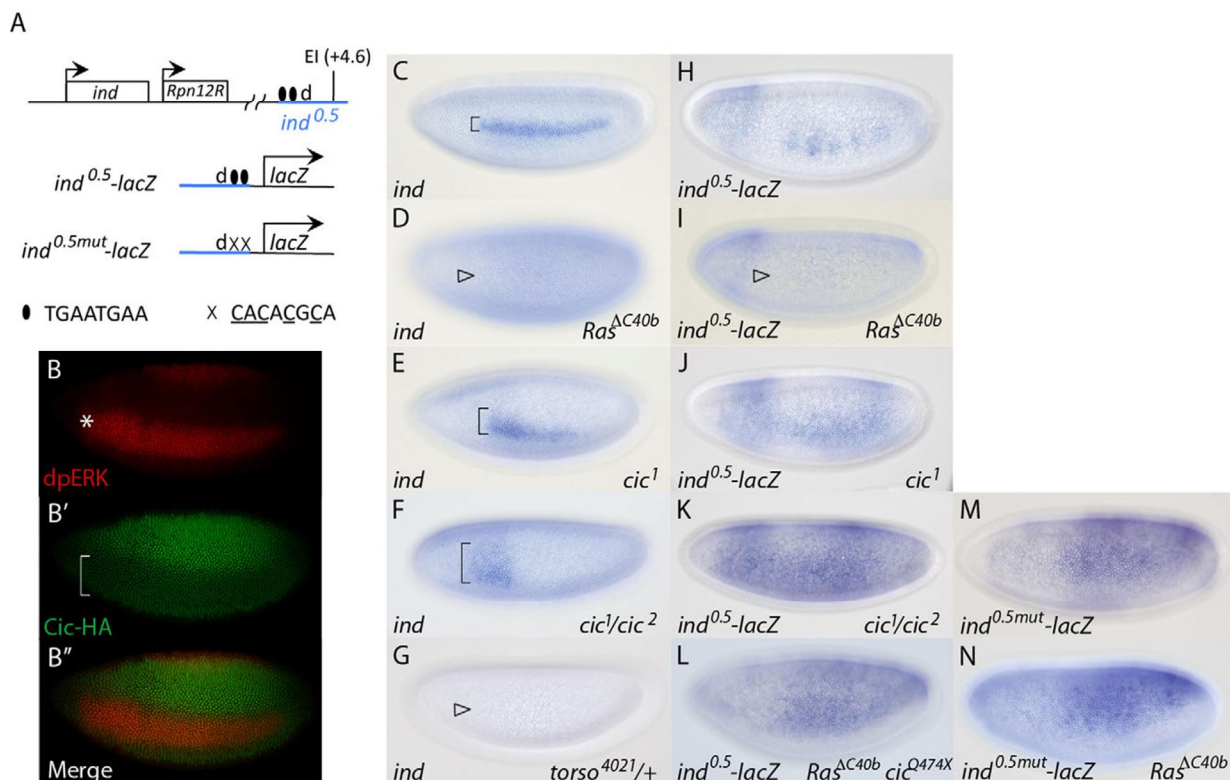


Fig. 4. EGFR induces *ind* expression by relieving Cic repression. (A) The *ind* locus showing the neighboring *Rpn12R* gene (predicted to encode a component of the proteasome) and the *ind^{0.5}* enhancer present in the 3'-flanking region (blue line). EI, EcoRI site present 4.6 kb downstream of the *ind* transcription start site. d, Dorsal-binding site (GGGAAATCCCC). *lacZ* reporters driven by *ind^{0.5}* enhancer sequences are also shown. (B-B'') Stage 5 *cic-HA*; *cic¹* embryo stained with anti-dpERK (red, B) and anti-HA (green, B') antibodies; the merged image is shown in B''. EGFR activation in the lateral neuroectoderm (asterisk in B) produces a corresponding downregulation of Cic levels in ventrolateral regions (bracket in B''). (C-N) *ind* (C-G), *ind^{0.5}-lacZ* (H-L) and *ind^{0.5mut}-lacZ* (M,N) mRNA expression patterns in wild-type (C,H,M), *Ras^{Δ40b}* (D,I,N), *cic¹* (E,J), *cic^{1/cic²}* (F,K), *Ras^{Δ40b} cic^{Q474X}* (L) and *torso^{4021/+}* (G) embryos. All images are lateral surface views of mid- to late-stage 5 embryos. Brackets in C,E,F indicate the maximal width of *ind* stripes. Open arrowheads in D,G,I indicate loss of *ind* and *ind^{0.5}-lacZ* expression in *Ras^{Δ40b}* and *torso⁴⁰²¹* backgrounds.

clones (Fig. 4L), implying that EGFR/Ras signaling normally induces *ind^{0.5}-lacZ* expression by downregulating Cic. Finally, mutation of the two *A-box* motifs in *ind^{0.5}* caused derepression throughout lateral and dorsal regions of the embryo (Fig. 4M), and this pattern was unaffected in embryos lacking EGFR/Ras activity (Fig. 4N), consistent with removal of Cic being sufficient for EGFR-dependent induction of the *ind^{0.5}* enhancer. Together, these results indicate that the *A-box* motifs in *ind* are binding sites for Cic protein that respond to EGFR regulation via Cic derepression.

Cic octamers mediate EGFR-dependent regulation during wing development

Previous analyses showed that Cic behaves as a repressor of *argos* expression in the wing imaginal disc (Roch et al., 2002). *argos* is an EGFR signaling target that encodes a feedback inhibitor of this pathway (Freeman et al., 1992; Golembo et al., 1996). During wing development, EGFR activity defines the position of wing veins and leads to downregulation of Cic in presumptive vein cells, particularly in two rows of cells running along the future wing margin and in prospective veins L3, L4 and L5 (Fig. 5A) (Roch et al., 2002). This pattern of Cic downregulation is markedly complementary to the expression of *argos*, as visualized with the *argos^{w11}* enhancer trap reporter (compare Fig. 5A with 5B) (Gabay

et al., 1997). Reduced Cic function in *cic²/cic^{fatE11}* discs causes *argos^{w11}* derepression in intervein cells at levels similar to those of endogenous wing margin and L5 stripes (Fig. 5C; see also Fig. S4 in the supplementary material) (Roch et al., 2002). This ectopic expression is weaker than in stripes L3 and L4, suggesting that these stripes are subject, at least in part, to Cic-independent regulation, an idea supported by the relatively normal development of the L3-L4 intervein region in *cic* mutant adults (Fig. S4 in the supplementary material). We also analyzed *argos^{w11}* expression in discs lacking both Cic and EGFR signaling activities, using the *cic²/cic^{fatE11}* background in combination with *rhomboid* (*rho*) and *vein* (*vn*) alleles that eliminate EGFR signaling in the wing disc (Martín-Blanco et al., 1999). This caused generalized *argos^{w11}* expression throughout the wing pouch without enhancement in stripes L3 and L4 (Fig. 5D), suggesting that EGFR signaling induces *argos^{w11}* expression in prospective veins by relieving Cic repression, and that an additional EGFR-dependent input reinforces this expression in stripes L3 and L4.

To investigate whether Cic represses *argos* directly through Cic octameric sites, we first identified several conserved TGAATG(G/A) motifs within the first intron of *argos* (Fig. 5E; data not shown). Next, we selected a 1.0 kb intron fragment containing five such sites (four TGAATGAA and one

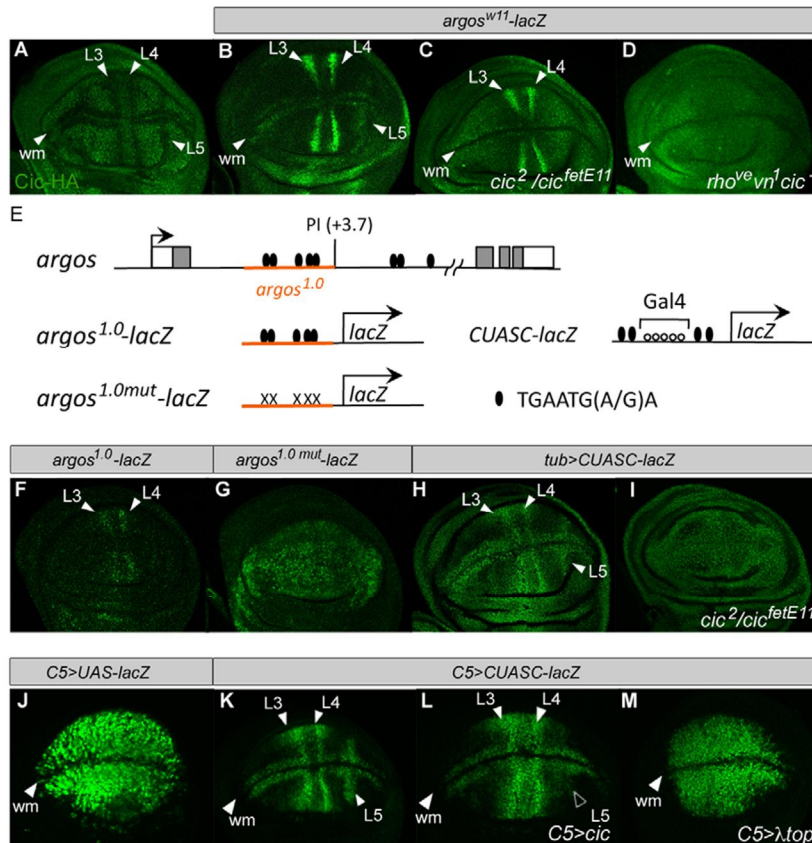


Fig. 5. EGFR signaling regulates *argos* expression through Cic octamers. (A) Staining of *cic*-HA third instar wing disc using anti-HA antibody; arrowheads indicate the stripes of Cic downregulation in response to EGFR signaling. wm, wing margin. (B-D) Anti-β-Gal staining of *argos*^{w11} expression in otherwise wild-type (B), *cic*²/*cic*^{fetE11} (C) and *rho*^{ve} *vn*¹ *cic*²/*rho*^{ve} *vn*¹ *cic*^{fetE11} (D) wing discs. (E) Diagram of the *argos* locus indicating the *argos*^{1.0} enhancer (orange); exons are depicted by boxes and coding sequences are shown in gray. PI, PstI site present 3.7 kb downstream of the transcription start site. The structure of *lacZ* reporters is shown below. (F,G) β-Gal expression patterns of *argos*^{1.0}-*lacZ* (F) and *argos*^{1.0mut}-*lacZ* (G) reporters in wing discs. (H,I) Anti-β-Gal staining of *tubulin-Gal4/CUASC-lacZ* imaginal discs from otherwise wild-type (H) or *cic*²/*cic*^{fetE11} (I) larvae. (J) β-Gal expression in *UAS-lacZ/+; C5-Gal4/+* imaginal disc. (K-M) β-Gal expression patterns resulting from *C5-Gal4*-directed activation of *CUASC-lacZ* in imaginal discs from otherwise wild-type (K), *UAS-cic* (L) or *UAS-λtop* (M) larvae. β-Gal expression is lost in prospective L5 vein cells after Cic overexpression (open arrowhead in L).

TGAATGGA motifs) and other conserved sequences. When placed upstream of a *lacZ* reporter, this fragment (designated *argos*^{1.0}) directs restricted expression in presumptive veins L3 and L4 (Fig. 5E,F), indicating that it mediates partial aspects of *argos* regulation. By contrast, the same fragment carrying mutated Cic sites drives widespread expression in the wing pouch and peripheral regions of the disc (Fig. 5E,G; data not shown). Thus, conserved Cic-binding sites in *argos* restrict its expression to prospective wing vein cells of the disc.

To test whether Cic-binding sites are sufficient to mediate EGFR-dependent regulation in the wing, we assayed an artificial enhancer containing five GAL4-binding sites flanked on either side by two tandem TGAATGAA motifs (construct *CUASC-lacZ*; Fig. 5E). Indeed, inducing ubiquitous GAL4 expression under the control of the *tubulin* or *hsp-70* promoters leads to localized activation of the *CUASC* enhancer in prospective veins (Fig. 5H and data not shown). This restricted pattern depends on Cic, as it becomes significantly derepressed in *cic* mutant discs (Fig. 5I). We also monitored *CUASC-lacZ* expression driven by the *C5-GAL4* line, which is active in the presumptive wing pouch (Fig. 5J) (Yeh et al., 1995). As shown in Fig. 5K, *C5-GAL4* activates *CUASC-lacZ* expression only in presumptive vein cells of the wing pouch. Co-expression of *CUASC-lacZ* and Cic [using an *UAS-cic* construct (Lam et al., 2006)] with the same driver resulted in loss of *lacZ* expression in presumptive vein L5 (Fig. 5L), which correlated with loss of vein L5 in adult wings (Fig. S4 in the supplementary material). Conversely, co-expressing *CUASC-lacZ* together with *UAS-λtop*, which encodes a constitutively active form of EGFR (Queenan et al., 1997), caused severe *lacZ*

derepression throughout the presumptive wing pouch (Fig. 5M; see also Fig. S4 in the supplementary material). This pattern recapitulates *C5-GAL4*-mediated activation of the standard *UAS* enhancer lacking Cic sites (Fig. 5J), and is therefore consistent with generalized downregulation of Cic in the *C5-GAL4>UAS-λtop* background. Thus, our results indicate that EGFR signaling controls *argos* expression through octameric Cic sites, and that such sites are sufficient to define a complex pattern of EGFR-mediated activation in the developing wing.

DISCUSSION

RTK signaling pathways play key functions in metazoan development, but the molecular mechanisms underlying RTK-initiated responses are not well understood. Until recently, it was generally assumed that Pointed and Yan were the only nuclear effectors of all RTK pathways in the fly [see, for example, Simon (Simon, 2000)]. However, several studies have identified the Cic repressor as an important sensor of some of these pathways (Jiménez et al., 2000; Goff et al., 2001; Astigarraga et al., 2007; Tseng et al., 2007). Here, we have shown that Cic regulatory functions downstream of Torso and EGFR signals depend on common TGAATGAA DNA octamers and that, at least in certain assays, these octamers are sufficient to induce localized RTK responses in vivo.

Our results show that regulation of *hkb* expression in response to Torso signaling crucially depends on conserved TGAATGAA elements recognized by Cic (Fig. 1). We also find that these elements combined with Bcd activator sequences are sufficient to establish localized reporter expression in the anterior pole of the

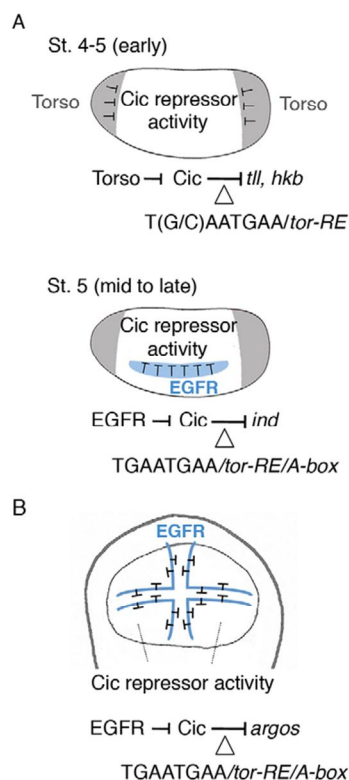


Fig. 6. Cic regulatory elements mediate Torso and EGFR responses. (A) Sequential activation of the Torso (gray) and EGFR (blue) RTK pathways downregulates Cic along the AP and DV embryonic axes. Both pathways relieve Cic repression mediated by common cis-regulatory elements. Developmental stages (St.) are indicated. (B) EGFR signaling (blue) induces *argos* expression via Cic sites; activation of the pathway in vein cells leads to downregulation of Cic repressor activity, thereby derepressing *argos* transcription.

embryo (Fig. 2). It thus appears that binding of Cic to specific sites in *hkb* is the key step for delimiting *hkb* expression in response to Torso activation. Therefore, although we cannot rule out that other (possibly redundant) Torso-dependent factors contribute to *hkb* regulation, we propose that this regulation largely depends on broadly distributed activators such as Bcd, Dorsal and Lilliputian (Reuter and Leptin, 1994; Häder et al., 2000; Tang et al., 2001) (Fig. 1G), and localized Cic repression.

