



HAL
open science

Pri a novel target of ecdysone for the temporal control of drosophila development

Azza Dib

► **To cite this version:**

Azza Dib. Pri a novel target of ecdysone for the temporal control of drosophila development. Vegetal Biology. Université Paul Sabatier - Toulouse III, 2016. English. NNT : 2016TOU30144 . tel-01537458

HAL Id: tel-01537458

<https://theses.hal.science/tel-01537458>

Submitted on 12 Jun 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par :

Azza DIB

le vendredi 23 septembre 2016

Titre :

pri a novel target of ecdysone for the temporal control of drosophila
development

École doctorale et discipline ou spécialité :

ED BSB : Biologie du développement

Unité de recherche :

Centre de Biologie du Développement

Directeur/trice(s) de Thèse :

Dr. François PAYRE

Jury :

Pr. David CRIBBS, président

Dr. Jacques MONTAGNE, rapporteur

Dr. Maria CAPOVILLA, rapporteur

Dr. Laurent PERRIN, rapporteur

Dr. Hélène CHANUT-DELALANDE, examinateur

Azza DIB
PhD manuscript

23 September 2016

*“Do not go where the path may lead;
Go instead where there is no path and
Leave a trail.”*

Ralph Waldo Emerson

Acknowledgement

Words fail me when I yearn to depict my profoundest feelings of gratitude for the people who have rendered invaluable help during my thesis years. Yet, I intend to make a sincere effort in portraying my deep sense of gratitude in the form of words.

This work would not have the spirit that it has without the invaluable academic, educational, human support and belief in me as a researcher provided by the following scholars and persons who in one way or another have contributed in making this study possible:

*I would like to express my gratitude towards my thesis jury **Pr. David Cribbs, Dr. Jacques Montagne, Dr. Maria Capovilla and Dr. Laurent Perrin** for the time they gave me despite, without doubt, the massive work they have, and also for their effort in checking and editing this manuscript.*

*My sincere thanks go towards the directors of the CBD; **Dr. Marc Haenlin and Dr. Fabienne Pituello**, for accepting me into this great research center and thus giving me the chance to do my thesis there.*

*In our days when role models have become fewer and fewer I had the privilege of being shaped by one of the most intelligent researchers who is awarded by a silver medal my thesis director, **Dr. Francois Payre**. You were a guru and not a mere teacher to me. You ironed out the rough edges by taking interest even in the minutest of the details. You cared about my every step on my stairway to the knowledge Heaven. You patiently and laboriously help me realize my dream.*

*My immeasurable appreciation and deepest gratitude for the help and support are extended to my supervisor **Dr. Helene Chanut-Delalande**, I'm very grateful to you for your advices, guidance, valuable comments, for your suggestions and for your pedagogical way in clarifying the ideas, beside you I learned how to always think with scientific spirit.*

*I gratefully acknowledge **Dr. Serge Plaza** whose passion for helping sets new standards for anyone involved in training and development, or any other endeavor in which one human being seeks to support the growth and improvement of another.*

I'm highly indebted to every member of our team who shared their knowledge with me and providing me necessary information

regarding this research. A special thanks to **Dr. Anne Pelissier - Monnier** for her kindness, support and words of encouragement that gave me an endless help to finish this manuscript. Other special thanks to **Mr. Philippe Valenti**, for transmitting some of his technical know-how in molecular biology, I believe he really deserves to be surnamed "the king of molecular biology".

I would like to thank our collaborators **Yuji Kageyama Team** in Japan, my thesis committee **Magalie Suzanne** from LBCMCP-Toulouse and **Bernard Moussian** from Tuebingen University - Germany, for giving us time and effort in terms of providing us all the needed information.

A special appreciation is extended to the first Prime Minister **Mr. Najib Mikati** and for "Azm and Saade" Association. Thank you for your magnanimity in providing me financial assistance which kept away the nagging sensation of financial instability. Our Leader; kindly accept my eternal fidelity and gratefulness. I also thank the **ANR** and **UPS** for awarding me a financial aid which was of great help in tiding over the monetary crunch.

I remember with gratitude people who believed in me and stayed besides me to accomplish this mission, **Mr. Mostafa Ghourani** and **Mr. Mohammad Saadieh**, all my appreciation full of love, respects and gratefulness to both of you, I will never forget your efforts and support.

Marie Claude Chatelain and **Raphael Arches**; my French family, no words can explain my gratitude not only for housing me but for providing me with tenderness and facilities which helped me in completion of this paper. You were a real family in absence of my parents who are in Lebanon. Likewise I extend my deepest thanks to all my host family including my friends there especially **Mouin Nasr**. Thank you for making my stay and studies in France more enjoyable.

Foremost, Many thanks and great appreciations also go to my colleagues and friends especially to **Hazar El Mouhammad**, **Maya El Haje**, **Noura El Haje** and **Mounira Kourjieh** who have willingly helped me out by encouraging me to stand up all over these last four years. To you I say "we meet to part but more importantly we part to meet".

The fruit of 28 years could not be but dedicated to **Mom and Dad**, who raised me up to believe in myself. I could never have done this without your faith, support and constant encouragement, thank you for

teaching me to believe in God, and in my dreams. You were my advisers for imparting your expertise in life to overcome any difficulty. I love you heaps, my idols!!!

My sister Nadia, My two brothers Omar and Ahmad I can't forget to send each one of you a big hug with big thanks for being by my side in every step, I love you so much. Thank you for being my thesis buddies by your encouragement and moral support.

My beloved and supportive husband, Ahmad who was always by my side the time I needed him most and offered huge help to accomplish this study. My lovable man, you served as inspiration to pursue this dream, thank you is not enough for all your understanding and patience.

Azza DIB

Abbreviations

aa	amino-acid
<i>AbdA</i>	<i>Abdominal A</i>
AP	Anterior-Posterior
APF	After Puparium Formation
BAC	Bacterial Artificial Chromosome
<i>bFTZ-F1</i>	<i>beta Fushi-TaraZu-F1</i>
bHLH	basic-Helix-Loop-Helix
<i>Br-C</i>	<i>Broad Complex</i>
BRE	B Recognition Element
ChIP-seq	Chromatin Immuno-Precipitation and sequencing
CRM	<i>Cis-Regulatory Modules</i>
<i>CS-1</i>	<i>Chitin Synthase-1</i>
CYP450	CYtochrome P450 mono-oxygenases
DBD	DNA Binding Domain
<i>DHR3</i>	<i>Drosophila Hormone Receptor 3</i>
<i>Dib</i>	<i>Disembodied</i>
DNA	Deoxyribo-Nucleic Acid
DPE	Downstream Promoter Element
DV	Dorsal-Ventral
<i>dyl</i>	<i>dusky-like</i>
EcR	Ecdysone Receptor
EcRDN	Dominant Negative of Ecdysone Receptor
EcRE	Ecdysone Receptor Elements
EcR-USP	Ecdysone Receptor- Ultraspiracle
<i>eng</i>	<i>Engrailed</i>
eve	even-skipped
GFP	Green Fluorescent Protein
<i>GstE14</i>	<i>Glutathione s transferase E14</i>
gz	growth zone
Hh	Hedhog
Hox	Homeobox
Inr	Initiator
kb	kilo base pairs
<i>kkv</i>	<i>krotzkopf verkehrt</i>
LBD	Ligand Binding Domain
mRNA	messenger Ribonucleic Acid
<i>Mst87F</i>	<i>Male-specific transcript at 87F</i>
ncRNA	non-coding Ribonucleic Acid
<i>Nm-g/ Sro</i>	<i>Non-molting glossy/Shroud</i>
NO	Nitric Oxide
nt	Nucleotide