We also find that association of Gro to the *hkb* enhancer requires the presence of intact Cic octamers in the enhancer. How does this association occur? Although Cic and Gro proteins interact in vitro, we have not yet demonstrated a direct correlation between such binding and Cic repressor activity in vivo (Jiménez et al., 2000; Astigarraga et al., 2007) (C.N. and G.J., unpublished). Our finding that Gro associates with sequences containing Dorsal and Retn sites is consistent with a role of these factors in recruiting Gro to the *hkb* enhancer, possibly through cooperative interactions with Cic. However, mutations in *dorsal* or *retn* do not cause clear derepression of *hkb^{0.4}-lacZ* or *hkb* expression (Fig. 1G) (Häder et al., 2000). It is also possible that local recruitment of Gro by Cic results in subsequent spreading of the co-repressor along the entire *hkb^{0.4}* enhancer, a mechanism that may involve oligomerization of Gro and binding to hypoacetylated histones (Courey and Jia, 2001; Song et al., 2004; Martínez and Amosti, 2008).

Our results indicate that patterning of the dorsal-ventral (DV) embryonic axis requires a mechanism of EGFR-mediated derepression that is similar to the role of Torso signaling in the anterior-posterior (AP) terminal system. In both cases, a local source of RTK activation downregulates the Cic repressor, thus inducing expression of Cic targets in restricted patterns (Fig. 6A). During DV patterning, the Dorsal morphogen activates the expression of several targets in ventral and lateral regions of the embryo, and it is believed that decreasing amounts of Dorsal protein help establish the dorsal limits of those expression domains. However, Dorsal nuclear levels appear rather uniform across the *ind* expression domain (Kanodia et al., 2009; Liberman et al., 2009), indicating that other mechanisms define the dorsal limit of *ind* expression. Indeed, previous studies have shown that EGFR signaling plays a key role in setting this border (Weiss et al., 1998; von Ohlen and Doe, 2000), and suggested the existence of unknown repressors restricting *ind* expression in dorsal regions (Stathopoulos and Levine, 2005). Our results indicate that these two events are linked through a mechanism of EGFR-mediated downregulation of Cic repressor activity.

During wing vein specification, there is a precise correlation between EGFR/MAPK signaling, Cic downregulation and *argos* transcription in prospective wing vein cells (Fig. 5A,B) (Gabay et al., 1997). Furthermore, our data show that Cic represses *argos* directly (Fig. 5E-G), and that Cic octamers alone are sufficient to interpret the EGFR activation signal to produce an *argos*-like response (Fig. 5H-M, Fig. 6B). However, the *CUASC-lacZ* reporter does not recapitulate all aspects of *argos* transcription, because only endogenous *argos* shows elevated expression in presumptive veins L3 and L4. This difference probably depends on localized determinants that regulate gene expression in the L3-L4 region (Blair, 2007), and do not affect *CUASC-lacZ*. Still, *argos* regulation during wing development appears largely dependent on EGFR-mediated downregulation of Cic as well as on positive input(s) by localized or ubiquitous activators, which may include the Osa/Eyelid factor (Terriente-Félix and de Celis, 2009). In addition, both loss- and gain-of-function experiments show strong correlation between Cic-dependent activity through TGAATGAA elements and differentiation of wing veins in the adult (Fig. 5 and see Fig. S4 in the supplementary material) (Goff et al., 2001; Roch et al., 2002), suggesting that Cic is an important sensor of EGFR signaling in this system. Cic probably controls additional EGFR targets involved in wing vein specification and other EGFR-regulated processes such as cell proliferation in imaginal discs (Tseng et al., 2007). Future studies will probably reveal new roles of Cic and its binding sites downstream of RTK signaling cascades.

In summary, Cic regulates multiple RTK signaling responses by binding to conserved octameric sites in target enhancers, indicating that conservation between these RTK pathways extends to specific response elements in cis-regulatory regions. Notably, these octamers are sufficient to translate RTK signaling inputs into localized transcriptional responses in different tissues: RTK signals produce complementary gradients (Torso) or boundaries (EGFR) of Cic downregulation that are then translated into complementary patterns of target gene expression through relief of Cic repression. This mechanism represents a particular case of 'default repression', a general strategy of developmental control whereby target genes induced by signaling pathways are maintained repressed in the absence of signaling (Barolo and Posakony, 2002). For example, a similar derepression switch occurs during TGF- β /Dpp-mediated induction of *optomotor-blind* transcription via relief of Brinker repression (Sivasankaran et al., 2000; Barolo and Posakony, 2002).

Finally, the human Cic protein binds octameric sequences related to those characterized here (Kawamura-Saito et al., 2006). The best-characterized targets of Cic in human cells are ETS genes of the *pea3* family (Kawamura-Saito et al., 2006), which are known to respond to FGF RTK activation in different vertebrate systems (e.g. Roehl and Nüsslein-Volhard, 2001; Raible and Brand, 2001). Therefore, it will be interesting to ascertain whether Cic octamers also mediate RTK responses in those systems.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Supplemental Information

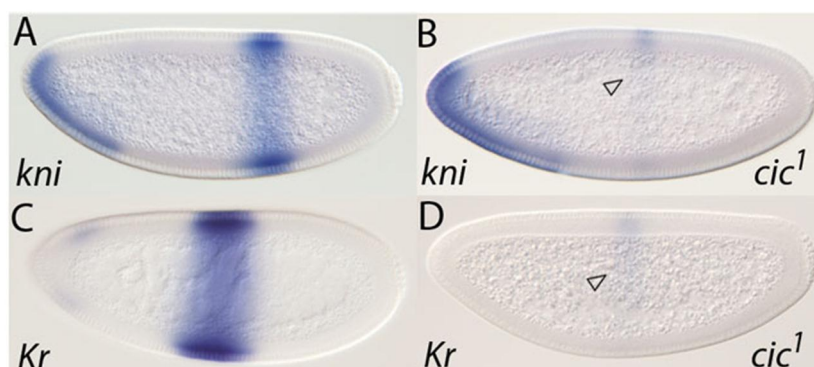


Figure S1. Cic is required for expression of the central gap genes *kni* and *Kr*. (A-D) mRNA expression patterns of *kni* (A,B) and *Kr* (C,D) in wild-type (A,C) and *cic*¹ (B,D) embryos. The central domains of *kni* and *Kr* expression appear severely reduced in the *cic*¹ background and only residual staining is observed (open arrowheads in B and D).

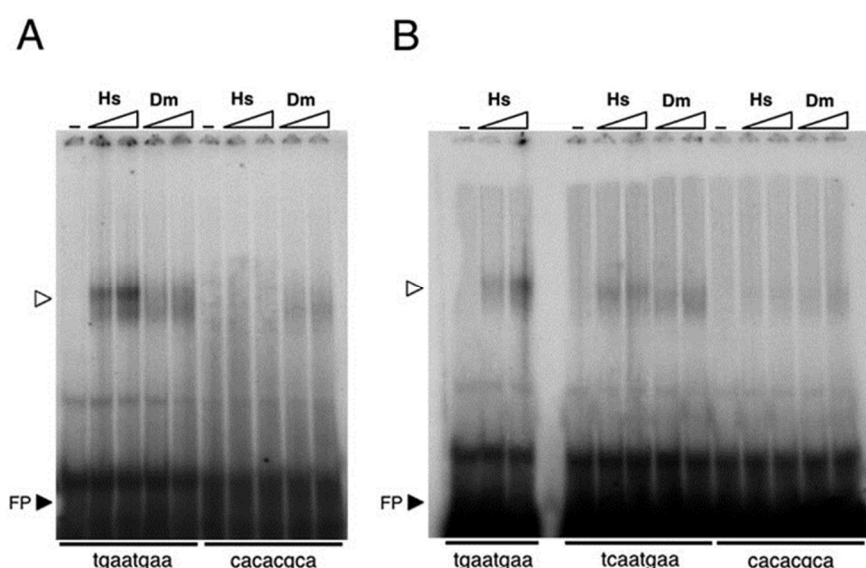


Figure S2. Cic binds T(G/C)AATGAA sequences in vitro. EMSA experiments using recombinant GST fusions containing the HMG-box regions of human (Hs) and *Drosophila* (Dm) Cic. Increasing amounts of protein (0.1 and 0.2 µg) used in the incubations are represented by a triangle. Control lanes without proteins are indicated by (-). Retarded bands are indicated by open arrows. The different wild-type t(g/c)aatgaa or mutant (cacacgca) Cic-binding sites are indicated beneath each section of the gel. FP, free probe. (A) EMSA using oligonucleotide probes containing one intact or mutated TGAATGAA site from the *hkb* upstream region. (B) EMSA using oligonucleotide probes containing one intact or mutant *TRE* site from the *tll* enhancer. Binding of the human HMG-box to the *hkb* TGAATGAA probe is shown on the left for comparison. The following ³²P-labelled oligonucleotide probes were used (only the upper strand of each probe is indicated): *hkb*(wt), 5'GAC AGT TTA TGA ATG AAT TTAC TA 3'N_j; *hkb*(mut), 5'GAC AGT TTA CAC ACG CAT TTA CTA 3'N_j; *tll*(wt), 5'GAT CCT TGC TCA ATG AAT TTT TCG 3'N_j; *tll*(mut), 5'GAT CCT TGC CAC ACG CAT TTT TCG 3'N_j.

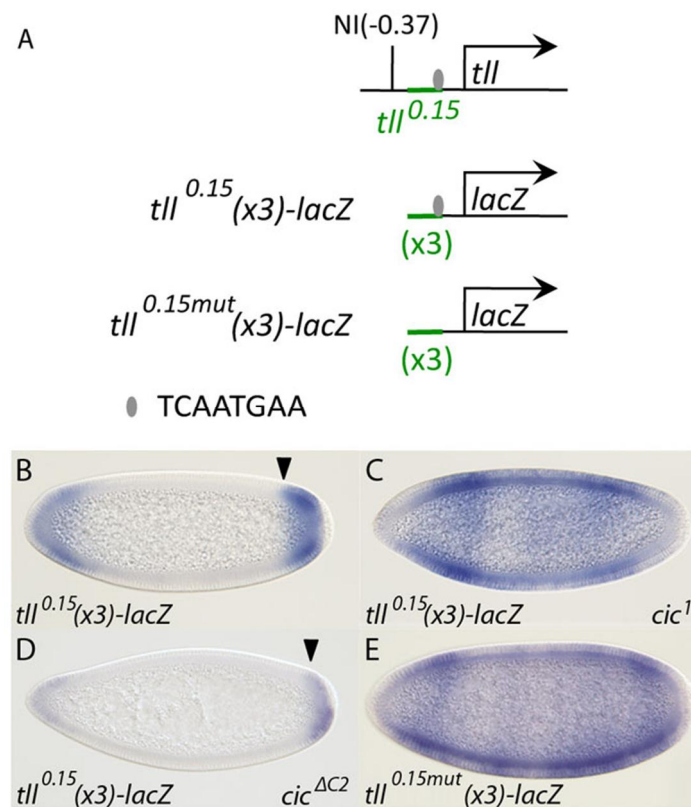


Figure S3. Cic represses a *tll* enhancer containing the *tor-RE*. (A) Diagram of the *tll* gene (top). NI, *Nco*I restriction site present 0.37 kb upstream of the transcription initiation site. The minimal 150 bp *tll* enhancer (*tll*^{0.15}) including a *tor-RE* (TGCTCAATGAA) at its proximal end is depicted in green. Reporters driven by three tandem copies of wild-type (*tll*^{0.15}-*lacZ*) and mutant (*tll*^{0.15mut}-*lacZ*) enhancer sequences are shown below; *tll*^{0.15mut}-*lacZ* lacks the *tor-RE*/TCAATGAA motif in each of the three regulatory modules. (B-E) mRNA expression patterns of *tll*^{0.15}(x3)-*lacZ* (B-D) and *tll*^{0.15mut}(x3)-*lacZ* (E) in otherwise wild-type (B, E), *cic*¹ (C) and *cic*^{ΔC2} (D) embryos (see main text). Arrowheads above the embryos indicate the anterior limits of *lacZ* expression. Note that *tll*^{0.15}(x3)-*lacZ* is expressed weakly in the anterior region of blastoderm embryos but does not form the sharp anterior-dorsal stripe characteristic of the *tll* pattern (B). However, this reporter recapitulates *tll* expression at the posterior pole of mid-blastoderm embryos, forming a cap of expression extending from 0 to 14% embryo length (EL; 0% being the posterior tip of the embryo; B). In *cic*¹ embryos, expression of *tll*^{0.15}(x3)-*lacZ* expands dramatically throughout the prospective abdomen and trunk regions (C). By contrast, *cic*^{ΔC2} embryos exhibit a narrow domain of posterior *tll*^{0.15}(x3)-*lacZ* expression spanning 0-9% EL (arrowhead in D). This reduced expression is comparable with that of endogenous *tll* in *cic*^{ΔC2} embryos (Astigarraga et al., 2007). *tll*^{0.15mut}(x3)-*lacZ* directs ubiquitous staining that is similar to the expression of *tll*^{0.15}(x3)-*lacZ* in *cic*¹ embryos (compare C with E). These results are consistent with the original analyses of *cis*-acting regulatory elements of *tll* (Liaw et al., 1995) and support the notion that Cic represses *tll* by directly binding to the *tor-RE* element.

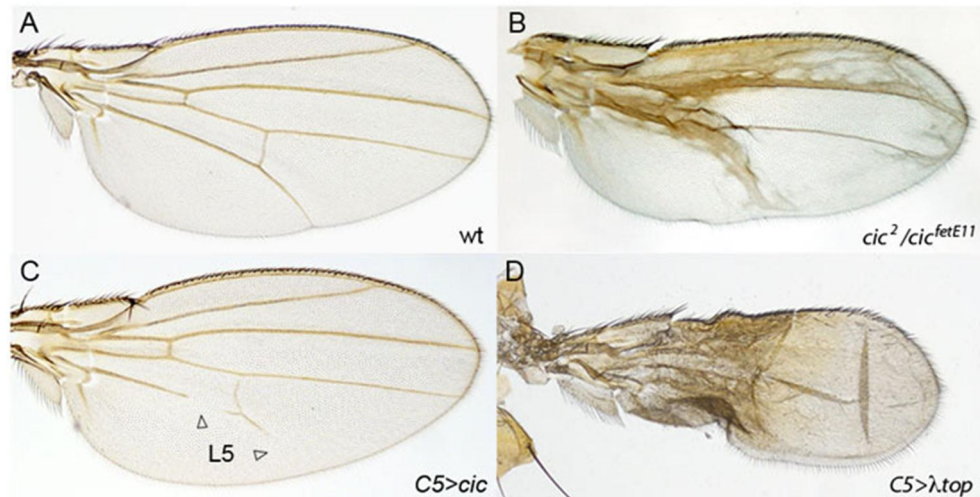


Figure S4. Wing vein phenotypes induced by alterations in Cic and EGFR function. (A) Wild-type wing. (B) Wing from *cic*²/*cic*^{fetE11} heterozygous fly displaying ectopic vein tissue and blistering. *cic*^{fetE11} is a hypomorphic allele that produces viable combinations with other *cic* alleles, including the *cic*². (C) Wing from *C5-GAL4/UAS-cic* fly showing gaps in vein L5 (open arrowheads). Male flies of this combination also tend to show gaps in vein L2 (not shown). (D) Wing from *C5-GAL4/UAS-λtop* fly exhibiting extra vein tissue and blisters.

ARTICLE 4: Gene Regulation by MAPK Substrate Competition

Authors

Yoosik Kim, **María José Andreu**, Bomyi Lim, Kwanghun Chung, Mark Terayama, Gerardo Jiménez, Celeste A. Berg, Hang Lu, and Stanislav Y. Shvartsman

Reference

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Summary (in Spanish)

La formación del patrón de los tejidos durante el desarrollo requiere la actividad coordinada de diferentes sistemas de señalización, los cuales pueden ser integrados mediante la unión de múltiples factores de transcripción a la misma región reguladora de un gen o mediante un único factor de transcripción el cual es modificado por múltiples enzimas. Basándonos en la combinación de experimentos genéticos y análisis de imagen en el embrión temprano de *Drosophila*, hemos descrito un mecanismo de integración de señales que no puede ser reducido a un elemento de regulación o un único factor de transcripción. Este mecanismo depende de una red enzimática formada por MAPK y sus sustratos. Específicamente, sustratos de MAPK localizados en la región anterior, como Bicoid, antagonizan la inhibición dependiente de MAPK del factor Capicua, el cual es un represor implicado en la regulación del eje dorso-ventral del embrión. Nuestros resultados apoyan un mecanismo por el que la competición de sustratos de MAPK controla el patrón de expresión génica mediante la interacción de los sistemas anterior, dorso-ventral y terminal. Finalmente, se ha formalizado este mecanismo en un modelo matemático que explica los patrones de expresión de genes, como *zerknüllt*, con polaridad tanto antero-posterior como dorso-ventral.

Gene Regulation by MAPK Substrate Competition

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SUMMARY

Developing tissues are patterned by coordinated activities of signaling systems, which can be integrated by a regulatory region of a gene that binds multiple transcription factors or by a transcription factor that is modified by multiple enzymes. Based on a combination of genetic and imaging experiments in the early *Drosophila* embryo, we describe a signal integration mechanism that cannot be reduced to a single gene regulatory element or a single transcription factor. This mechanism relies on an enzymatic network formed by mitogen-activated protein kinase (MAPK) and its substrates. Specifically, anteriorly localized MAPK substrates, such as Bicoid, antagonize MAPK-dependent downregulation of Capicua, a repressor that is involved in gene regulation along the dorsoventral axis of the embryo. MAPK substrate competition provides a basis for ternary interaction of the anterior, dorsoventral, and terminal patterning systems. A mathematical model of this interaction can explain gene expression patterns with both anteroposterior and dorsoventral polarities.

INTRODUCTION

Similar to other multicellular organisms, *Drosophila* uses its extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway throughout embryogenesis. It is first used downstream of Torso receptor tyrosine kinase (RTK), to specify the nonsegmented terminal regions of the future larva. Locally activated Torso establishes a two-peaked pattern of activated, double-phosphorylated ERK/MAPK (dpERK) (Coppey et al., 2008; Gabay et al., 1997). One MAPK substrate is an HMG-box transcriptional repressor Capicua (Cic), which is uniformly expressed in the embryo (Jiménez et al., 2000). In a process essential for the development of the terminal structures, Cic is degraded at the poles in direct response to its phosphorylation by MAPK (Ajuria et al., 2011; Astigarraga et al., 2007; Jiménez et al.,

2000). Another substrate of MAPK is an anteriorly localized homeodomain protein Bicoid (Bcd), which determines the anterior pattern of the embryo (Janody et al., 2000; Ronchi et al., 1993).

Together with another anteriorly localized MAPK substrate, Hunchback (Hb), Bcd antagonizes MAPK-dependent downregulation of Cic (Kim et al., 2010). Our earlier work supports a model whereby MAPK substrates indirectly affect each other by competing for their common regulator (Kim et al., 2010). Competitive effects of this type should be common in large-scale biomolecular networks where one regulator controls a large number of targets. Computational studies of mass-action networks demonstrate that, in general, competitive effects decay with distance (the number of interaction edges) from perturbed nodes (Maslov and Ispolatov, 2007). In other words, competitive effects could be localized to a specific node and thus might not significantly affect more distant network components. In the context of the terminal patterning system, anteriorly localized MAPK substrates can decrease the level of Cic downregulation, but the effect may be too small to impact Cic-dependent gene regulation.

Here, we present evidence to the contrary and propose that MAPK substrate competition controls gene expression in the early embryo. This conclusion is based on the analysis of the expression pattern of *zerknüllt* (*zen*), which encodes a homeodomain transcription factor and is expressed in a complex pattern that depends on the joint action of the terminal and dorsoventral (DV) patterning systems (Figure 1). *zen* expression is broadly activated by Zelda and possibly other maternal factors, and repressed in the ventral and lateral regions by Dorsal (Dl), a transcription factor that patterns the DV axis (Figures 1A and 1B) (Doyle et al., 1989; Jiang et al., 1993; Liang et al., 2008; Ratnaparkhi et al., 2006). At the poles, Dl-mediated repression of *zen* is antagonized by Torso signaling (Rusch and Levine, 1994).

The precise mechanism of *zen* derepression by the terminal system has been unclear, but it is likely to depend on MAPK-dependent downregulation of Cic (Astigarraga et al., 2007; Goff et al., 2001; Jiménez et al., 2000). We demonstrate that the antirepressive effect of MAPK signaling on *zen* is modulated by the anteriorly localized MAPK substrates. Specifically, removing anterior MAPK substrates makes *zen* expression symmetric along the anteroposterior (AP) axis. Our results support a mechanism whereby MAPK substrate competition controls spatial patterns of gene expression by coordinating the actions of the anterior, DV, and terminal systems. We formalize this mechanism in a mathematical

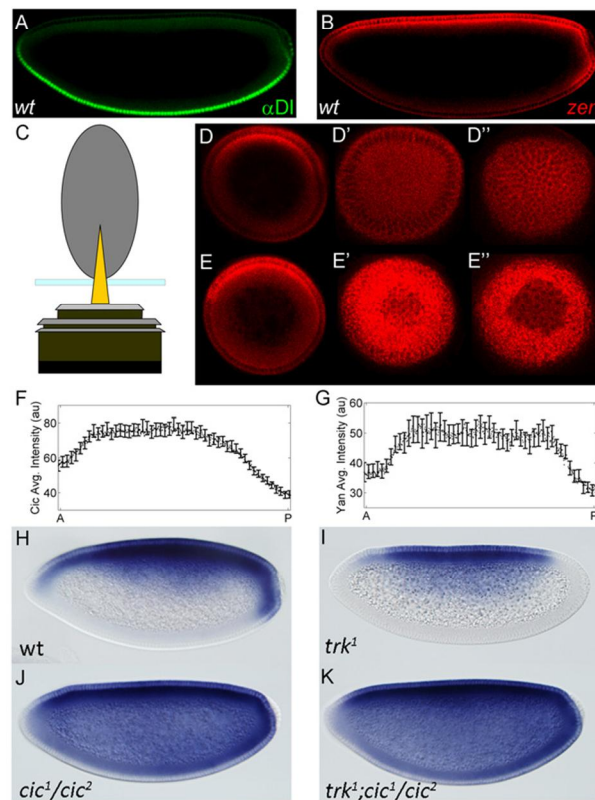


Figure 1. Expression of *zen* mRNA Exhibits AP Asymmetry

(A and B) Spatial patterns of Dl protein (green) and *zen* mRNA (red) in a wild-type embryo. *zen* transcripts were detected by FISH (see [Experimental Procedures](#)). The image is oriented with anterior left and dorsal side up.

(C) Schematic of end-on imaging.

(D) Optical sections of an embryo stained with *zen* mRNA at $\sim 70 \mu\text{m}$ (D), $\sim 7 \mu\text{m}$ (D'), and $0 \mu\text{m}$ from the anterior pole (D''). These images are oriented with the dorsal side up.

(E) *zen* expression at the posterior pole: optical sections at $\sim 70 \mu\text{m}$ (E), $\sim 7 \mu\text{m}$ (E'), and $0 \mu\text{m}$ from the posterior pole (E'').

(F) AP gradient of nuclear Cic in wild-type embryos. Solid line is the average gradient, based on 29 embryos. Error bars indicate standard error of the mean (SEM). The nuclear level of Cic is significantly higher at the anterior pole, suggesting weaker MAPK enzymatic activity in this region ($p < 0.001$).

(G) Enzymatic activity of MAPK can also be assessed by quantifying the spatial pattern of ectopically expressed Yan. Similar to Cic, Yan downregulation by MAPK is significantly weaker in the anterior region ($p < 0.001$). Solid line is the average gradient, based on 23 embryos; error bars indicate SEM.

(H–K) *zen* mRNA in a wild-type embryo (H), *trk* embryo (I), *cic* embryo (J), and *trk cic* double-mutant embryo (K). *zen* transcripts are detected using alkaline phosphatase staining.

model and demonstrate that this model correctly predicts *zen* expression in multiple genetic backgrounds.

RESULTS

Cic Downregulation Is Required for *zen* Expression at the Poles

zen expression pattern displays a pronounced AP asymmetry: *zen* transcripts are present at the posterior but not at the anterior

pole. This asymmetry is particularly clear when embryos are imaged in an upright position (Figure 1C). As shown in Figures 1D and 1E, *zen* transcript levels are markedly different at the anterior versus the posterior pole of the embryo. Below, we argue that AP asymmetry of *zen* expression can be explained by a mechanism that relies on MAPK substrate competition. Briefly, we propose that MAPK-dependent downregulation of Cic is responsible for *zen* derepression at the poles. Anteriorly localized MAPK substrates inhibit Cic downregulation, increasing the level of Cic and thus the strength of Dl-dependent *zen* repression at the anterior pole.

As a first step in evaluating this model, we asked whether Cic downregulation by Torso signaling contributes to *zen* expression at the poles of the embryo. Previous studies (Jiménez et al., 2000; Astigarraga et al., 2007) suggested that Cic is involved in Dl-dependent ventral repression of *zen* (Figures 1H–1J). To test whether downregulation of Cic is also involved in Torso-dependent control of *zen* at the poles, we examined *zen* expression in embryos derived from *trunk* (*trk*) and *cic* double-mutant females. *trk* encodes a Torso ligand, which is required for MAPK activation at the poles (Casanova et al., 1995) as well as the posterior expression of *zen* (Figure 1I). If the latter effect is due to the lack of Cic downregulation, then posterior expression of *zen* should be restored in embryos that lack both Cic and Trk. Consistent with this prediction, we found that *zen* is significantly derepressed in embryos derived from *trk cic* double-mutant flies (Figure 1K).

Taken together with the results of previous studies, our data support a model where Cic downregulation is necessary for *zen* expression at the poles. Based on the previously characterized AP asymmetry of Cic downregulation (Figure 1F), we propose that higher levels of Cic at the anterior pole are responsible, at least in part, for reduced expression of *zen* in this region. We found that both of these asymmetries are seen in other *Drosophilids* (see Figure S1 available online), suggesting that the proposed mechanism is conserved and might be functionally significant.

Mathematical Model Describes *zen* Expression

Based on the above results, we formulated a mathematical model of *zen* regulation: *zen* is activated by spatially uniform activators, such as Zelda (U), and repressed by the ventral-to-dorsal nuclear gradient of Dl. Repression of *zen* by Dl requires a cofactor (R), which is downregulated at the poles by the terminal signal (E, which models locally activated MAPK). At the anterior pole, the enzymatic activity of E toward R is inhibited by the anteriorly localized substrates of E (B, which models the combined effects of Bcd and Hb) (Figure 2B). In the simplest case, R can be viewed as Cic, which is clearly involved in Dl-dependent *zen* repression (Figure 1).

We note that our model does not rely on the assumption that Cic is the only MAPK substrate required for Dl-dependent repression of *zen* (Dubnicoff et al., 1997; Jiang et al., 1993; Jiménez et al., 2000). Thus, R can represent a collective effect of spatially uniform MAPK substrates that are involved in Dl-dependent *zen* repression. We expect that these substrates, some of which are yet to be identified, will also be subject to competitive effects of anteriorly localized MAPK substrates. This idea is supported by the following experiment, which used nuclear

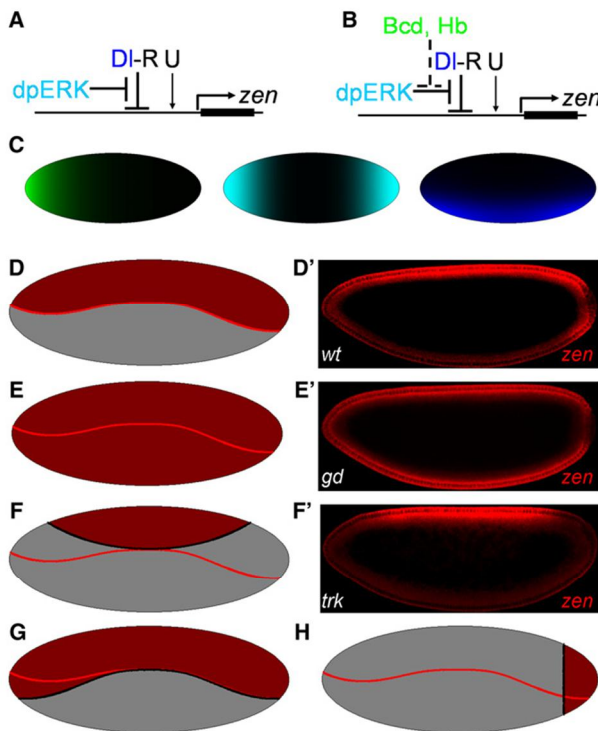


Figure 2. Mathematical Model of *zen* Regulation by MAPK Substrate Competition

(A) A model for the early pattern of *zen* expression (Rusch and Levine, 1994): *zen* is induced by uniform activators (U) and repressed in the ventral region of the embryo by the nuclear DI-R gradient (DI-R). DI-mediated repression is antagonized at the poles by MAPK.

(B) Revised model: the anteriorly localized substrates of MAPK (Bcd, Hb) competitively inhibit MAPK-mediated *zen* derepression.

(C) Schematic representation of the spatial distribution of maternal factors contributing to the regulation of *zen*: Bcd+Hb (green), dpERK (cyan), and DI (blue).

(D–F) The model successfully predicts *zen* expression in mutants. The boundary of the wild-type pattern is indicated by a solid red line. *zen* in wild-type embryos (D), embryos with no DI (E), and no terminal signaling (F).

(G and H) The model can also predict *zen* expression pattern in multiple genetic backgrounds. The model predicts ectopic expression pattern of *zen* in the absence of anterior substrates of MAPK (G) and posterior expression in embryos with uniform nuclear of DI (H).

accumulation of ectopically expressed Yan as a Cic-independent reporter of MAPK activity. Similar to Cic, Yan is degraded in response to MAPK phosphorylation (Figure S2) (Lai and Rubin, 1992; Rebay and Rubin, 1995). We found that the spatial pattern of Yan downregulation is also asymmetric: nuclear levels of Yan at the anterior pole were higher than at the posterior pole (Figure 1G). This suggests that MAPK activity toward all of its uniformly distributed substrates is lower in this region of the embryo.

According to our model, *zen* repression requires a critical value of both DI, distributed in a ventral-to-dorsal pattern, and R, distributed in an AP pattern. To find this pattern, we used an enzyme kinetics model where anteriorly localized substrates of E competitively inhibit R downregulation at the poles. Starting

from the experimentally observed shape of the nuclear DI gradient (Chung et al., 2011; Kanodia et al., 2009), the AP asymmetry of Cic downregulation (Kim et al., 2010), and the expression boundary of *zen* in the midbody of the embryo (Chung et al., 2011), we derived an analytical expression that describes *zen* expression boundary on the surface of a spheroid that approximates the three-dimensional shape of the embryo (see Experimental Procedures for a more detailed presentation of the model).