<i>nvd</i>	Neverland
ORF	Open Reading Frame
OSC	Ovarian Somatic Cells
PCR	Polymerase Chain Reaction
PG	Prothoracic Gland
<i>phm</i>	<i>Phantom</i>
pri	polished rice
PSM	Presomitic Mesoderm
PTTH	Prothoracicotrope Hormone
PWMs	Position Weight Matrixes
RG	Ring Gland
RNA	Ribonucleic Acid
RXR	Retinoic X Receptor
<i>Sad</i>	<i>Shadow</i>
<i>shd</i>	<i>Shade</i>
smORF	small Open Reading Frame
<i>spk</i>	<i>Spook</i>
<i>Spok</i>	<i>Spookier</i>
Srp	Serpent
STARR-seq	Self-Transcribing Active Regulatory Region sequencing
<i>svb</i>	<i>Shavenbaby</i>
<i>tal</i>	<i>tarsal-less</i>
TBP	TATA box Binding Protein
TF	Transcription Factor
TFBs	Transcription Factor Binding-sites
TFIIBRE	Transcription Factor II B Recognition Element
tRNA	transfer Ribonucleic Acid
UAS	Upstream Activating System
Ubx	<i>Ultrabithorax</i>
USP	Ultraspiracle
UTR	Untranslated Regions
<i>wg</i>	<i>Wingless</i>

Table of content

INTRODUCTION	10
1. THE PHYSICAL ELEMENTS REGULATING EUKARYOTIC GENE TRANSCRIPTION	11
▪ CIS-REGULATORY MODULES	11
▪ TRANSCRIPTION FACTORS	14
2. DEVELOPMENT RELIES ON SPATIOTEMPORAL REGULATION OF GENE EXPRESSION	16
3. <i>DROSOPHILA</i> AS A FRUITFUL MODEL OF ANIMAL DEVELOPMENT	20
▪ AN OVERVIEW OF EMBRYONIC DEVELOPMENT	20
▪ SEGMENTATION	22
▪ LARVAL DEVELOPMENT	24
▪ METAMORPHOSIS	25
4. THE ECDYSONE STEROID HORMONE TIMES <i>DROSOPHILA</i> DEVELOPMENT	27
▪ ECDYSONE : MAKING THE SIGNAL	27
▪ THE ECDYSONE RECEPTOR (ECR)	30
▪ KNOWN TARGETS OF THE ECDYSONE PATHWAY: MULTITIERED TFs	31
▪ A NEGLECTED ROLE OF ECDYSONE IN EMBRYONIC DEVELOPMENT	37
5. EPIDERMIS DEVELOPMENT	39
▪ CUTICLE COMPOSITION AND SECRETION	40
▪ ESTABLISHING THE PATTERN OF EPIDERMAL TRICHOMES	41
▪ <i>PRI</i> PEPTIDES	46
RESULTS	52
6. <i>PRI</i> PEPTIDES ARE MEDIATORS OF ECDYSONE FOR THE TEMPORAL CONTROL OF DEVELOPMENT	52
▪ SUMMARY	52
▪ ARTICLE AND SUPPLEMENTAL INFORMATION	53
7. MULTIPLE ENHANCERS DRIVE THE SPATIO-TEMPORAL EXPRESSION OF <i>POLISHED RICE</i>, INVOLVING DIRECT CONTROL BY THE ECDYSONE RECEPTOR	53
▪ SUMMARY	53
▪ MANUSCRIPT AND SUPPLEMENTAL INFORMATION	54
DISCUSSION	96
▪ SPATIO-TEMPORAL EXPRESSION OF <i>PRI</i> DURING <i>DROSOPHILA</i> DEVELOPMENT	96
▪ <i>PRI</i> REGULATION AND THE CROSSTALK WITH THE ECDYSONE HORMONE.	98
BIBLIOGRAPHY	104

Introduction

The development of a single cell to an entire organism is a fascinating process that begs several basic questions: how subsets of cells regularly adopt a specific behavior, while their neighbors concurrently adopt others? At what time of development cells know that they should execute a given program of differentiation? Which are the mechanisms orchestrating different developmental processes across the whole body?

It is now well established that development primarily relies on the elaboration of fine patterns of gene expression, which progressively define the fate of specific groups of cells. Therefore a main issue is to understand the mechanisms underlying the spatiotemporal regulation of genome transcription. While we have a wealth of information to explain the regulatory interactions that build sophisticated patterns of spatial expression, little remains known on the mechanisms ensuring temporal control of development. Indeed, time is a fundamental aspect of development that is involved at each scale of a biological system. From the cycle of cell division to differentiation, the organization of tissues and organs, and finally organism-wide integration and appropriate final body size, all these aspects require a proper timing that should be regulated by internal and external cues.

Various intrinsic timing parameters may be involved in controlling development such as the rate of protein production, diffusion, degradation, etc... Many studies have shown the existence of molecular circuits, where the half-life of regulatory factors and their feedback interactions produce oscillating transcriptional outputs, as well illustrated by the circadian clock (Hirayama and Sassone-Corsi, 2005; Webb and Oates, 2016), or the regular production of somites in vertebrates (Gomez et al., 2008; Keyte and Smith, 2014). Another striking case is provided by recent work on neurogenesis in flies. Proliferating neural stem cells utilize transcription factor cascades as a generic mechanism for temporal patterning, providing remarkable diversity of cell types in the developing nervous system (Yasugi and Nishimura, 2016).

A broader systemic control is yet to consider for understanding how different programs are synchronized across tissues and evolve over embryonic and postembryonic development. It is known that these two latter actions are regulated by several mechanisms. An Open Reading Frame (ORF) is a messenger RNA sequence (mRNA) (corresponding to a gene), translated by ribosomes to a protein. A small Open Reading Frame (smORF) is translated to a small peptide < 100 amino acids. Among these smORFs there are those who are localized on noncoding RNAs since they have no known ORF. Groundbreaking studies have shown the role of noncoding RNAs in regulating developmental timing both in plants and in animals. The so-called heterochronic mutations in nematodes lead to premature or delayed differentiation, desynchronizing development. For example, *lin-28* mutants start producing an adult cuticle, while animals are still in a sexually immature juvenile stage (Faunes and Larrain, 2016; Rougvie, 2001). Likewise, external cues such as nutrition or environment may control the duration of developmental intervals. A growing body of evidence shows that modifications in human dietary may have adverse impacts on the proper timing of puberty, becoming a serious health concern (Rogol et al., 2000).

Therefore, an interesting problem is to figure out how genetically encoded developmental programs are connected with a systemic control of timing, integrating both internal and external cues. Through studying the mechanisms regulating the transcription of an apparently noncoding RNA that actually produces small peptides, we addressed this question for the development of epidermal derivatives in flies.

1. The physical elements regulating eukaryotic gene transcription

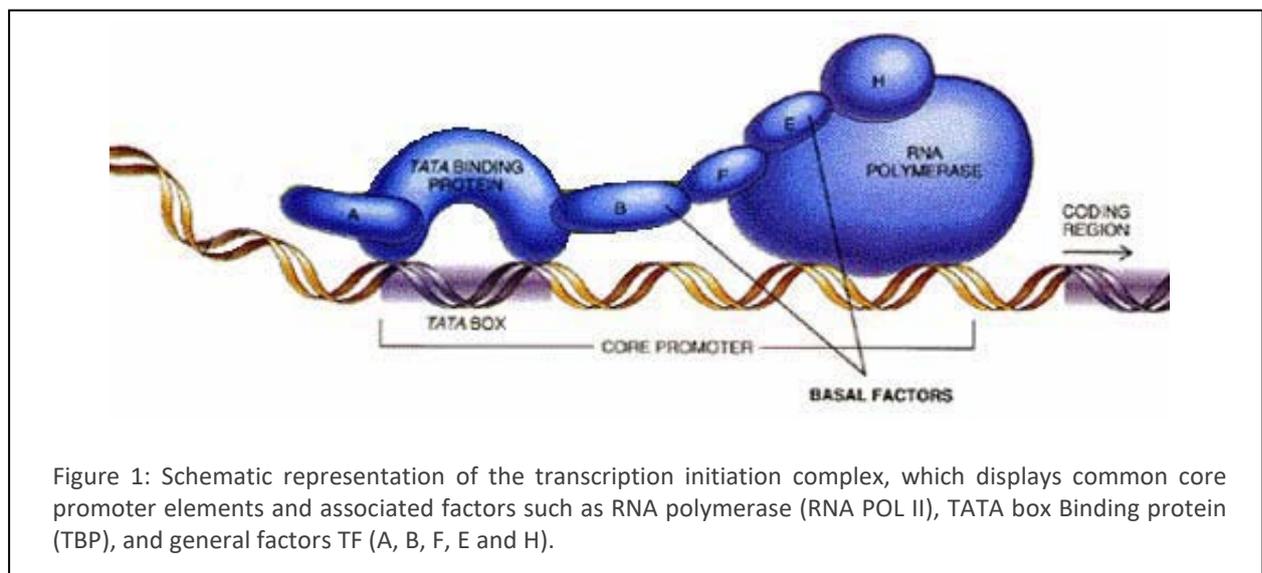
Gene expression is a multistep process that decodes the information carried by a given gene and translated into an active protein. In eukaryotes, each individual step is finely regulated from the chromatin level, to the transcriptional and post-transcriptional levels, and finally translational and post-translational levels. A major dimension of developmental control relies on the specific patterns of gene transcription.