Remarkably, this simple model can account for changes of *zen* expression in response to multiple perturbations of maternal patterning systems (Figures 2D–2H). For instance, removal of anterior MAPK substrates leads to ectopic *zen* expression at the anterior pole (Figure 2G), making the pattern symmetric along the AP axis. This model also successfully predicts the persistence of *zen* expression at the posterior pole of embryos with uniform nuclear DI (Figure 2H), in agreement with *zen* expression in embryos derived from *cactus* mutant females (Rushlow et al., 1987). Thus, this model parsimoniously explains how *zen* expression changes in multiple mutant backgrounds. Finally, this model makes a number of predictions, which motivated our experiments presented below.

Experimental Tests of Model Predictions

A substrate competition model for *zen* regulation predicts that *zen* expression should become symmetric in embryos that lack anteriorly localized MAPK substrates (Figure 2G). To test this prediction, we examined *zen* expression in embryos that lack both Bcd and maternally contributed Hb, which is also distributed in an AP pattern and phosphorylated by MAPK. In these embryos, both MAPK phosphorylation and signaling activity, reported by Cic downregulation, are essentially symmetric (Figures 3C and 3D). Furthermore, a target gene of MAPK signaling, *tailless* (*tl*), is expressed as two symmetric caps at the poles (Figure 3F). Thus, MAPK signaling and its transcriptional effects are indeed symmetric in these embryos. Importantly, *zen* expression also becomes symmetric (Figures 3H–3J and S3B–S3D).

These observations are consistent with the notion that the AP asymmetry of the wild-type *zen* expression pattern is generated by anteriorly localized MAPK substrates. Furthermore, these observations suggest that Bcd and Hb constitute a significant fraction of all MAPK substrates as the anterior pole. Moreover, loss of *bcd* alone increases *zen* expression at the anterior pole (Figures 4N–4P). Thus, removal of a single binding partner of MAPK can generate a significant downstream effect in a larger patterning network.

Another prediction of our model is that the inhibitory effects of Bcd on MAPK signaling and *zen* expression do not depend on its transcriptional activity. To test this prediction, we used Bcd-A₉, a (K50L) form of Bcd that does not bind to Bcd's DNA recognition sequences and thus cannot activate endogenous transcriptional targets of Bcd (Hanes et al., 1994). We found that expression of two copies of this construct, driven by Bcd endogenous promoter in the *bcd* homozygous embryos (*Bcd-A₉;bcd*), does not activate several *bcd* targets (Figures 4E–4H), yet does rescue both MAPK phosphorylation and Cic downregulation gradients (Figures 4A–4D). Importantly, *zen* expression is greatly reduced at the anterior pole in these embryos (Figures 4J–4L). Thus,

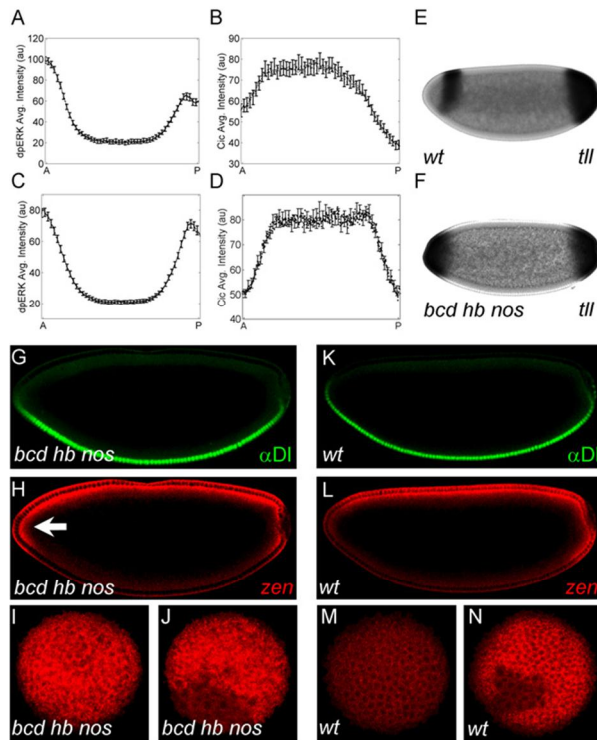


Figure 3. Anterior Repression of *zen* Requires Bcd and Hb

(A and B) Quantified gradient of MAPK phosphorylation (A) and Cic downregulation (B) in wild-type embryos ($n = 27$ for A and $n = 29$ for B; error bars are SEM). Cic downregulation gradient is reproduced from Figure 1F. (C and D) Quantified gradients of MAPK phosphorylation (C) and Cic downregulation (D) in embryos derived from *bcd hb nos* germline clone flies ($n = 34$ for both gradients; error bars are SEM). The anterior and posterior levels of both MAPK phosphorylation and nuclear Cic are nearly symmetric ($p > 0.1$). (E and F) Expression of *tll* in wild-type (E) and *bcd hb nos* (F) embryos. (G and H) DI protein and *zen* mRNA in a *bcd hb nos* embryo. In the absence of Bcd, Hb, and Nos, *zen* is expressed throughout the anterior pole (arrow). (I and J) Optical images of *zen* at the anterior (I) and posterior (J) poles. Similar to Figure 3H, *zen* is expressed at both poles in the absence of anterior substrates of MAPK. (K and L) DI protein and *zen* mRNA in a wild-type embryo. (M and N) End-on images of *zen* mRNA at the anterior (M) and posterior (N) poles of wild-type embryos.

transcriptionally inert Bcd can inhibit MAPK-dependent downregulation of Cic, and this effect propagates to the DI-dependent gene expression.

Another argument supporting our model of gene regulation by MAPK substrate competition is based on the analysis of the expression of *hb*, which is transcriptionally regulated by both anterior and terminal systems. In embryos derived from *bcd* homozygous flies, *hb* is expressed at the poles, reflecting its activation by the terminal system (Margolis et al., 1995). However, the anterior domain of this pattern is eliminated by expressing two copies of *Bcd-A₉* (Figure 4F). This suggests that transcriptionally inert Bcd weakens terminal signaling at the anterior region, leading to a drastic reduction of the anterior *hb* expression. This conclusion is further strengthened by the fact that, in the

same background, the anterior expression of *hkb* and *tll*, both of which depend on MAPK signaling, is also attenuated, even when compared to their expression in embryos derived from *bcd* homozygous females (Figure 4H; data not shown).

Taken together with the wild-type pattern of *zen* expression and its changes in mutant backgrounds, our results support a nontranscriptional mechanism, according to which the anterior repression of *zen* depends on competitive inhibition of Cic downregulation by anteriorly localized MAPK substrates. Although our proof of this mechanism is not exhaustive, we cannot formulate a viable alternative that would have a similar explanatory and predictive power. For instance, we could rule out the alternative models that rely on the AP asymmetry of the DI gradient or on the more indirect, transcriptional, effect of the terminal system (see Figures S4A–S4H).

DISCUSSION

Patterning of the anterior region of the *Drosophila* embryo depends on the concentration gradient of Bcd, the nuclear localization gradient of DI, and the phosphorylation gradient of MAPK (Casanova, 1990; Driever and Nüsslein-Volhard, 1988; Rushlow et al., 1989; St Johnston and Nüsslein-Volhard, 1992). Previous studies revealed several pairwise interactions between these signals (Pignoni et al., 1992; Ronchi et al., 1993; Rusch and Levine, 1994). In particular, Bcd and DI synergistically regulate a large number of genes by binding to common *cis*-regulatory regions of those genes (Papatsenko et al., 2009). Similarly, the concentration gradient of Cic, established by MAPK signaling, controls the posterior expression borders of genes activated by Bcd (Löhr et al., 2009).

We discovered an additional, ternary interaction of the AP, DV, and terminal systems. This mechanism cannot be reduced to a particular regulatory region on DNA or to a single protein. Instead, it is based on competitive effects in a network formed by MAPK and its substrates. Based on the asymmetry of the wild-type pattern of *zen* mRNA, we propose that the anteriorly localized substrates of MAPK act as competitive inhibitors in the process of MAPK-dependent gene derepression. The same mechanism can regulate other genes that are repressed by the DI gradient. For example, *decapentaplegic* (*dpp*), another gene expressed in the dorsal region, is regulated by the joint action of the DV and terminal systems (Casanova, 1991; Huang et al., 1993). Similar to *zen*, the expression of *dpp* is weaker at the anterior pole than at the posterior (data not shown).

At this point, the main evidence supporting the notion that MAPK substrate competition controls gene expression in the embryo is provided by experiments that perturb the expression levels of MAPK substrates. In the future, we plan to complement these experiments by perturbations of the docking domains involved in MAPK interactions with its binding partners (Torii et al., 2004; Shaul and Seger, 2007; Goldsmith et al., 2007; Bardwell, 2006). For instance, according to our model, the spatial pattern of *zen* should become symmetric in embryos in which the wild-type Bcd protein is replaced by a Bcd variant that lacks MAPK docking domain. To test this prediction, we plan to identify the docking domains involved in MAPK/Bcd and other MAPK-dependent interactions in the early embryo.

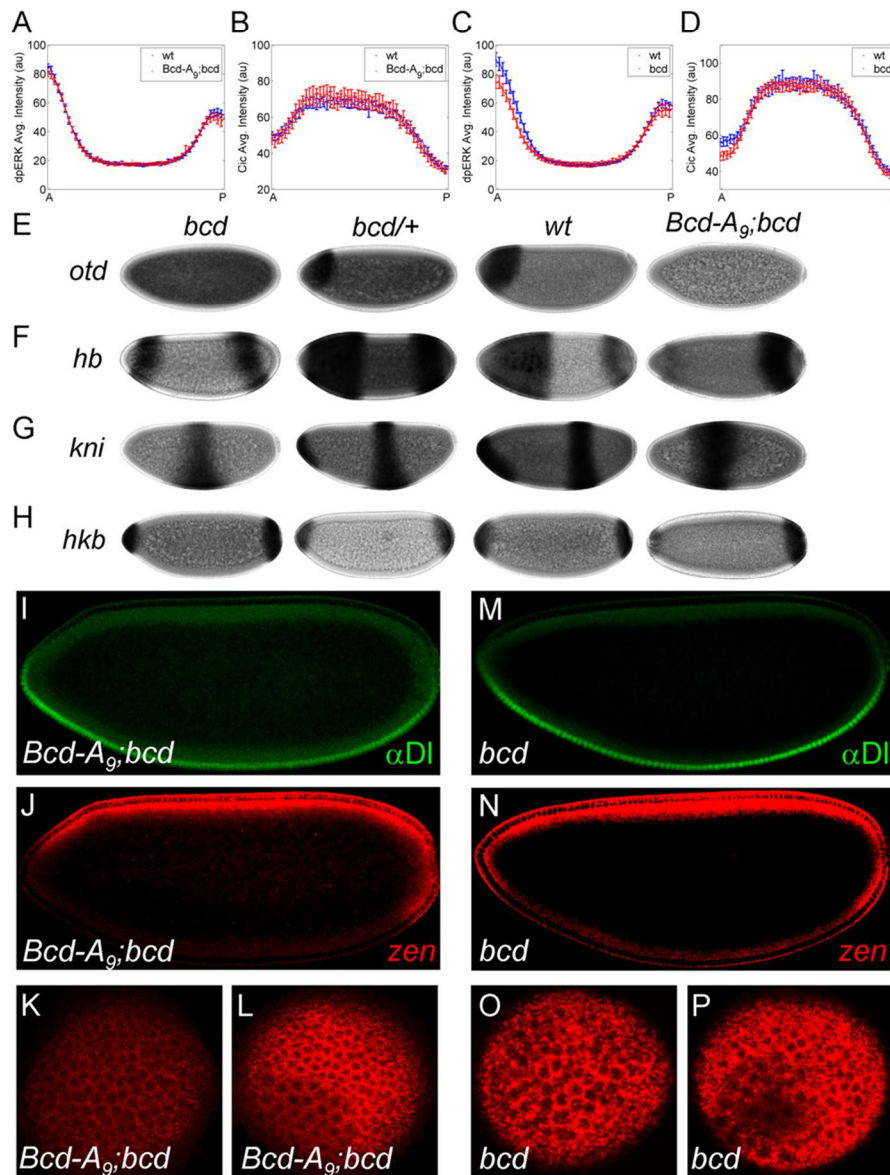


Figure 4. Anterior Repression of *zen* Does Not Require Transcriptional Activity of *Bcd*

(A and B) Expression of two copies of *Bcd-A₉* construct driven by *Bcd* promoter in the *bcd* null embryos (*Bcd-A₉;bcd*) results in MAPK phosphorylation (A) and nuclear Cic (B) gradients that are indistinguishable from those in wild-type embryo (error bars are SEM). The numbers of embryos used in the analysis are: $N_{WT} = 32$, $N_{mutant} = 19$ for (A) and $N_{WT} = 28$, $N_{mutant} = 24$ for (B).

(C and D) Quantified gradients of MAPK phosphorylation (C) and Cic downregulation (D) in *bcd* null embryos (error bars are SEM). $N_{WT} = 28$, $N_{mutant} = 28$ for (C) and $N_{WT} = 39$, $N_{mutant} = 38$ for (D).

(E–H) Expression of *otd* (E), *hb* (F), *kni* (G), and *hkb* (H) in embryos derived from *bcd* homozygous (first column), *bcd* heterozygous (second column), wild-type (third column), and *bcd* homozygous with *Bcd-A₉* (fourth column) flies. Anterior expression of these genes is significantly reduced in *Bcd-A₉;bcd* embryos.

(I and J) Nuclear Dl and *zen* mRNA in a *Bcd-A₉;bcd* embryo: similar to wild-type embryos, *zen* is repressed at the anterior pole.

(K and L) *zen* expressions at the anterior (K) and posterior (L) poles also show that *zen* is not expressed at the anterior pole in this mutant background.

(M and N) Nuclear Dl and *zen* mRNA in a *bcd* null embryo.

(O and P) *zen* expression at the anterior (O) and posterior (P) poles of embryos from *bcd* null flies.

Identification of these domains should shed light on the functional significance of MAPK substrate competition in the early embryo and other stages of development.

Competitive effects are likely to be a general feature of biochemical networks, which are commonly organized around hubs, regulators that can interact with a large number of targets.

For instance, in posttranscriptional regulation by microRNAs, different targets of a given microRNA can compete for their common regulator. Indeed, a number of recent studies suggest that mRNA competition in microRNA networks can lead to indirect inhibitory interactions between transcripts (Ebert et al., 2007). As a consequence, a given mRNA can control translation of other transcripts by regulating activity of their common microRNAs, in a way that is independent of its protein-coding function (Poliseno et al., 2010). Exploring network-level consequences of such effects requires a quantitative approach, similar to the one used in our study of MAPK signaling in *Drosophila* embryo.

EXPERIMENTAL PROCEDURES

In Situ Hybridization and Immunohistochemistry

Fluorescence in situ hybridization (FISH) was performed as described elsewhere (Kosman et al., 2004). Embryos were dechorionated in 50% bleach and fixed in 8% formaldehyde in PBS for 20 min. Embryos were then incubated in 90% xylene for 1 hr and then treated with 80% acetone for 10 min at -20°C . Next, embryos were hybridized overnight at 60°C with antisense probes labeled with digoxigenin (DIG) or fluorescein. Embryos with labeled probes were visualized using standard immunofluorescence technique. The following primary antibodies were used in this study: sheep anti-DIG (Roche; 1:200), mouse anti-dpERK (Sigma; 1:100), mouse anti-Dorsal (DSHB; 1:100), rabbit anti-Cic (see Supplemental Experimental Procedures; 1:2000), mouse anti-Yan (DSHB; 1:100), rat anti-Hkb (Ashyraliyev et al., 2009), and rabbit anti-fluorescein (Invitrogen; 1:200). The Alexa Fluor-conjugated secondary antibodies were from Invitrogen (1:500). DAPI (1:10,000; Vector Laboratories) was used to visualize nuclei.

Microscopy and Image Processing

Imaging was done on a Zeiss LSM510 confocal microscope, with a Zeiss C-Apo 20 $\times$ objective (NA = 0.6). High-resolution images (512 $\times$ 512 pixels, 12 bits depth) were obtained from a focal plane in the mid-horizontal cross section of the embryo. Images of individual embryos were automatically extracted from raw confocal files and reoriented as described elsewhere (Coppey et al., 2008). For end-on imaging, embryos were oriented using previously described microfluidic device (Chung et al., 2011). Zeiss LSM510 confocal microscope with a C-Apo 40 $\times$ water-immersion objective (NA = 1.2) was used for end-on imaging.

Gradient Quantification and Statistical Analysis

Quantification of spatial gradients of a protein of interest (dpERK, Cic, or Yan) was performed as described previously (Coppey et al., 2008). Confocal images of embryos stained with antibodies detecting the protein of interest were analyzed using a custom-made MATLAB image processing program. A Student's *t* test was used to determine the statistical significance in the asymmetry of Cic and Yan gradients. Briefly, a quantified gradient from a single embryo was first separated into anterior and posterior regions; these two regions were then independently approximated by a Gaussian with negative normalization (constant minus a Gaussian). The minima of the two fitted curves provide the anterior and posterior levels of Cic (or Yan) in this embryo. This process was repeated for gradients extracted from 20–30 embryos. A Student's *t* test was used to test whether the mean of this sample is significantly different from one.

Mathematical Model of *zen* Regulation

We explain our model using a rectangular embryo, where the *x* and *y* directions represent the AP and DV axes of the actual embryo. We assume that the AP and terminal signals are uniform along the DV axis. The rectangular case is presented here for simplicity because it contains all of the essential features of our model, and leads to a simple algebraic expression that describes the boundary of *zen* expression. The same model applies for an embryo shaped as a prolate spheroid and was used to generate images in Figure 2.

Let *R* denote a factor, such as Cic, that acts together with DI to repress *zen*. The joint repressive action of DI and *R* can be modeled by the product of DI

and *R* concentrations, DI $\cdot$ *R*. In the model, *zen* is expressed when this product is below some threshold, denoted by Θ . Thus, the level of *zen* expression, denoted by *Z*, is given by $Z(x, y) = H(\Theta - DI(y) \times R(x))$, where *H*(*u*) is the Heaviside step function: $H(u) = 1$, when $u > 0$ and zero otherwise. The boundary of *zen* expression, denoted by $y_Z(x)$, is then given by the implicit function: $\Theta = DI(y) \times R(x)$. This equation can be related to the experimentally observed profiles of the Bcd, DI, and Cic gradients.

We assume that repressor *R* is produced at a constant rate, denoted by S_R , and degraded via two parallel channels: constitutive degradation throughout the embryo and faster degradation that depends on the terminal signal. A steady-state model for repressor level then becomes $S_R = k_c R + V(R)$, where k_c is the rate constant of constitutive degradation, and $V(R)$ is the rate law for the enzymatic degradation that depends on the terminal signal. We assume that the spatial pattern of active enzyme, denoted by $E(x)$ (phosphorylated MAPK), is uniform along the DV axis. The spatial pattern of enzyme distribution $E(x)$ is given by the product of the amplitude E_0 and a symmetric function $f_E(x)$ that is equal to one at the poles and close to zero in the middle of embryo.

We assume that degradation of *R* follows Michaelis-Menten kinetics, with constants k_{cat} and K_M . We also assume that Bcd competitively inhibits MAPK-dependent repressor degradation. This leads to the following expression for the rate of signal-induced repressor degradation: $V(R) = k_{cat} E(x) R(x) / (K_M + R(x) + K_M B(x) / K_I)$, where K_I is the equilibrium constant of enzyme-Bcd interaction.

To simplify the algebra, we assume that the enzyme is not saturated by *R*. Under this assumption, $V(R) = k_{cat} E(x) R / (K_M (1 + B(x) / K_I))$. Substituting this into the mass balance for repressor levels, we get the following expression for the spatial pattern of *R*:

$$R(x) = \frac{S/k_c}{(1 + \alpha f_E(x)) / (1 + f_B(x) \beta)}$$

where α and β are defined as: $\alpha = k_{cat} E_0 / k_c K_M$, $\beta = B_0 / K_I$. These parameters can be estimated from the wild-type pattern of Cic downregulation: At the posterior pole, where $f_E(x) = 1$, but the concentration of anteriorly localized Bcd is zero ($f_B(x) = 0$), Cic is downregulated to 10% of its level in the midbody of the embryo, where $f_E(x) = 0$. From this result, we find that $\alpha \approx 9$. At the same time, at the anterior of the embryo, where $f_B(x) = 1$, Cic is downregulated only to $\sim 50\%$ of its midbody level. Based on this result, and on the estimate for α , we get that $\beta \approx 4$. Combining these estimates with the shape of the Bcd gradient and the shape of the terminal signal, we can predict how *R* is distributed throughout the AP axis of the embryo.

The equation for the boundary of *zen* expression then takes the following form:

$$D_0 f_D(y) \times \frac{S/k_c}{(1 + \alpha f_E(x)) / (1 + f_B(x) \beta)} = \Theta,$$

where D_0 is the amplitude of the nuclear DI gradient and $f_D(y)$ is the shape that characterizes its distribution along the embryo.

Introducing one more dimensionless group $\gamma = \Theta k_c / (S \times D_0)$, we get the following equation for the *zen* expression boundary:

$$\frac{f_D(y)}{1 + \alpha f_E(x) / (1 + \beta f_B(x))} = \gamma.$$

The remaining parameter of the model, γ , can be estimated from the location of the *zen* boundary in the midbody region of the embryo, where $f_E(x) \approx 0$, which implies that $\gamma = f_D(y_{Zm})$, where y_{Zm} denotes the position of the *zen* boundary at the midbody region of the embryo. Based on our previous imaging results (Chung et al., 2011; Kanodia et al., 2009), we estimate that $\gamma \approx 0.1$.

Putting everything together, we get the following equation for the expression boundary of the wild-type pattern:

$$\frac{f_D(y)}{(1 + 9 f_E(x) / (1 + 4 f_B(x)))} = 0.1.$$

Note that the values of the three dimensionless groups in the model were obtained from the asymmetries of the wild-type pattern of Cic downregulation, the location of the *zen* expression boundary in the midbody region of the embryo, and the spatial distribution of the nuclear DI gradient in the midbody region.

We can now combine the values of α , β , and γ with the empirically determined distributions for the patterning signals $f_D(y)$, $f_E(x)$, and $f_B(x)$, to plot the wild-type pattern of *zen* expression. This model predicts how the *zen* expression boundary “bends” in response to variations in the levels of anterior, terminal, and DV signals. For example, removing Bcd makes MAPK more available for *R*, lowering a corepressor that acts together with DI in *zen* repression, and results in ectopic *zen* at the anterior pole (Figure 2G).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2011.05.009.

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Supplemental Information

Gene Regulation by MAPK Substrate Competition

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Inventory of Supplemental Information

Supplemental Figures

Figure S1, related to Figure 1. Spatial pattern of the terminal MAPK signaling and *zen* expression are conserved in different *Drosophila* species

Figure S2, related to Figure 1. Yan and Cic expression in syncytial blastoderm embryos

Figure S3, related to Figure 3. Anterior substrates of MAPK are required for anterior repression of *zen*

Figure S4, related to Figure 4. Alternative mechanisms for the anterior repression of *zen*

Supplemental Experimental Procedures

Supplemental References

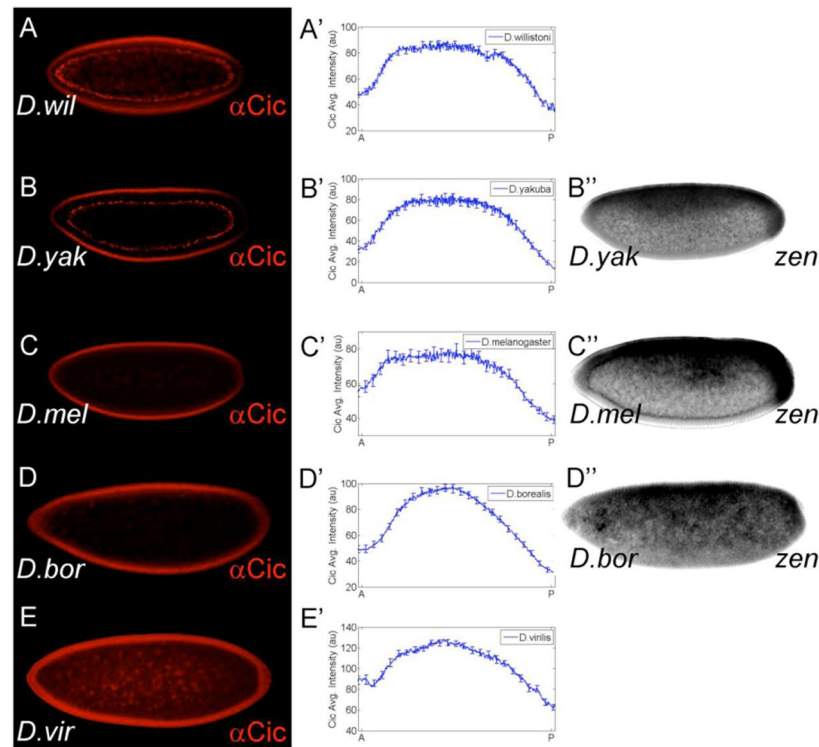


Figure S1, Related to Figure 1. Spatial Pattern of the Terminal MAPK Signaling and *zen* Pattern Are Conserved in Different *Drosophila* Species
 (Left) Pattern of Cic protein in *D. willistoni* (A), *D. yakuba* (B), *D. melanogaster* (C), *D. borealis* (D), and *D. virilis* (E). In all five *Drosophila* species, Cic is downregulated at the poles by MAPK signaling. (middle) Quantified AP profile of nuclear Cic gradients in five *Drosophila* species. For (A'-E'), N=31, 40, 29 41, and 19 respectively, and error bars are s.e.m. Note that in all five species, nuclear levels of Cic are higher at the anterior pole, suggesting weaker MAPK signaling activity in this region.
 (Right) AP asymmetric spatial patterns of *zen* are also conserved in different fly species. Since MAPK substrate competition plays an essential role in defining expression boundaries of *zen* in *D. melanogaster*, we argue that a similar mechanism will control the expression pattern of these genes in other fly species.

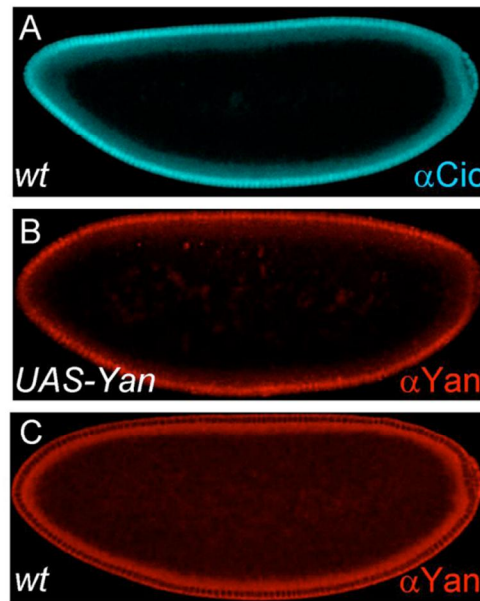


Figure S2, Related to Figure 1. Yan and Cic Expression in Syncytial Blastoderm Embryos

(A) Expression pattern of Cic in the early stage-5 embryo.

(B) Expression pattern of Yan in an embryo with ectopic expression of Yan using a maternal Gal4 driver (see Supplemental Experimental Procedures). Yan is expressed throughout the embryo and is downregulated at the poles by MAPK.

(C) Expression pattern of Yan in a wild type embryo at the same stage as panel (B). The absence of nuclear signal suggests that Yan is not active at this stage of embryogenesis.

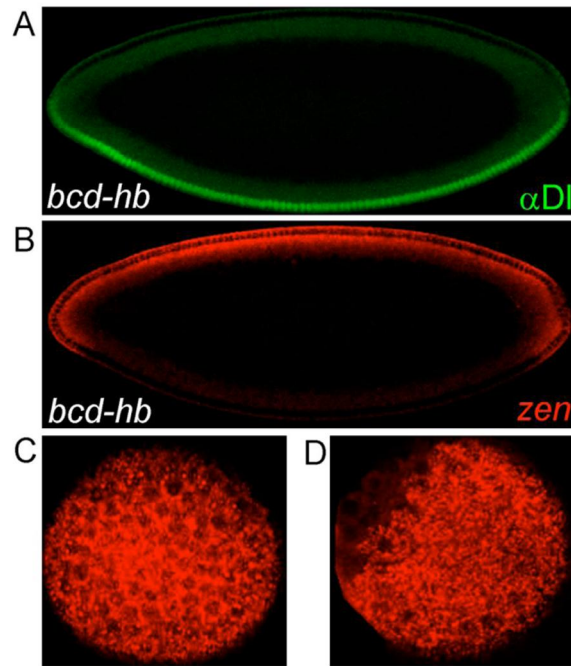


Figure S3, Related to Figure 3. Anterior Substrates of MAPK Are Required for Anterior Repression of *zen*

(A, B) Expression pattern of Dl protein and *zen* mRNA in an embryo derived from *bcd hb ts/* germline clone flies. Since *torsolike (ts/)* is provided by the follicle cells rather than by the germline, these mutant embryos still express terminal MAPK signaling. Similar to embryos from *bcd hb nos* germline clone flies from Figure 3 *bcd hb ts/* embryos also express *zen* throughout the anterior pole.

(C, D) Optical images of *zen* mRNA at the anterior (C) and posterior (D) poles showing expression of *zen* at both poles.

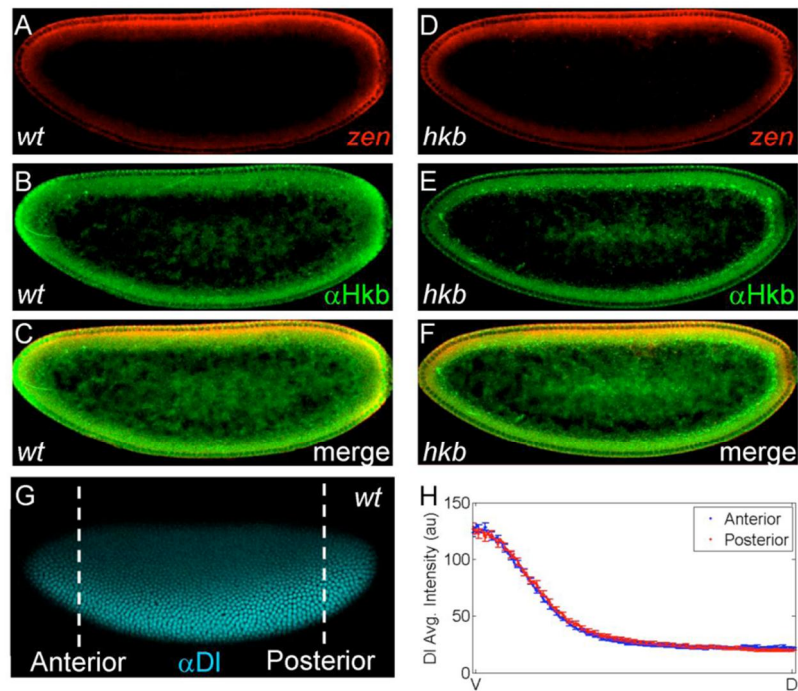


Figure S4, Related to Figure 4. Alternative Mechanisms for the Anterior Repression of *zen*

(A-C) Spatial pattern of *zen* mRNA and Hkb protein in a wild type embryo. The two expression patterns partially overlap in the dorsal-anterior region (C), suggesting that Hkb is unlikely to be the anterior specific repressor of *zen*.

(D-F) Expression pattern of *zen* mRNA and Hkb protein in embryos that lack Hkb. Even in the absence of Hkb, *zen* is still repressed at the anterior pole.