A first lock consists on the DNA structure that is wrapped around histones with a high compaction level, making the DNA sequences hardly accessible for proteins to induce transcription. Transcription is yet fundamentally regulated by two major elements: *cis*-regulatory DNA sequences and their associated *trans*-regulatory factors. A same gene may be expressed from several alternative promoters that produce different transcripts and/or proteins (Landry et al., 2003; Zavolan et al., 2002). Eukaryotic promoters are typically composed by a combination of two kinds of *cis*-regulatory sequences on which various transcriptional factors will bind. The first type of these sequences is implicated in the transcription initiation and found across most promoters. The second type gathers specific regulatory elements for a promoter category and builds specific patterns of expression, according to tissues and developmental stages.

Thereby a package of different structures is responsible to orchestrate gene transcription necessary for development.

▪ *Cis*-regulatory modules

Cis-regulatory modules (CRM) are key sequences distal to promoters that serve as a podium for the attachment of transcription factors (TFs) and thereby control the transcription. CRMs may be localized upstream or downstream of a gene, even very far away from it, or within the transcribed part of a gene such as untranslated regions of the RNA (UTR) or introns (Fig.1).



CRMs play a crucial role in development by regulating gene expression. They gather the effects of signaling and transcriptional networks and translate them into highly specific expression patterns (Bulger and Groudine, 2011). Depending on their role in genetic

expression regulation, CRMs were initially separated in two classes. Enhancers stand at first for regulatory regions that activate target gene expression, while silencers repress it, as observed by artificial constructs containing regulatory elements in front of a reporter gene, in culture cells or in transgenic animals. However, depending on cell types a same regulatory region may act either as enhancer or silencer. In addition, the specific output of a given CRM often incorporates both positive and negative regulatory inputs, in the same cell type. Nowadays, the term enhancer is often used as a synonymous of CRM. As pioneered by studies on the *eve* gene in flies (Halfon et al., 2000; Hare et al., 2008; Small et al., 1992; Wilson and Odom, 2009), one developmental gene may otherwise have many distinct CRM/enhancers driving specific expressions in different tissues. Thus, the different enhancers of the same gene can be seen as a regulatory network, integrating different spatial and temporal information to output a finely controlled pattern of gene expression (Buchler et al., 2003).

Enhancers often contain multiple sites for the binding of TFs as well illustrated for the first enhancer discovered, that of simian virus 40 (Schirm et al., 1987). Such clustering of Transcription Factor Binding Sites (TFBs) is indeed widespread in developmental enhancers, with multiple binding sites for a given TF (homotypic clustering) and/or several TFs (heterotypic clustering) (Aerts, 2012). According to the respective organization of these binding sites and to the mode of TF assembling on the enhancer, different types of architectures are generally distinguished.

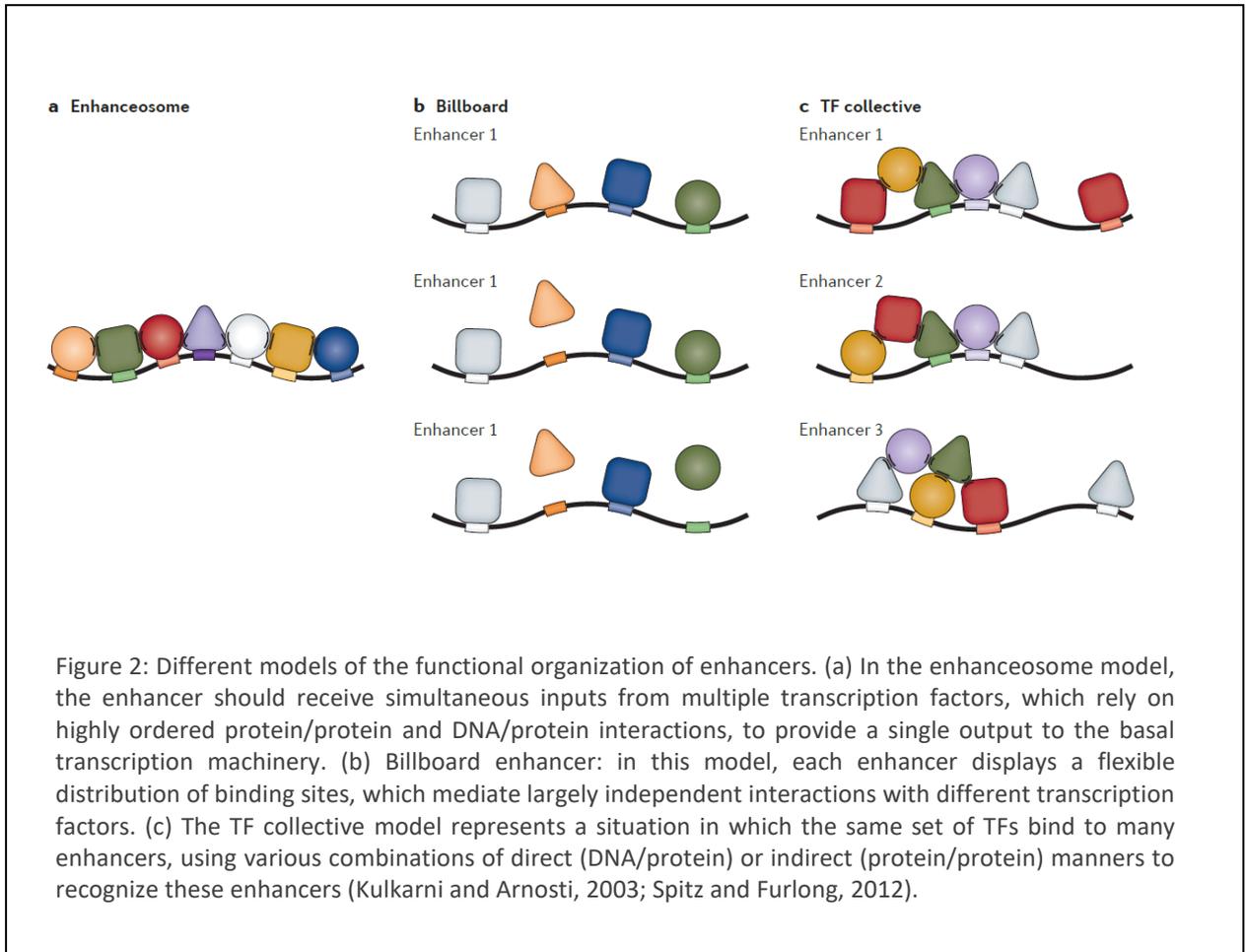
In the so-called “**enhanceosome**” model, the binding site architecture is critical for enhancer function. The paradigm of the enhanceosome is an enhancer from the human *interferon-beta* gene, which requires the cooperative binding of at least eight TFs (Panne, 2008). To be active, the enhancer must recruit all individual TFs. Their binding sites are precisely arranged along the enhancer sequence that acts as a DNA scaffold, creating the final complex of TFs fastened to DNA and acting as a single control unit (fig. 2A). So this kind of assembly depends on constrained interactions between TFs and DNA sequences, and thus a strict spacing or orientation of TFBs within the enhancer is crucial for its activity.

On the other hand, the “**Billboard**” or “information display” model of enhancers corresponds to a largely flexible architecture regarding the order, orientation or spacing of the TFBs, even if it may require a relatively fixed composition of motifs (Kulkarni and Arnosti, 2003). In this case, the position of TFBs within the enhancer displays no strict spacing or orientation rules (fig. 2B), and the lack of a given TF or the disruption of a TFB is generally not sufficient to inactivate the enhancer. For example, loss of cell type-specific repressive input often leads to ectopic target gene expression, but not to a breakdown of the enhancer (Slattery et al., 2014).

A third model called “**TF collective**” has been proposed to explain the regulatory logic underlying *Drosophila* heart development (Erceg et al., 2014; Junion et al., 2012; Slattery et al., 2014). A same set of TFs regulates multiple enhancers showing largely flexible organization and composition of motifs, since the TFs can use different manners to recognize their targets (fig. 2C). This occurs either through direct DNA-binding or through specific protein/protein interactions between TFs. In that case, both DNA and proteins act as scaffold and contribute to cooperative binding (Slattery et al., 2014); therefore enhancers harboring strikingly different combinations of TFBs can generate similar outputs for regulating gene expression.

Instead of following one or the other model, it is likely that TFs assemble on enhancers in a continuum manner, from a strict grammar to a highly flexible organization of regulatory motifs. Nevertheless, results from high-throughput discovery and dissection of CRMs

(Patwardhan et al., 2012; Smith et al., 2013) suggest that most animal enhancers do not use a constrained grammar of motifs and generally handle varying levels of flexibility in their functional organization (Slattery et al., 2014)



▪ Transcription factors

Transcription factors are proteins able to bind the DNA in a sequence-specific manner and their activity is responsible for the genome expression in particular cells and at a particular time. There are several kinds of transcription factors according to their DNA binding domains, including: Helix-Loop-Helix, Homeobox, Zinc Fingers, etc... To regulate gene expression, each TF binds to a specific short DNA motif, which often tolerates varying degrees of deviations from the consensus sequence. Therefore, TFBs are generally pictured as position weight matrixes (PWMs) that represent the frequency of nucleotides at each position of the consensus, as defined by compiling known binding sites for a given TF (Santolini et al., 2014). Strikingly, TFs bind only some of their potential binding sites, given their big number in the genome. In addition, the development of genome-wide profiling of TF-bound sequences (*e.g.*, Chromatin Immuno-Precipitation and sequencing, ChIP-seq) shows that a substantial proportion of binding events do not necessarily represent functional enhancers.

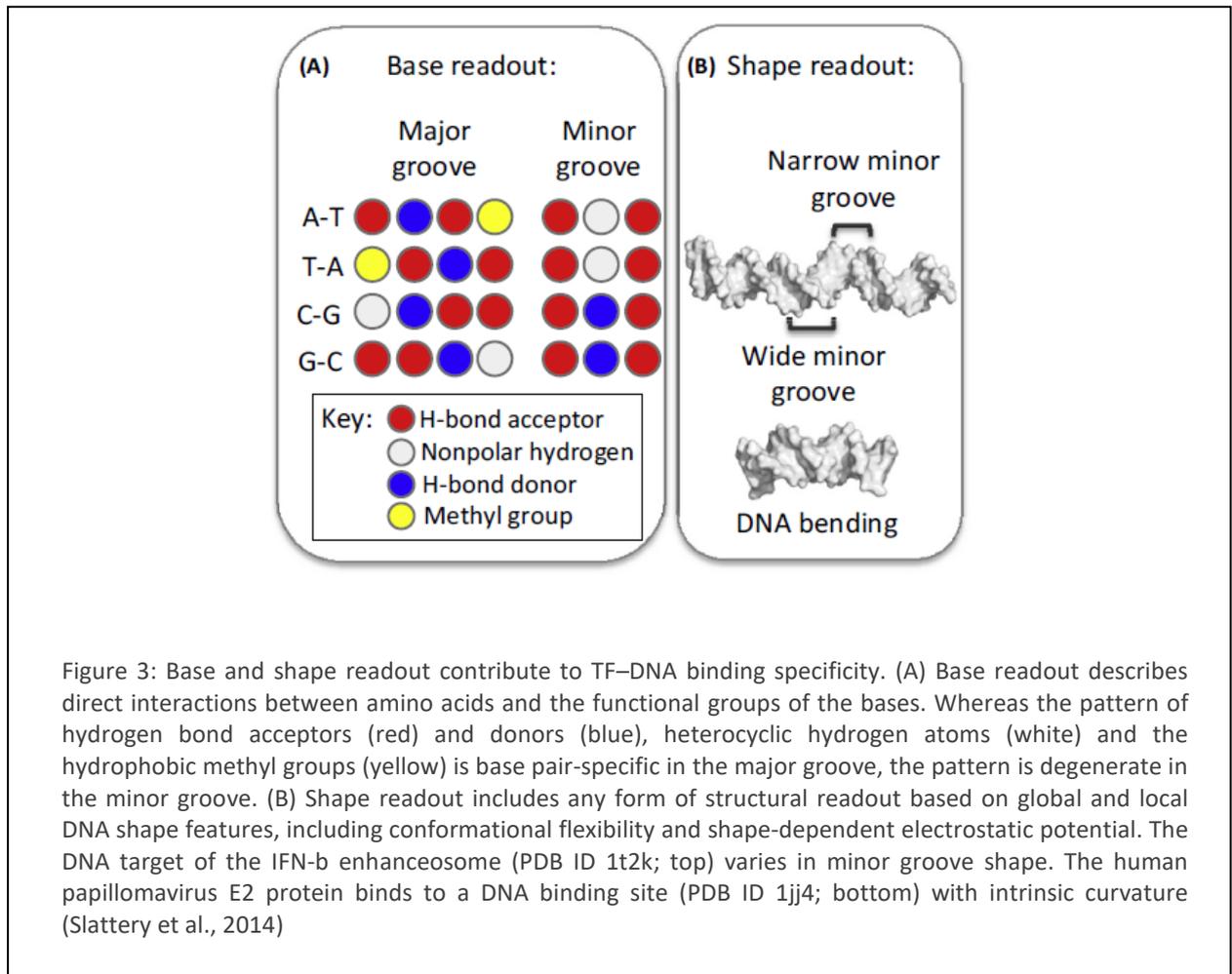
Therefore, it remains difficult to predict the location of enhancers from the genome-wide distribution of putative TFBs, or even that of ChIP peaks. Several studies have shown that combining data for several TFs that cooperate for regulating the expression of a category of genes may help predicting animal enhancers. A nice illustration is provided by the parallel mapping of genome-wide binding events for five TFs, at successive times throughout embryonic development, leading to the definition of TFBs signatures that can predict spatiotemporal activity of some enhancers in mesodermal derivatives (Bonn et al., 2012; Zinzen et al., 2009). Several bioinformatic pipelines similarly search for the local accumulation of TFBs, arranged in homo and/or heterotypic clusters, as a way to increase the accuracy of enhancer prediction (Aerts, 2012; Markstein et al., 2002). In most cases, however, analysis of DNA motif composition remains poorly efficient to predict functional enhancers.

Several parameters contribute to the difficulty of sequence-based enhancer detection. First, as mentioned above, *cis*-regulatory modules are often located far away from their target genes, dispersed throughout noncoding intergenic or intronic DNA, and there is no efficient means to prefigure the enhancer location. Second, we still don't know the DNA-binding specificity for most eukaryotic TFs, and several studies have suggested that PWMs defined from *in vitro* assays may not accurately represent TFBs active *in vivo*. For example, TFs can bind *in vivo* to degenerate sequences, which display very weak if any binding *in vitro*, and therefore are not well represented by PWMs (Croker et al, 2015).