(G) Surface image of an embryo stained with anti-Dl antibody. The two dashed lines represent the approximate locations at which we obtained the optical sections to compare anterior and posterior nuclear Dl gradients.

(H) Quantified ventral-to-dorsal nuclear gradient of Dl protein at the poles. Averaged gradients of $N_{\text{Anterior}} = 50$ and $N_{\text{Posterior}} = 40$ are plotted with error bars indicating s.e.m. Note that the two gradients are indistinguishable from each other, suggesting that differences in the nuclear Dl gradient cannot account for the asymmetry in *zen* expression pattern.

Supplemental Experimental Procedures

Drosophila Stocks

The following stocks were used in this study: *OreR*, *trk¹*, *gd⁷*, *bcd⁶*, *cic¹*, *cic^{D49}*, *Hist-GFP* (Coppey et al., 2008), *UAS-Yan*, *mata4-Gal4-VP16* (Hacker and Perrimon, 1998), *Bcd-A9* (Hanes et al., 1994), and *hkb^{XM9}*. *FRT82B bcd⁶ hb¹⁵ nos^{BN}* and *FRT82B bcd⁶ hb¹⁵ tsl¹* flies were kindly provided by E. Weischaus. Flies were grown and embryos were collected at 25°C.

To make germline clone of *bcd hb nos* and *bcd hb tsl*, standard FLP-DFS technique was used. Males with *hsFLP* and *FRT82B ovo^D* were crossed with females carrying the mutation of interest. The resulting progenies were heat shocked at 37.5°C for 2 hours at 3rd instar larva stage.

Anti-Capicua Antibody

DNA near the 5' end of the *capicua* gene was used to screen an ovary cDNA library (Steinhauer et al., 1989). One cDNA clone, K6, was full-length and matched the intron-exon boundaries of the *cic-RA* transcript described by FlyBase. A 1.4-kb *Bgl*III fragment from the 3' portion of the cDNA was subcloned into the pRSETc expression vector (Invitrogen) to create plasmid RCB1.4. The cloned fragment contains coding DNA from K6 (*cic-RA*) between the genomic identifiers 3R AE014297 16122211 16125976, including 10 nucleotides of exon five, a small portion of exon six, all of exons 7–11, and part of exon 12. Importantly, this region does not include the SOX DNA-binding domain. The expressed peptide was purified using the 6XHis tag and sent for injection into rabbits to R&R Rabbitry (Stanwood, WA). The specificity of the antibody was tested by antibody stainings of the egg chambers, where the spatial pattern of Cic expression is known (Astigarraga et al., 2007). Immuno-histochemistry of *Drosophila* egg chambers using anti-Cic sera revealed nuclear stain that was absent in clones lacking the *cic* gene (data not shown).

Supplemental References

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DISCUSSION

Formation and patterning of the body plan depends on complex genetic networks and cell signaling pathways, which are temporally and spatially regulated during embryogenesis. Only a limited number of signaling pathways are involved in this process and they are reiteratively used throughout development to mediate communication between cells. However, the molecular mechanisms by which commonly used signaling pathways are able to elicit specific responses is an important but poorly understood problem.

To address this issue, we have focused on RTK/Ras/MAPK signaling, which is highly conserved in the eukaryotes and regulates several cellular responses during development and in adult homeostasis. During development, RTK/Ras/MAPK pathway controls a broad spectrum of biological processes as cell proliferation, survival, differentiation, cell migration and cell fate specification. To advance in the understanding of how the same signaling pathway is interpreted differently in distinct tissues, we have used DV axis specification in *Drosophila* as a model to study the mechanisms underlying RTK/Ras/MAPK-initiated responses. Specifically, we have studied how EGFR signaling initiates DV polarization during oogenesis and the role of the RTK-effector Cic in this process. We have also studied Cic function in response to Torso and EGFR signaling during DV subdivision of the early embryo.

In this thesis, we show that different RTK/Ras/MAPK pathways with key roles in *Drosophila* development operate through common mechanisms that involve the post-transcriptional downregulation of Cic. By downregulating Cic activity, RTK signals create gradients or boundaries of Cic repressor activity that are then translated into complementary patterns of target gene expression. Since different Cic targets are regulated in different contexts, our results support the view that RTK specificity arises mainly at the level of downstream enhancers responding to general RTK effectors (including Cic) and additional ubiquitous and tissue-specific factors.

As many of these mechanisms of RTK signaling interpretation are likely conserved during evolution, studies of *Drosophila* EGFR and Torso signaling will also eventually contribute to our understanding of vertebrate RTK signaling.

1. Mechanism of DV axis polarization by EGFR signaling

In *Drosophila*, first DV asymmetries arise during mid-oogenesis through activation of EGFR in dorsal follicle cells. Localized activity of EGFR leads to the patterning of dorsal structures of the eggshell and the orientation of the future DV axis of the embryo by restricting of *pipe* expression to the ventral region of the egg chamber. Pipe activity in ventral follicle cells is crucial for transferring DV polarity from the egg chamber to the embryo. Pipe acts as a sulfotransferase enzyme involved in the modification of extracellular matrix components that, after fertilization, activates a proteolytic cascade in the perivitelline space which culminates in the activation of receptor Toll at embryonic membrane. Finally, ventral activation of Toll results in a ventral-to-dorsal patterning gradient of Dorsal protein in the early embryo.

The mechanism by which EGFR signaling regulates *pipe* expression in the follicular epithelium has been a subject of study for years. An important feature is that while *pipe* is expressed only in the ventral-most third of the follicular epithelium, the expression of genes activated by EGFR signal is detected only in the dorsal-most third of the epithelium. Some studies indicate that it is the gradient of Grk/EGFR signaling along the DV axis what directly controls *pipe* expression (Pai et al., 2000; Goentoro et al., 2006). Supporting this model, loss of EGFR/Ras/MAPK activity causes cell-autonomous derepression of *pipe* in all dorsal and lateral positions (James et al., 2002; Peri et al., 2002), which rules out a mechanism of repression based on secondary diffusible signals (Jordan et al., 2000; Zhao et al., 2000). On the other hand, some studies show that ectopic expression of Mirr, a homeodomain transcription factor induced by EGFR signaling, represses *pipe* expression (Jordan et al., 2000; Zhao et al., 2000a). However, clonal analyses indicate that loss of *mirr* function does not affect normal expression of *pipe* (Peri et al., 2002). We reasoned that Mirr function could be masked by redundant activities of the two other components of the *iroquois* complex (Iro-C), *araucan* and *caupolican*, which encode homeodomain proteins highly related to Mirr.

1.1 Mirr mediates cell-autonomous repression of *pipe* in dorsal follicle cells

In this thesis, we report that Mirr is the only member of the Iro-C complex which is essential for repression of *pipe* in dorsal follicle cells (Andreu et al., 2012; Figure 1). Loss-of-function clones of *mirr* show cell-autonomous derepression of *pipe-lacZ* expression at all positions where *pipe* is normally off. This repression occurs even in lateral and dorsal-posterior cells close to the normal *pipe* domain, where Mirr protein levels are very low or undetectable. This high sensitivity of *pipe* to Mirr repression is consistent with the evidence that low levels of EGFR/Ras/MAPK signaling mediate *pipe* repression in lateral and dorsal-posterior cells (Pai et al., 2000; James et al., 2002; Peri et al., 2002). We reason that previous negative results were caused by residual Mirr activity of the *mirr^{e48}* allele (Peri et al., 2002) since loss-of-function clones of the hypomorphic allele *mirr¹⁸³⁵* displayed ectopic expression of *pipe-lacZ* only in a fraction of the clones nearby the endogenous *pipe* domain (Andreu et al., 2012; Figure S1).

Our work also shows that Mirr mediates *pipe* repression by binding to a conserved element (*r1*) in its regulatory region. We have defined a minimal enhancer module (*M2-lacZ*) which responds to EGFR signaling and includes the *r1* element. Moreover, we have found that *pipe* regulation depends crucially on the *r1* sequence, since mutation of this element results in dorsal derepression of reporter constructs (Andreu et al., 2012; Figure 2A-G). These mutant constructs, particularly *M2^{r1mut}-lacZ*, exhibit weaker and more variable expression than their analogous wild-type constructs throughout the follicular epithelium, raising the possibility that the *r1* element includes sequences required for both repression in DA cells and full activation in ventral cells. In addition, we found in EMSA experiments that recombinant Mirr protein binds strongly to the *r1* sequence but not to the mutated *r1mut* sequence that causes dorsal derepression of reporter constructs *in vivo* (Andreu et al., 2012; Figure 2I). Furthermore, Mirr binds preferentially to a site containing the sequence ACACGA, which resembles the previously defined minimal Mirr-binding element *in vitro* (Billioni et al., 2005).

These results are consistent with analyses of the regulatory region of the *pipe* gene done in parallel by other groups. Technau and colleagues have defined a *cis*-

Discussion

regulatory module of *pipe* with a repressor element that contains the *r1* sequence (Technau et al., 2012). Interestingly, Fuchs and colleagues have identified related Mirr-responsive elements in *pipe* and *broad* genes, an EGFR-regulated target involved in dorsal appendage formation. They found that Mirr represses both genes in dorsal follicle cells during stage 9-10A, whereas by stage 10B Mirr plays a role in the activation of a late *broad* enhancer (Fuchs et al., 2012).

Therefore, our work shows that the activity of Mirr as a mediator of EGFR signaling during oogenesis is not only required for the eggshell morphogenesis (Atkey et al., 2006; Boisclar et al., 2009), but also for the establishment of embryonic polarity. Nevertheless, the effect of Mirr on the eggshell and the embryo involves two different sensitivities to its level of expression as analyses of different *mirr* mutant phenotypes show that some heteroallelic combinations only affect the eggshell but not the embryonic cuticle (Zhao et al., 2000a). Therefore, high levels of Mirr are required for a proper eggshell patterning while low or undetectable levels of Mirr protein are enough to repress *pipe* gene.

1.2. Cic supports *pipe* expression by repressing *mirr*

Previous studies have shown that Cic is required for *pipe* expression in ventral follicle cells; accordingly, embryos derived from *cic* mutant females are severely dorsalized (Goff et al., 2001). However, the mechanism by which Cic controls DV patterning was unknown. Cic functions as a repressor generally downstream of RTK signaling pathways in different contexts (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Astigarraga et al., 2007; Tseng et al., 2007). Moreover, Cic has been proposed to restrict appendage-producing follicle cell fate by blocking induction of *mirr* by anterior positional cues (Atkey et al., 2006). Therefore, we hypothesized that Cic could support *pipe* transcription indirectly by repressing *mirr* in ventral follicle cells.

Our studies show that Cic is required cell-autonomously for *pipe* expression and that it acts downstream of EGFR/Ras/MAPK signaling, as mosaic analyses of *cic* mutant clones and *ras cic* double mutant clones lack *pipe* expression in all positions. On the contrary, *ras* mutant clones exhibit ectopic *pipe* expression in dorsal cells without

affecting its ventral domain (Andreu et al., 2012; Figure 3A-C). Hence suggesting that differently to mutations in negative regulators of EGFR pathway, such as Cbl, Cic does not cause ventral *pipe* repression by upregulating EGFR signaling (Pai et al., 2000).

Consistent with the observation that both *grk cic* and *cic* mutant ovaries exhibit expanded *mirr* expression only in the anterior half of the follicular epithelium (Atkey et al., 2006), *cic* and *ras cic* double mutant clones only cause ectopic expression of *mirr* in the anterior region (Andreu et al., 2012; Figure S2). This supports a model in which *mirr* is normally activated by EGFR signaling and by an independent anterior signal, with Cic being required to counteract this later input and repress *mirr* in ventral-anterior follicle cells. Nevertheless, there is an apparent discrepancy: although Cic is essential for *pipe* expression in both anterior and posterior follicle cells, loss of Cic derepresses *mirr* only in anterior cells. Given that Mirr normally represses *pipe* in lateral and dorsal-posterior follicle cells where Mirr is undetectable, we reasoned that loss of *cic* function in ventral-posterior cells may give rise to very low levels of Mirr expression that are sufficient to repress *pipe*.

Indeed, whereas Cic activity is dispensable for the expression of mutant *pipe* enhancers lacking the Mirr-binding motif (*M1^{r1mut}-lacZ* and *M2^{r1mut}-lacZ*) in both anterior and posterior follicle cells, the reporter constructs which contain the *r1* element (*M1-lacZ* and *M2-lacZ*) require Cic activity for their expression in ventral follicle cells (Andreu et al., 2012; Figure 3D and E). This strongly suggests that Cic supports *pipe* expression by acting indirectly through the Mirr-binding motif (*r1*). Furthermore, we confirmed that Mirr represses *pipe* in the absence of Cic by generating *mirr* mutant clones in *cic* mutant ovaries (Andreu et al., 2012; Figure 3F). We found that *pipe* expression is upregulated, independently of the position, in all mutant clones throughout the follicular epithelium, indicating that *cic* loss of function in ventral-posterior cells may give rise to very low levels of *mirr* expression that are nevertheless sufficient to repress *pipe*.

1.3. Significance of EGFR-dependent downregulation of Cic

In several contexts, Ras/MAPK activation leads to downregulation of Cic nuclear levels (Jiménez et al., 2012). Previous studies in our group showed that EGFR signaling downregulates Cic nuclear levels in DA cells, causing partial re-localization of Cic protein towards the cytoplasm (Astigarraga et al., 2007). This may contribute to *mirr* induction through relief of repression, but, as mentioned above, EGFR signaling has a positive input on *mirr* expression independent of Cic. Thus, although Cic is essential for *pipe* expression during DV axis formation, the contribution of EGFR-dependent downregulation of Cic in this process was unclear.

Using a reporter of Cic-mediated repression (*CUASC-lacZ*), we show that Cic repression activity is downregulated by EGFR signaling in dorsal follicle cells (Andreu et al., 2012b; Figure 1). Interestingly, Cic downregulation is sensitive to low levels of graded EGFR activation as high levels of the reporter expression extends along dorsal-posterior follicle cells. This observation is consistent with recent studies done in collaboration with our group, where it is reported that EGFR/MAPK signaling can relieve transcriptional repression of direct targets of Cic before reducing Cic levels in the nucleus (Lim et al. 2013).

Our study also shows that EGFR signaling modulates Cic-mediated repression of *mirr* to determine the precise position of the border of *pipe* expression in lateral and dorso-lateral follicle cells. Consistent with the idea that *mirr* expression is not just a consequence of Cic downregulation, overexpression of Cic protein induces *pipe* transcription only in lateral regions close to the endogenous *pipe* domain without causing detectable changes in *mirr* expression. Similarly, when we analyze a Cic mutant derivative insensitive to MAPK-dependent downregulation (Cic^{ΔC2}) no repression of *mirr* is observed although it causes moderate expansion of *pipe* domain. Only high-level expression of Cic^{ΔC2} produces full repression of *mirr* and the corresponding derepression of *pipe* (Andreu et al., 2012b; Figure 2). These results suggest a competition mechanism between EGFR-dependent and EGFR-independent activation of *mirr* and Cic-mediated repression. As low or undetectable levels of Mirr are sufficient to mediate repression of *pipe*, downregulation of Cic can

influence the pattern of *Mirr* distribution within the region of low *mirr* expression, thereby affecting *pipe* transcription.

We propose a model where EGFR signaling regulates the size of *pipe* expression domain through *Cic*-dependent and -independent mechanisms (Andreu et al., 2012b; Figure 3). In DA cells, EGFR signaling induces *Mirr* expression which then represses *pipe*. In this position downregulation of *Cic* may contribute to *Mirr* induction but other EGFR-dependent mechanisms are also involved (Atkey et al., 2006; Astigarraga et al., 2007). In lateral and dorsal-posterior cells, low levels of EGFR and *Mirr* reach a threshold where *pipe* is no longer repressed and define its expression border. EGFR-mediated downregulation of *Cic* influences the precise position of the border of *pipe* expression by facilitating *mirr* expression in this region and placing this boundary more ventrally. Finally, in ventral follicle cells, very low EGFR signaling together with other positive input(s) in the anterior region may activate *mirror* but are counteracted by *Cic*, so that *mirr* is repressed and *pipe* is transcribed.

However, despite having progressed in the understanding of the role of *Cic* in *pipe* regulation, further studies should be done to characterize the molecular mechanisms involved in the process. Although we have shown that *Cic* supports *pipe* expression in ventral follicle cells by repressing *mirr*, it remains unclear whether this repression is direct or whether *Cic* regulates an intermediate factor, which controls *mirr* transcription. Another unsolved aspect is the identification of the anterior EGFR-independent activator of *mirr* that *Cic* is required to counteract permitting *pipe* transcription in ventral region. It has been proposed that the anterior signaling molecule required for *mirr* expression is Dpp, which is produced by the anterior-most follicle cells and regulates gene expression along the AP axis (Twombly et al., 1996; Deng and Bownes, 1997; Peri and Roth, 2000; Atkey et al., 2006). Consistent with this hypothesis, preliminar experiments in our group show that overexpression of the constitutively active receptor of Dpp, *thickveins* (*tkv*), in *cic* loss-of-function clones causes ectopic expression of *mirr* detectable in more posterior positions than in a *cic* loss-of-function background.

Discussion

In addition, we have noted that the regulatory circuit linking EGFR signaling to *pipe* expression shares basic functional principles with other signal-regulated responses. This patterning network illustrates two basic mechanisms common to several signaling pathways for the control of gene expression: ‘cooperative activation’ and ‘default repression’ (Barolo and Ponsakony, 2002). On the one hand, EGFR functions cooperatively with other positive input(s) to activate *mirr* expression. On the other hand, Cic functions as a default repressor to prevent *mirr* activation by low levels of EGFR signaling and weak activation input from anterior positional cues. Remarkably, there are other cases in development where morphogenetic signals are translated into antagonistic repressor gradients, which in turn are responsible for spatial expression of downstream target genes. For example, Dpp signaling in the *Drosophila* wing disc generates a complementary gradient of Brinker activity that represses Dpp targets such as *optomotor-blind* and *spalt* (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Müller et al., 2003). Similarly, in the vertebrate neural tube repressive interactions among downstream transcriptional factors of Sonic Hedgehog signaling gradient results in the proper tissue pattern (Balaskas et al. 2012).

2. Cic as a general sensor downstream of RTK signaling

For years, two members of the ETS-domain transcription factor superfamily, the activator Pointed-P2 and the repressor Yan, have been considered to be the most prominent effectors of RTK/Ras/MAPK signaling pathway in *Drosophila* and vertebrates (Simon, 2000; Yordy and Muise-Helmericks, 2000). Both are directly phosphorylated by MAPK upon RTK activation, which activates Pointed-P2 and inhibits Yan. Since Pointed-P2 and Yan have similar binding preferences, the activation of MAPK is thought to promote transcription by causing a switch of activated Pointed-P2 for Yan at ETS sites (O’Neill et al., 1994; Gabay et al., 1996; Tootle and Rebay, 2005). Nevertheless, several studies in the last decade have identified the HMG-box factor Cic as a general sensor of RTK signaling in the induction of cellular responses during *Drosophila* development (Jiménez et al., 2000; Goff et al., 2001; Atkey et al., 2006; Astigarraga et al., 2007; Tseng et al., 2007). Cic

protein has been reported to act as a repressor of RTK-responsive genes, silencing them in the absence of signaling. However, direct binding of Cic to the regulatory regions of these genes had not been shown and the mechanism by which Cic interprets RTK signaling was unknown.

Our work shows that Torso and EGFR signaling pathways control expression of downstream genes through common octameric sequences, which are binding sites for Cic, in several developmental contexts (Ajuria et al., 2011; Figure 6). Indeed, Cic protein binds to these octameric sequences in vitro, T(G/C)AATGAA (Ajuria et al., 2011; Figure S2), which are similar to the motifs characterized in human as Cic-binding sites, TGAATG(G/A)A (Kawamura-Saito et al., 2006).

We have analyzed the role of Cic in repression of different target genes of RTK signaling pathways during development. In the early embryo, Torso signaling regulates *tll* and *hkb* expression at the poles via the octameric repressor elements by downregulating Cic protein in this region. This octameric sequences are similar to the *tor-RE* described as a regulatory element in the *tll* enhancer (Liaw et al., 1995; Löhr et al., 2009). Similarly, EGFR signaling induces *ind* expression by relieving Cic repression in the neuroectodermal region of the embryo. Conserved Cic-binding sites are present within the *A-box* elements, which were previously identified as a repressor element in the enhancer of *ind* (Stathopoulos and Levine, 2005). Moreover, Cic octamers are also found in the first intron of *aos*, which is expressed in the prospective vein cells of the wing disc and is also regulated by EGFR signaling. During wing development, EGFR signal downregulates Cic in the presumptive vein cells permitting the expression of *aos* in those cells. Remarkably, Cic octameric sites are sufficient to translate RTK signaling into restricted patterns, as the simple combination of activator-binding sites and Cic octamers is able to establish localized reporter expression in response to Torso or EGFR signaling (Ajuria et al., 2011; Figures 2 and 5H-M). Still, regulation of these target gene expression depends not only on RTK-mediated Cic downregulation, but also on additional ubiquitous or localized activators.

Interestingly, Cic activity in the early embryo resembles to its function in the ovary (Andreu et al., 2012; Figure 4B), where Cic supports *pipe* expression in the ventral

Discussion

region by repressing *mirr*. EGFR signaling in the dorsal region downregulates Cic but also contributes to the activation of *mirr*. In the early embryo, Cic supports the expression of central gap genes such as *Krüppel* (*Kr*) or *knirps* (*kni*). In this case, Torso signaling at the poles of the embryo downregulates Cic activity to induce gap gene repressors, such as *tll* or *hkb*, which are repressed by Cic in the center of the embryo (Paroush et al., 1997; Jiménez et al., 2000; Goff et al., 2001; Morán and Jiménez, 2006; Löhr et al., 2009; Ajuria et al., 2011). Therefore, similar interactions between Cic and RTK-induced repressors (Mirr and Tll) are essential for the early subdivision of both DV and AP embryonic axes. However, downregulation of Cic plays a more critical role in inducing *tll* expression than it does during EGFR-dependent activation of *mirr*, as Torso signaling functions by simply relieving Cic repression (Astigarraga et al., 2007; Cinnamon et al., 2008).

With Cic octameric sites already characterized, future studies will reveal new target genes and, presumably, new roles of Cic during development in the regulation of RTK signaling responses. Indeed, to analyze whether *mirr* Cic-mediated repression in ventral follicle cells of the egg chamber is direct or not, it would be useful to search for Cic-binding sites in its regulatory region. For this reason, we have started the identification of conserved DNA sequences that might be controlling *mirr* expression in dorsal follicle cells.

In addition, the activity of Gro co-repressor is required for repression of *tll* and *hkb* (Paroush et al., 1997; Ajuria et al., 2011) and our studies show that the Cic-binding motifs are essential for Gro recruitment in vivo to the *hkb* enhancer. As most transcriptional repressors require one or more co-repressors to inhibit the expression of their target genes (Courey and Jia 2001; Gray and Levine, 1996b; Payankulam et al., 2010), we favor a model in which Cic recruits Gro to mediate repression. However, it remains unclear whether Cic and Gro interact directly in vivo or there are additional factors involved. Moreover, Cic-mediated repression does not require Gro activity in all contexts. In the ovary, for example, Gro is dispensable for Cic function as *gro* loss-of function do not affect *mirr* or *pipe* expression patterns (our unpublished results; Technau et al., 2012). Therefore, further studies need to be done to understand the molecular aspects of the relationship between Cic and Gro in the repression of *tll* and *hkb*, but also, to

determine if Cic repressor mechanism requires the presence of Gro for the regulation of other genes in the early embryo and in other tissues, such as *ind* in the neuroectoderm and *aos* in the wing disc.

3. Gene regulation during DV axis formation by MAPK substrate competition

MAPK is the last component of the protein kinase cascade triggered by RTK signaling. Often, MAPK is present in the cytoplasm in an inactive state and when it is phosphorylated becomes active and enters the nucleus. There, it phosphorylates serine and threonine residues of its multiple substrates, generally transcription factors, to induce cellular responses. Phosphorylated MAPK is controlled by multiple enzymes, adaptors and scaffolds proteins that regulate its catalytic activity, interaction partners and subcellular localization. Recent studies have reported that substrates phosphorylated by MAPK can also interfere with the processes that control phosphorylation of MAPK itself, such as MAPK phosphatases, by direct competitive interactions (Bardwell et al., 2003; Grewal et al., 2006; von Kriegsheim et al., 2009; Kim et al., 2011a). This is surprising given that control of MAPK phosphorylation is generally considered to be independent of its substrates, which are viewed as simple downstream effectors of MAPK activity.

One MAPK substrate is Cic protein, which is uniformly expressed in the embryo and degraded at the poles in response to its phosphorylation by MAPK (Astigarraga et al., 2007). Interestingly, the distribution of Cic protein is asymmetrical with higher levels at the anterior pole. This is due to the presence in the anterior region of the maternal determinants Hunchback (Hb) and Bicoid (Bd) that antagonize MAPK-dependent downregulation of Cic (Kim et al., 2010). However, it is not clear whether the effects of MAPK substrate competition have a real impact on Cic-dependent gene regulation.

In collaboration with the group of Stanislav Shvartsman, we have studied gene expression changes that occur in response to competitive interactions between Cic and other MAPK substrates that are present in the embryo. Specifically, we have analyzed the expression pattern of one particular Cic target, *zerknüllt* (*zen*), which depends on the action of the terminal and DV patterning systems and displays a

pronounced AP asymmetry. *zen* expression is controlled in lateral and ventral regions by Dl-mediated repression but at the poles this repression is antagonized by Torso signaling (Rusch and Levine, 1994). Although the precise mechanism of *zen* derepression at the poles is unknown, it is likely to depend on MAPK-dependent downregulation of Cic (Jiménez et al., 2000; Goff et al., 2001; Astigarraga et al., 2007).

We found that anteriorly localized MAPK substrates, such as Bcd, that counteract the activity of MAPK towards Cic, enhance Cic-mediated repression contributing to asymmetric expression of *zen* in the anterior versus the posterior pole. Cic downregulation and *zen* expression become symmetric in embryos lacking Bcd and Hb (Kim et al., 2011b; Figures 3D and 3H). In addition, expression of a form of Bcd, which is unable to bind to DNA, in *bcd* mutant embryos rescues Cic downregulation gradient and *zen* expression is reduced at the anterior pole (Kim et al., 2011b; Figures 4D and 4J).

Since Bcd is a key regulator of anterior embryonic patterning, and *zen* is a target of the DV and the terminal system, our results have revealed a new mechanism of signal integration between the anterior, DV and Torso regulatory systems. Our work also shows that MAPK substrate competition can modulate gene expression in the early embryo. Furthermore, it has been reported that a given mRNA can regulate translation of other transcripts indirectly by competing for a common microRNA (Erbert et al., 2007; Poliseno et al., 2010). Therefore, competitive interactions are likely to be a general control strategy in complex signaling networks where regulatory factors, such as MAPK and microRNAs, interact with multiple targets.

4. Role of Cic in Dorsal-mediated repression downstream of Torso signaling

The nuclear concentration gradient of Dl determines the DV patterning of the early embryo. Dl protein can function as both activator and repressor of transcription to regulate cell fate along the DV axis (Jiang et al. 1992; Pan and Courey, 1992). Dl-mediated repression requires the recruitment of co-repressor Gro and is mediated by conserved DNA sequences, ventral repressor elements (VRE), which are present adjacent to Dl binding sites within the regulatory region of *zen* and *dpp* genes (Jiang

et al., 1993; Kirov et al., 1993; Huang et al., 1993; Dubnicoff et al., 1997). The VRE of *zen* contains Df binding sites as well as AT-rich elements which are essential for its restriction to the dorsal region of the embryo. Likely, ventral repression requires the formation of a multiprotein complex at the VRE, which includes Df, Gro and additional DNA binding proteins. Significantly, repression by Dorsal is inhibited by Torso/Ras/MAPK signaling at the poles of the embryo, suggesting that one or more components of the Dorsal repressor complex are downregulated by this signal (Rusch and Levine, 1994).

It has been reported that *cic* mutant embryos exhibit derepression of *zen* in ventral regions (Jiménez et al., 2000). Additionally, dorsal-specific expression of *zen* retracts in *cic^{AC2}* embryos, being almost absent at the posterior pole (Astigarraga et al., 2007). Consistent with that *trk* mutant embryos lack *zen* expression at the poles (Kim et al., 2011; Figure 1I) while in *trk cic* double mutant embryos *zen* is significantly derepressed (Kim et al., 2011; Figure 1K). Together these results suggest that relief of Dorsal-mediated repression by Torso signaling occurs through downregulation of Cic.

Furthermore, Cic octameric sequences resemble specific AT-rich sites in *zen* enhancer. Taking all the evidence together, we hypothesize that Cic acts as a co-repressor of Dorsal in DV patterning. It should be noted that such role of Cic should be regulated to avoid constitutively repression of Cic in dorsal regions. One possibility would be that Cic binds weakly to the AT-rich sites and this binding is stabilized by Dorsal. To test our hypothesis, we have started to generate synthetic enhancers with different combinations of AT-rich sites and Df binding sites to analyze them in different mutant backgrounds.

CONCLUSIONS

1. EGFR signaling restricts *pipe* expression to ventral follicle cells through Mirr, a repressor induced by the pathway in dorsal follicle cells.
2. Mirr represses *pipe* expression by binding directly to a conserved element (*r1* motif) in its regulatory region.
3. Mirr is a common mediator of EGFR signaling responses in the specification of DV polarity of both eggshell and embryo.
4. Cic supports *pipe* expression indirectly by repressing *mirr* in ventral follicle cells; in the absence of Cic function, low-level derepression of *mirr* is sufficient to prevent *pipe* expression.
5. Cic repressor activity is downregulated by EGFR signaling in dorsal follicle cells.
6. EGFR-mediated downregulation of Cic modulates the spatial distribution of Mirr in lateral follicle cells, thus contributing to define the position of the *pipe* expression border.
7. The resulting EGFR-dependent network controlling DV axis specification in the ovary resembles the role of Torso signaling and Cic repression in regulating AP patterning in the early embryo.
8. Cic repression downstream of RTK signaling occurs through direct binding to the octameric sequence T(G/C)AATGAA in the regulatory region of target genes.
9. Asymmetric expression of *zen* at the poles of the embryo depends on competitive inhibition of Cic downregulation by anteriorly localized MAPK substrates.
10. MAPK substrate competition can modulate gene expression in the early embryo and integrate the actions the anterior, DV and Torso regulatory systems.

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SUMMARY IN SPANISH

INTRODUCCIÓN

La embriogénesis animal es un proceso de gran complejidad y precisión mediante el cual tiene lugar la transformación de una única célula, el cigoto, en un individuo maduro compuesto por multitud de células distintas. En los metazoos con simetría bilateral, este proceso supone el establecimiento y diferenciación de dos ejes, antero-posterior (AP) y dorso-ventral (DV), que han de ser definidos molecularmente y que definen el plan corporal del futuro individuo.

Solamente una pequeña fracción de todos los genes contenidos en el genoma de un determinado animal son necesarios para la formación de su plan corporal. Dos clases de productos génicos constituyen el kit genético básico para el desarrollo: los factores de transcripción, los cuales regulan la expresión de otros genes, y los miembros de las vías de señalización, los cuales median las interacciones intercelulares. Este kit genético básico se encuentra altamente conservado en todos los organismos eucariotas. Los eventos de señalización celular junto con la expresión de factores de transcripción específicos controlan la formación, la identidad y el patrón de las principales características del plan corporal animal. Existen un número reducido de vías de señalización que, sin embargo, son usadas reiteradamente durante el desarrollo de los metazoos: la vía de TGF β /BMP, la vía de Notch, la vía de Wnt/ β catenin, la vía de Hedgehog y las vías Receptor Tirosina Quinasa (RTK, siglas en inglés), entre otras.