Finally, mounting evidence supports that both base and shape readout can contribute to the recognition of DNA by the TFs. A base readout describes interactions between the amino acids and the functional groups of the bases. Namely, this relation includes direct hydrogen bonds, water-mediated hydrogen bonds, and hydrophobic contacts (Kitayner et al., 2010; Slattery et al., 2014) (fig. 3A). On the other hand, shape readout is based on the recognition of TFs to the structural features of their binding sites (fig. 3B). Both the global shape of the DNA helix (*e.g.*, an overall bend) and local shape of individual base pairs (*e.g.*, a local kink or narrow minor groove) can impact on TF/DNA recognition and binding (Hancock et al., 2013; Slattery et al., 2014; Stella et al., 2010). These findings highlight the importance of the three dimensional structure of both macromolecules for the interactions between them. Therefore, the DNA structure or topology may be as important as protein structure, in mediating a functional DNA/protein interaction (Parker et al., 2009). Based on a growing number of structures for TF/DNA complexes, it has been proposed that whereas specificity between different families of TFs frequently involves base readout in the major

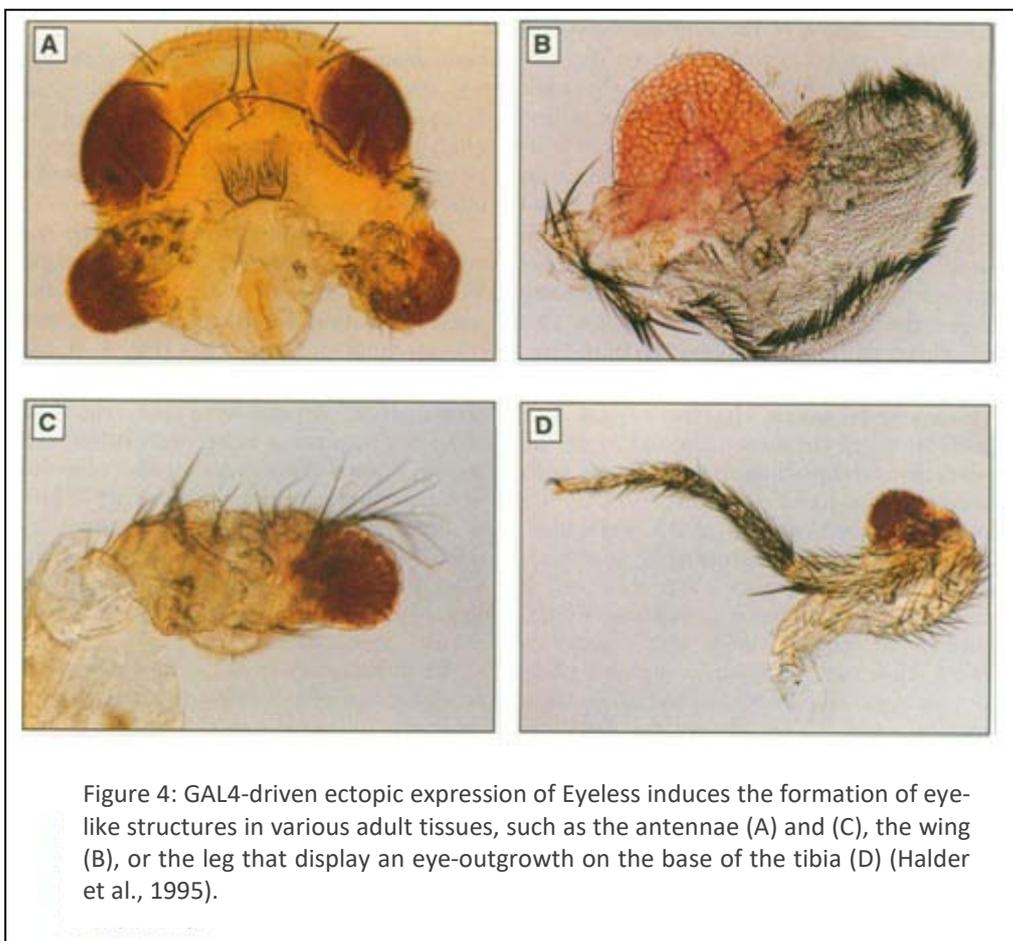
groove, the different parameters of shape readout may provide an additional level of high resolution specificity to distinguish between transcription factors harboring the same family of DNA-binding domains (Rohs et al., 2010). New methods have been developed to predict DNA structural features (*e.g.*, minor groove width, roll, twist, ...) and are amenable to the analysis of both DNA-sequence and shape for genome-wide data such as ChIP-seq (Zhou et al., 2013).

In sum, for all these reasons, enhancer discovery and delineation still generally relies on time-consuming experimental approaches, making large use of *ex* and *in vivo* reporter assays.



2. Development relies on spatiotemporal regulation of gene expression

Many studies have well demonstrated that specific spatiotemporal domains of gene expression play crucial roles in driving developmental progression. *Drosophila* genetics has provided good support to this notion, as nicely illustrated by the striking phenotypes resulting from the misregulation of homeotic gene expression. One of the most spectacular demonstration that experimental manipulation of gene expression can deeply modify the fate of developing tissues may be the work on Eyeless, a transcription factor that function as a “master regulator” of eye morphogenesis in *Drosophila* (Halder et al., 1995) (fig. 4). This seminal paper has shown that targeting the ectopic expression of Eyeless in various tissues, at early stages of development, is sufficient to induce the formation of ectopic “eyes” in wings, legs or in the antennae of adult *Drosophila*.



Likewise, the control of time seems to be crucial in order to mature at a suitable step. Studies in several model species have focused on the importance of timing during development crowned by three ways of timing biological processes: ordering, interval timing and rhythm (Webb and Oates, 2016). In vertebrates, the so-called segmentation clock manages the timing

of formation of somite boundaries from cells within the presomitic mesoderm (PSM) in the embryo (Keyte and Smith, 2014; Pourquie, 2011). Seminal work using the chicken embryo as a model system has shown that *c-hairy1*, encoding a transcription factor, displays a striking cycling expression during somitogenesis (Palmeirim et al., 1997). *c-hairy1* expression is first activated in the posterior PSM, then moves progressively towards the anterior, and finally restricts to the posterior-most cells of the newborn somite. The same cycle of spatiotemporal patterns of *c-hairy1* expression regularly occurs for the successive formation of each somite (fig. 5).

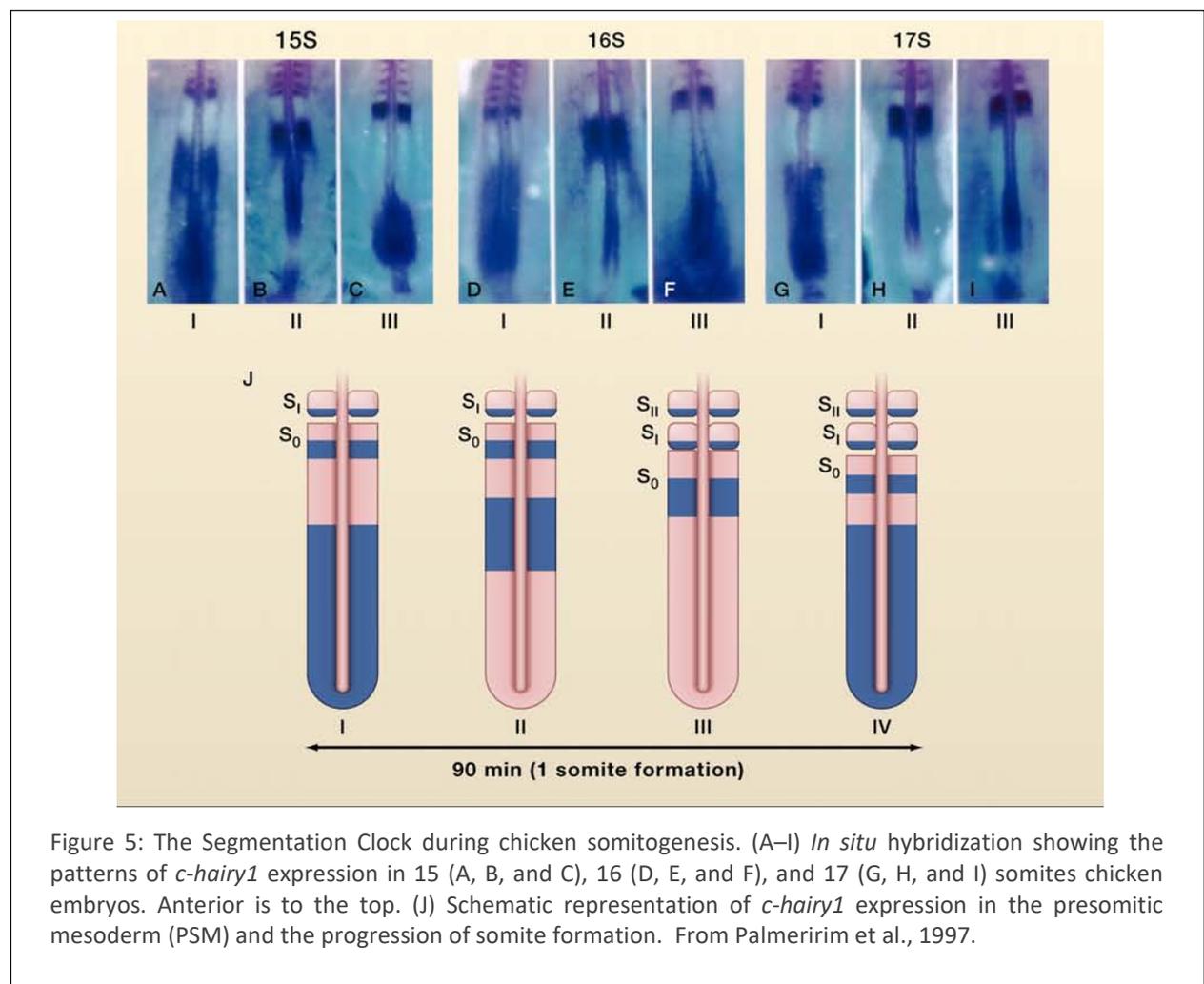


Figure 5: The Segmentation Clock during chicken somitogenesis. (A–I) *In situ* hybridization showing the patterns of *c-hairy1* expression in 15 (A, B, and C), 16 (D, E, and F), and 17 (G, H, and I) somites chicken embryos. Anterior is to the top. (J) Schematic representation of *c-hairy1* expression in the presomitic mesoderm (PSM) and the progression of somite formation. From Palmeririm et al., 1997.

Similar oscillating expression of transcription factors belonging to the Hes/Her/Hairy family were also reported across vertebrates species, showing the evolutionary conservation of this mechanism. Additional genes display a rhythmic expression pattern in the PSM, including members of the Wnt, FGF and Notch signaling pathways, the latter likely representing a key determinant of the vertebrate segmentation clock (Dequeant and Pourquie, 2008).

Although the precise function of the segmentation clock clearly involves complex interactions between dozens of regulatory factors that may have evolved across species, a general notion is the importance of molecular feedbacks to generate oscillating outputs (Pourquie, 2011).

For example, a simple model of the zebrafish oscillator involves a negative feedback loop, with transcriptional delay, to control the periodic expression of DeltaC, a ligand of the Notch

pathway (Lewis, 2003). Her1/7 transcription factors display a cyclic expression in the PSM, which is indispensable for proper segmentation (Holley et al., 2002). Notch activation triggers the expression of *Her1/7* and *DeltaC* and, once produced, Her1/7 TFs shutdown the transcription of *DeltaC* and finally of their own transcription (Giudicelli et al., 2007; Holley et al., 2002). This circuitry thus endows oscillating production of the DeltaC ligand, which in turn results in periodic activation of Notch in the neighboring cells (fig. 6), and therefore may act as a central pacemaker of the segmentation clock of the zebrafish (Giudicelli et al., 2007).

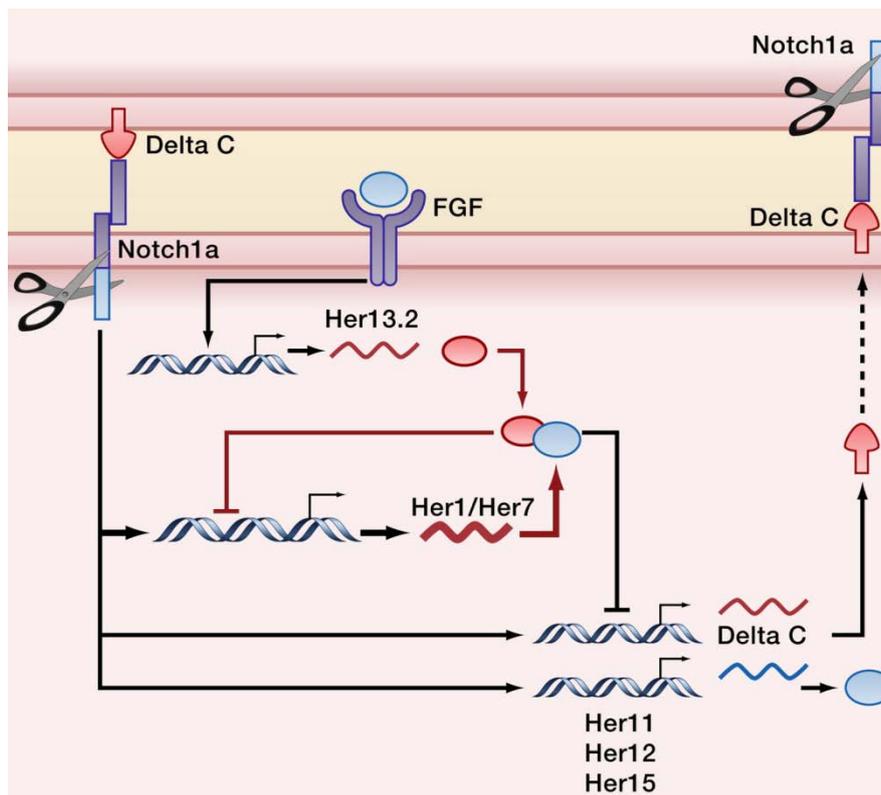
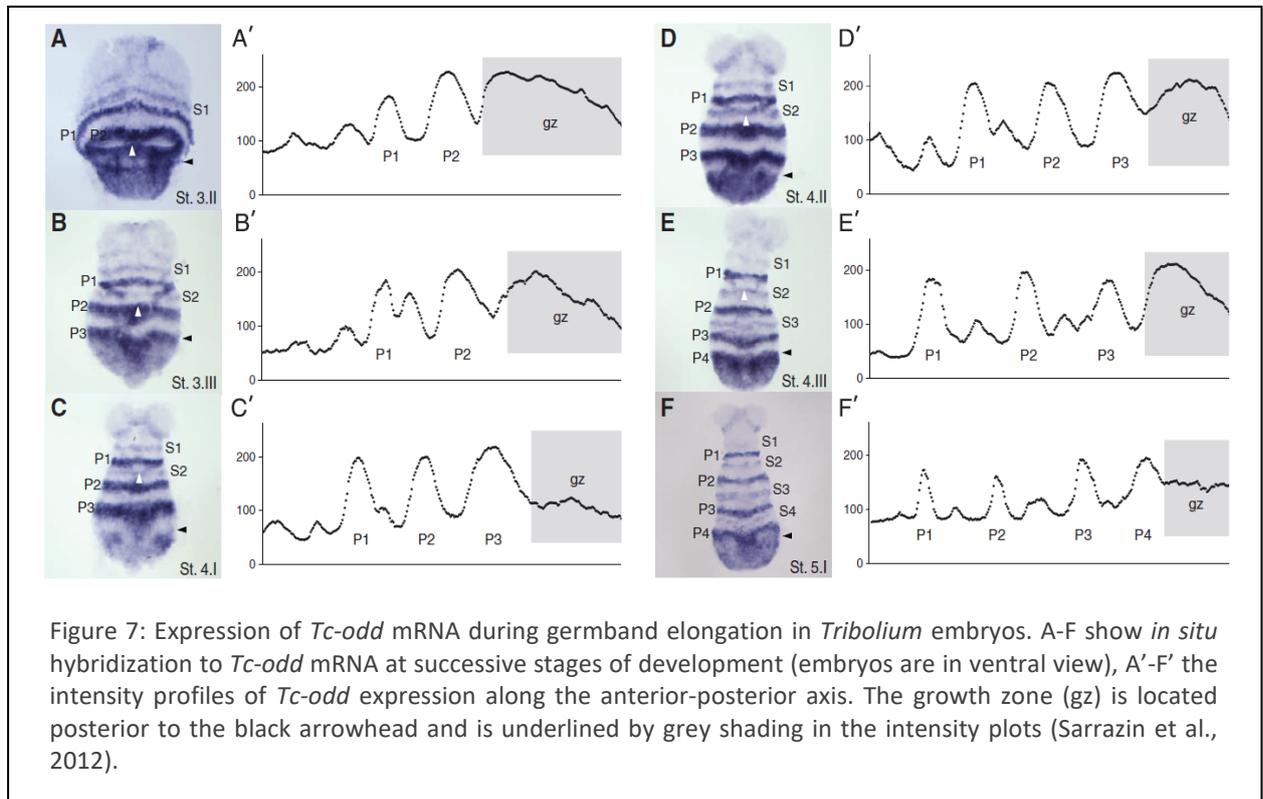


Figure 6: Model of the zebrafish oscillator. In response to the Notch pathway, the inhibitory activity of Her1/Her7 transcription factors controls the periodic repression of DeltaC, allowing the synchronous activation of Notch signaling in adjacent cells. Through activating the expression of Her13.2, a functional partner of Her1/Her7, FGF signaling provides further fine control of clock-synchronized oscillations (from Pourquié, 2011).