En esta tesis, nos hemos centrado en el estudio de las vías de señalización RTK, las cuales regulan un amplio abanico de procesos biológicos en los metazoos desde el desarrollo embrionario hasta la homeostasis del individuo adulto. Concretamente, hemos investigado los mecanismos por los cuales las vías RTK controlan la expresión génica. Para ello, hemos aprovechado las herramientas genéticas que nos ofrece *Drosophila* como organismo modelo y hemos estudiado la especificación del eje DV donde la vía RTK/Ras/MAPK juega un papel crucial en la regulación de la expresión localizada de genes encargados de determinar destino celular.

En el ovario de *Drosophila*, la señal de EGFR es necesaria para el establecimiento del eje DV de la cámara ovárica y es la responsable de la polaridad tanto del corion como del futuro embrión. El establecimiento de dicho eje requiere la activación de

forma localizada de la vía de EGFR/Ras/MAPK en el epitelio folicular que envuelve al oocito. Durante etapas intermedias de la ovogénesis, el núcleo del oocito situado en la futura región dorso-anterior da lugar a la traducción local del mRNA de *grk*. Grk es un factor del tipo TGF α que se acumula alrededor del núcleo del oocito y cuya secreción activa la señal de EGFR en las células foliculares adyacentes. La activación de EGFR confiere a dicho epitelio destino dorsal e inhibe la síntesis de Pipe en esa región. La expresión de *pipe* en las células foliculares ventrales es crucial para la correcta formación del patrón DV del embrión. El mecanismo por el cual la señal de EGFR restringe la expresión de *pipe* a las células foliculares ventrales ha sido objeto de estudio durante años pero todavía sigue siendo una incógnita. A pesar de que en *Drosophila* los efectores mejor caracterizados de la vía Ras/MAPK son los factores de transcripción ETS Pointed y Yan, han surgido factores de transcripción adicionales que actúan como mediadores de las señales RTK. En particular, el factor HMG-box Capicua (Cic) se ha descrito que actúa como represor transcripcional por debajo de dos vías RTK/Ras/MAPK, la vía de Torso y la vía de EGFR, en distintos contextos. De hecho, la actividad de Cic es esencial para la expresión de *pipe* en las células foliculares ventrales del ovario.

Pipe actúa como una enzima sulfotransferasa modificando diferentes componentes de la membrana vitelina. Una vez sulfonados, dichos componentes actúan como determinantes ventrales poniendo en marcha, tras la fertilización, una cascada proteolítica que culmina con la activación del receptor Toll en la superficie ventral del embrión. A continuación, la señal de Toll inducirá la translocación de Dorsal (Dl) al núcleo en forma de gradiente con niveles más elevados en la región ventral del embrión e indetectables en la región dorsal. Finalmente, será el gradiente de proteína Dl el que permitirá la especificación de las diferentes regiones del embrión a lo largo del eje DV. Dl puede actuar como activador o como represor para regular la expresión de los distintos genes cigóticos. Para la represión en ventral de genes como *zerknüllt* (*zen*) es esencial la asociación de Dl con el correpresor Groucho (Gro) y otros co-represores. Este complejo represor se encuentra regulado negativamente por la vía de Torso en los polos del embrión permitiendo la expresión de *zen* en esa región. Por lo tanto, el patrón de expresión de *zen* en el embrión temprano depende de la acción conjunta del sistema DV y terminal. Algunos estudios sugieren que Cic

participa en la regulación del patrón de *zen* y, además, se ha descrito que en el contexto del embrión temprano Cic actúa junto con Gro para reprimir genes como *tailless (tll)* y *huckebein (hkb)* en respuesta a la señal de Torso.

Por otro lado, para la activación del gen *intermediate neurogenic defective (ind)* en la región del neuroectodermo del embrión son necesarias la contribución de la proteína Dl y la vía de EGFR. El patrón de expresión de *ind* en forma de dos bandas en los dominios laterales del embrión depende del represor *ventral neuroblast defective (vnd)* en la región ventral y de un factor desconocido hasta el momento en la región dorsal.

OBJETIVOS

El objetivo general de la tesis es la caracterización de los mecanismos de regulación génica de las vías RTK en el contexto de la formación del patrón DV en *Drosophila*. Específicamente, los objetivos planteados en este trabajo son los siguientes:

1. Caracterizar el mecanismo por el que la señal de EGFR controla la expresión ventral del gen *pipe* en el ovario.
2. Analizar el papel de Cic en la regulación del gen *pipe* durante la oogénesis.
3. Estudiar la función de Cic en respuesta a las vías de Torso y EGFR durante la subdivisión del eje DV en el embrión.

RESULTADOS Y DISCUSIÓN

1. Mecanismo de represión transcripcional de *pipe*

Como se ha comentado anteriormente, el mecanismo por el cual la señal de EGFR regula la expresión de *pipe* en el epitelio folicular del ovario era desconocido. Un aspecto característico de dicha regulación es que mientras *pipe* se expresa únicamente en el tercio más ventral de dicho epitelio, la señal de EGFR sólo es detectada en el tercio más dorsal. Algunos estudios indican que es el gradiente de señalización de Grk/EGFR a lo largo del eje DV el que controla de forma directa la

expresión de *pipe* y descartan cualquier mecanismo de represión basado en señales secundarias difusibles. Por otro lado, otros trabajos muestran que la expresión ectópica de Mirror (Mirr), un factor de transcripción con homeodominio inducido por la vía de EGFR, reprime la expresión de *pipe*. Sin embargo, análisis clonales indican que la pérdida de función de *mirr* no afecta al patrón de expresión normal de *pipe*.

En esta tesis, mostramos que Mirr media la represión de *pipe* en las células foliculares dorsales uniéndose a un elemento conservado (*r1*) de su región reguladora. La pérdida de función de Mirr da lugar a la desrepresión de *pipe* incluso en regiones laterales y dorsales donde los niveles de proteína Mirr son muy bajos o indetectables. Probablemente los resultados negativos de trabajos previos son debidos a la actividad residual del alelo hipomorfo utilizado, *mirr^{e48}*. Definimos un *enhancer* mínimo de *pipe* (*M2-lacZ*) que responde a la señal de EGFR y reproduce el patrón de expresión del gen. Dicho transgén contiene el motivo conservado *r1*, el cual al ser mutado da lugar a desrepresión en la región dorsal (*M2^{r1mut}-lacZ*). Además, ensayos de retardo en gel muestran que la proteína recombinante Mirr se une preferentemente a la secuencia ACACGA del motivo *r1* pero no a las secuencias mutantes. Por lo tanto, nuestros resultados indican que la actividad de Mirr como mediador de la señal de EGFR durante la oogénesis no es únicamente requerida para la morfogénesis del corion, como se había descrito anteriormente, sino también para el establecimiento de la polaridad del futuro embrión.

Como se ha mencionado en la introducción, Cic es necesario para la expresión ventral de *pipe* aunque el mecanismo por el cual controla la formación del patrón DV continuaba sin conocerse. Teniendo en cuenta que Cic funciona en general como represor por debajo de las vías RTK y que algunos estudios sugieren que puede estar involucrado en la regulación de *mirr* en la formación de los apéndices del corion, nuestra hipótesis de trabajo era que Cic mantiene la expresión de *pipe* de forma indirecta reprimiendo a *mirr* en las células foliculares ventrales.

Nuestros estudios muestran que Cic es necesario de forma autónoma celular para la expresión de *pipe* y que actúa por debajo de la vía de EGFR/Ras/MAPK para reprimir a *mirr* en la región ventral. Clones mutantes *cic* y dobles *ras cic* pierden la

expresión de *pipe*. Por el contrario, clones mutantes *ras* exhiben expresión ectópica de *pipe* en la región dorsal sin afectar su dominio ventral normal. Además, la actividad de Cic es dispensable para la expresión de los transgenes que contienen el motivo de unión a Mirr mutado; mientras que para aquellos que contienen el elemento *r1* intacto, la actividad de Cic es esencial. Finalmente, para confirmar que Mirr reprime a *pipe* en la ausencia de Cic generamos clones mutantes *mirr* en ovarios mutantes para *cic* y observamos que la expresión de *pipe* se encontraba desreprimida en todos los clones independientemente de su posición.

2. Relevancia de la downregulación de Cic en la regulación de *pipe*

En diversos contextos, la activación de Ras/MAPK conlleva la regulación negativa de los niveles nucleares de proteína Cic. Estudios previos en nuestro grupo muestran que la señal de EGFR inactiva a Cic en la células dorso-anteriores provocando la relocalización de la proteína Cic en el citoplasma. A pesar de que Cic es esencial para la expresión de *pipe* durante la formación del patrón del eje DV, la contribución de la regulación negativa por EGFR de Cic en este proceso no estaba clara.

Usando una construcción reportera de la actividad represora de Cic (*CUASC-lacZ*), mostramos que dicha actividad es regulada negativamente por la vía de EGFR en las células foliculares dorsales. Cabe destacar que la regulación negativa de Cic es sensible a niveles bajos de activación de EGFR, ya que niveles altos de expresión del transgén se extienden hasta la región dorso-posterior del epitelio folicular. Nuestros estudios muestran también que la señal de EGFR modula la represión de *mirr* mediada por Cic para determinar la posición precisa del borde de expresión de *pipe* en las células foliculares laterales y dorso-laterales. La expresión de *mirr* no es consecuencia únicamente de la regulación negativa de Cic sino que la vía de EGFR también contribuye a su inducción de una forma independiente de Cic. Por esta razón, únicamente la sobreexpresión de formas mutantes de Cic insensibles a la regulación negativa dependiente de MAPK (*Cic^{ΔC2}*) dan lugar a una completa represión de *mirr* y la correspondiente desrepresión de *pipe* incluso en las regiones más dorsales.

3. Cic como sensor general de las vías RTK

A pesar de que en la última década Cic ha surgido como efector de las vías RTK actuando como represor transcripcional en diferentes contextos, se desconocía su mecanismo de acción y la unión a las secuencias reguladoras de sus genes diana no se había demostrado.

Nuestro trabajo muestra que las vías de Torso y EGFR controlan la expresión de sus genes diana a través de secuencias octaméricas comunes, que son lugares de unión a Cic. De hecho, la proteína Cic se une directamente *in vitro* a dichos octámeros, T(G/C)AATGAA, los cuales se asemejan a los ya anteriormente caracterizados en humanos, TGAATG(G/A)A. En el embrión temprano, la vía de Torso controla la expresión de *tll* y *hkb* en los polos regulando negativamente a Cic en esta región. Más tarde, la activación de la vía de EGFR en el neuroectodermo alivia la represión mediada por Cic induciendo la expresión de *ind*. Finalmente, durante el desarrollo del ala, la vía de EGFR inactiva a Cic en las células que formarán las venas permitiendo la expresión de *argos*. Las regiones reguladoras de los genes analizados contienen en todos los casos octámeros de Cic, los cuales son suficientes para transformar las señales RTK en patrones de expresión restringidos.

Cabe destacar que la actividad de Cic en el embrión temprano se asemeja a su función en el ovario donde Cic mantiene la expresión de *pipe* en la región ventral reprimiendo a *mirr*. En este caso, la señal de EGFR en la región dorsal inactiva a Cic y de forma independiente contribuye al mismo tiempo a la activación de *mirr*. En el embrión temprano, la señal de Torso inactiva Cic en los polos del embrión induciendo represores, como *tll* y *hkb*, que son reprimidos por Cic en la región central del embrión permitiendo la expresión de genes *gap* como *knirps* y *Kruppel*.

4. Regulación de la expresión génica por competición entre sustratos de MAPK durante la formación del eje DV

MAPK es el último componente de la cascada de quinasas iniciada por las vías RTK. Una vez fosforilada, MAPK a su vez fosforila residuos de serina y treonina presentes en sus múltiples sustratos, generalmente factores de transcripción, e

induce respuestas celulares. A pesar de que se consideraba que el control de la fosforilación de MAPK es independiente de sus sustratos, estudios recientes muestran que los sustratos fosforilados por MAPK pueden interferir también en el control de la fosforilación de la misma MAPK por competición directa.

Como hemos visto, la proteína Cic es un sustrato de MAPK. Cic se expresa en el embrión y es degradada en los polos donde presenta una distribución asimétrica con niveles más elevados en el polo anterior. Esto es debido a la presencia de otros sustratos de MAPK localizados en la región anterior que antagonizan la regulación negativa de Cic. Sin embargo, se desconoce si esta distribución asimétrica de Cic, consecuencia de la competición entre sustratos de MAPK, tiene un impacto real en la regulación de sus genes diana.

En colaboración con el grupo de Stanislav Shvartsman, hemos analizado el patrón de expresión de un gen diana de Cic en particular, *zerknüllt* (*zen*), el cual depende de la acción conjunta de los sistemas DV y terminal, y presenta una pronunciada asimetría AP. Observamos que sustratos de MAPK localizados en la región anterior, como Bicoid (Bcd) y Hunchback (Hb), contrarrestan la actividad de MAPK hacia Cic. En consecuencia, la represión mediada por Cic es potenciada contribuyendo a la expresión asimétrica de *zen*. La distribución de la proteína Cic y la expresión de *zen* se vuelven simétricas en embriones mutantes *bcd* y *hb*. En cambio, formas de Bcd incapaces de unirse a ADN expresadas en embriones mutantes *bcd* rescatan el gradiente de distribución de Cic y la expresión de *zen* se ve reducida en el polo anterior.

Como Bcd es un regulador clave del patrón anterior del embrión y *zen* es un gen diana de los sistemas DV y terminal, nuestros resultados revelan un nuevo mecanismo de integración de señales de los sistemas anterior, DV y terminal. Además, nuestro trabajo también muestra que la competición entre sustratos de MAPK puede modular la expresión génica en el embrión temprano.

CONCLUSIONES

1. La señal de EGFR restringe la expresión de *pipe* a las células foliculares ventrales a través de Mirr, un represor inducido por la vía en la células foliculares dorsales.
2. Mirr reprime la expresión de *pipe* uniéndose directamente a un elemento conservado (motivo *r1*) presente en la región reguladora de *pipe*.
3. Mirr es un mediador común de las respuestas de la vía EGFR en la especificación de la polaridad DV tanto del corion como del embrión.
4. Cic mantiene la expresión de *pipe* de forma indirecta mediante la represión de *mirr* en las células foliculares ventrales; en ausencia de la función de Cic, niveles bajos de desrepresión de *mirr* son suficientes para impedir la expresión de *pipe*.
5. La actividad represora de Cic se encuentra regulada negativamente por la vía de EGFR en las células foliculares dorsales.
6. La regulación negativa de Cic mediada por EGFR modula la distribución espacial de Mirr en las células foliculares laterales contribuyendo a definir la posición del borde de expresión de *pipe*.
7. La red de interacciones dependiente de EGFR que controla la especificación del eje DV en el ovario se asemeja al papel de la vía de Torso y la represión mediada por Cic en la regulación de la formación del patrón AP en el embrión temprano.
8. La represión mediada por Cic por debajo de las vías RTK tiene lugar mediante la unión directa a las secuencia octaméricas T(G/C)AATGAA presentes en las regiones reguladoras de sus genes diana.
9. La expresión asimétrica de *zen* en los polos del embrión depende de la inhibición competitiva de la regulación negativa de Cic por otros sustratos de MAPK localizados en la región anterior.
10. La competición entre sustratos de MAPK puede modular la expresión génica en el embrión temprano e integrar las acciones de los sistemas anterior, DV y terminal.