The periodic timing of gene expression has also been involved in the segmentation of arthropod species (Chipman and Akam, 2008; Schoppmeier and Damen, 2005).

In the *Tribolium castaneum* beetle, posterior segments are progressively added over time, from a posterior growth zone region; this mode of development, called short germband, likely being the ancestral mode of insect segmentation. The expression of *Tc-odd*, the ortholog of the pair-rule gene *odd-skipped* in flies, is expressed in sequential stripes, with a double segment periodicity, and inactivation of *Tc-odd* results in dramatic and almost asegmental phenotypes (Choe et al., 2006). Further work has recently shown *Tc-odd* cycles with periodic waves of expression (fig. 7). Strong evidence of this conclusion comes from

experimental anteroposterior bisection of developing embryos, and *in vitro* culture of the two halves during different times before being simultaneously subjected to *in situ* hybridization. This elegant approach (previously been taken by (Palmeirim et al., 1997) for the chicken segmentation clock) clearly showed that *Tc-odd* expression in the growth zone displays temporal oscillation during the production of primary stripes (Sarrazin et al., 2012), for the proper segmentation of the beetle embryo.



Taken together, these iconic examples well illustrate that specific control of gene expression, both in space and time, is of fundamental importance to underwrite developmental programs in animals.

3. *Drosophila* as a fruitful model of animal development

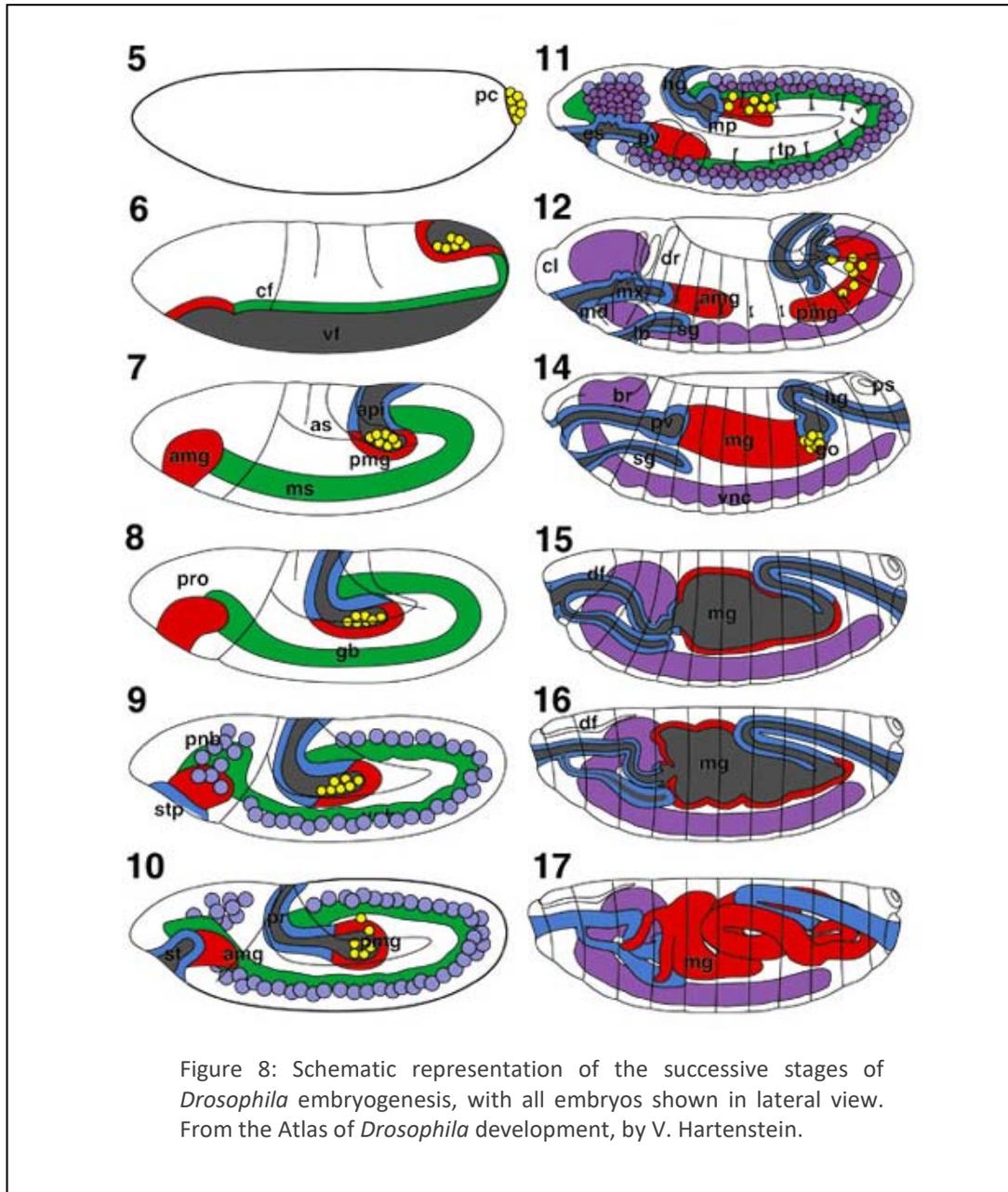
Drosophila species are insects of the *Diptera* order, *i.e.* provided with a single pair of wings. They are holometabolous animals, which have an indirect development, passing through three larval stages, before undergoing a metamorphosis that gives the fertile adult form. *Drosophila melanogaster* is widespread in the world and its cosmopolitan distribution is quite probably related to human activities and increased trade that allowed it to migrate over long distances (Lachaise D., 1988). The female of *Drosophila melanogaster* lays her eggs on a medium that will serve as food substrate to larvae that emerge. The female can lay several hundred eggs during her life. Depending on food substrate and temperature, development time of newly laid individuals will vary showing the influence of environmental inputs for the temporal control of *Drosophila* development. For example, the entire development will be in about 10 days at 25 °C, whereas development will last about 20 days if the temperature is only lowered to 18 °C (Thompson et al., 1977).

▪ An overview of embryonic development

The egg laid measures a millimeter in length, where the yolk that it contains is stored in its center. In 1985, Campos-Ortega and Hartenstein divided the different morphogenetic events of *Drosophila* embryogenesis into 17 successive stages (fig. 8). Embryonic development begins with a series of 13 fast nucleus divisions giving rise to a syncytial blastoderm, which will undergo cellularization three hours after fertilization. After cellularization, comes the gastrulation, an important process, where a whole set of movements implements the digestive structures of the animal. It is the first manifestation of cell fate and cell differentiation program besides the early individualization of pole cells. The embryo is divided into three presumptive territories: the ectoderm that will give rise to the nervous system and the epidermis, the mesoderm that will give rise to muscles and interstitial tissues, whilst endoderm represents the future digestive system. Approximately 3 hours after fertilization, the germinal band starts growing rapidly. It is placed at the origin in the ventral part of the embryo and grows first in a posterior-anterior dorsal direction that doubles its length and reduces its width twice. There are two types of cells involved in the extension of the germ band, the epidermal cells that start to divide before this process and stop at its beginning, and the neurogenic cells that do not divide, but get bigger. This germ band will give rise to three thoracic segments and nine abdominal segments and each segment has epidermal, neural and mesodermal constituents (Irvine and Wieschaus, 1994). Neurogenesis starts forty minutes later, with neural progenitors migrating from the ectoderm. The two hours that follow are marked by a period of growth with the appearance of furrows that will mark the limit of the different segments in the epidermis. Seven hours after fertilization, the germinal band stops its extension and begins shortening. This will result in several morphogenetic movements that will participate in the functional establishment of the different structures of the animal. Thus, the posterior portion of the intestine will be found at the posterior pole of the animal. The anterior and posterior part of the midgut move toward each other, forming two bands of cells on each side of the yolk that remains at the center of the embryo. While midgut closure continues, at the same time begins involution of the head that involves complex morphogenetic movements allowing implementation of the mouthparts and other head structures.

Shortening the germband leaves a membrane in the dorsal part, the aminoserosa, which will gradually be covered by lateral epidermis that progresses dorsally on each side to be merged (Schock and Perrimon, 2003). Dorsal closure involves interactions between integrins and the

extracellular matrix, contributing to epidermal cell migration over the amnioserosa (Schock and Perrimon, 2003).



▪ Segmentation

The anteroposterior axis of the insect body is characterized by the serial repetition of a large morphological unit, called segments. Genes that regulate the *Drosophila* segmentation were initially discovered from large scale genetic screening (Nusslein-Volhard and Wieschaus, 1980). Further work has shown that there are many players, connected in a successive series of regulatory cascades involving: maternal genes, gap genes, pair-rule genes, segment polarity genes and ultimately the homeotic genes that differentiate individual segments along the anteroposterior axis. I will here briefly remind the general principles of their action.

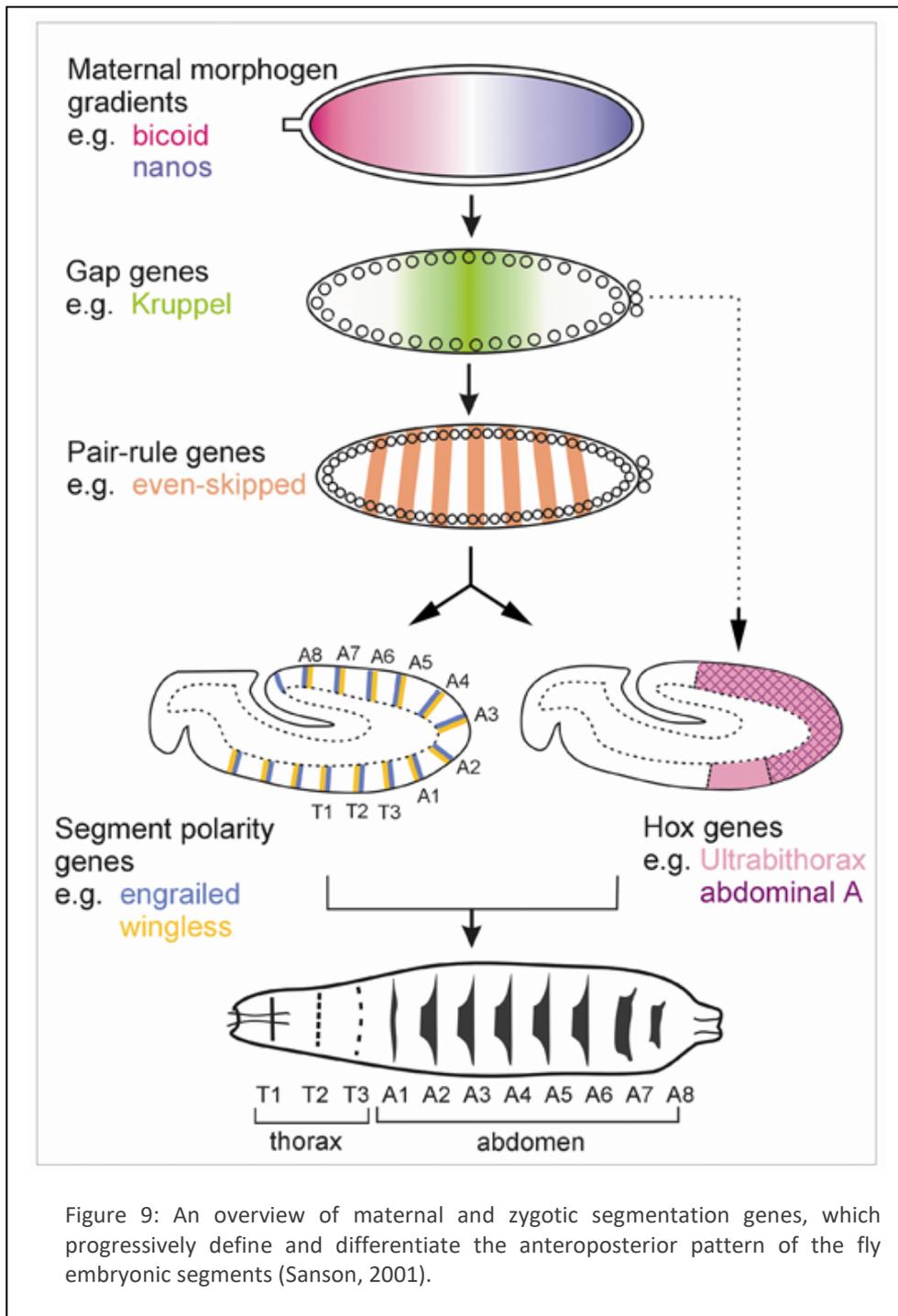
In *Drosophila* and other long germband insects, determination of the anterior-posterior (AP) and dorsal-ventral (DV) axes is controlled by maternal genes. These genes are expressed by the mother and their products are deposited in the oocyte by ovarian cells before fertilization. Along the AP axis, there are in the anterior part *bicoid* and *hunchback* which determine the head and thorax, on the other hand there are *nanos* and *caudal* in the posterior part determining the abdomen. Following their translation from a localized source of RNA, the diffusion of maternal proteins forms concentration gradients that act in a dose-dependent manner to activate specific targets, while repressing the reciprocal gradient. For example, *nanos* and *bicoid* mutually repress their translation. The terminal regions are additionally specified by the cell signaling pathway of the Torso receptor tyrosine kinase, which is locally activated at each pole of the embryo (Baek and Lee, 1999). Maternal TFs will then activate the transcription of gap genes (Jackle and Sauer, 1993).

Gap genes are the first zygotic segmentation genes expressed in wide transversal stripes, as readout of maternal gradients. Inactivation of a gap gene causes a characteristic phenotype where mutant embryos lack a set of adjacent segments. Gap genes are thus essential to specify large regions along the AP axis. They include *orthodenticle*, *empty spiracle* and *button head* to define the future embryonic head, zygotic *hunchback*, the gnathal and thoracic segments, *krüppel*, the thorax and first abdominal segments, *knirps*, the abdomen and *giant*, expressed in the head and the posterior part of the abdomen. In the terminal regions, *huckebein* and *tailless* are expressed in response to the Torso pathway.

In response to gap genes, the next tier of regulatory factors is pair-rule genes. They are expressed in seven AP stripes with a double segment register. Accordingly, inactivation of a pair rule gene leads to abnormal embryos where one segment over two disappears in the trunk. Several studies have demonstrated that the expression of pair-rule genes is driven by an array of largely independent enhancers, each incorporating various inputs from upstream segmentation genes. For example, the 6th stripe of *Hairy* expression is driven by a short enhancer, directly bound and activated by Giant and Knirps TFs (Howard and Struhl, 1990; Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991). In addition, Krüppel and Tailless provide negative regulatory inputs, specifying the anterior and posterior border of stripe 6. *Hairy* is a transcription factor of the basic-helix-loop-helix (bHLH) family and it associates with the Groucho corepressor, via a protein-protein binding domain (WRPW), to repress the transcription of other target segmentation genes, such as *fushi tarazu* (Poortinga et al., 1998). *fushi tarazu* is representative of the so-called secondary pair-rule genes, since its expression depends not only from maternal and gap genes but also from primary pair-rule genes. Indeed, the expression of *fushi tarazu* is initially activated in a uniform manner in the embryo, then repressed by a combination of primary pair rules including *hairy*, *runt* and *even-skipped*, to produce seven AP stripes. *Fushi tarazu* is a homeodomain TF that directly interacts with the aFtz-f1 nuclear receptor to regulate target gene expression (Florence et al., 1997).

During *Drosophila* development, there are two distinct segmentation units that overlap: segments and parasegments. Segments correspond to the units observed in adults, with 3 segments composing the head, 3 the thorax and 9 the abdomen. In embryos, the parasegments are delimited by transient mesodermal and ectodermal folds; the posterior part of each parasegment corresponds to the anterior compartment of a segment. Soon after cellularization, segment polarity genes are expressed and divide the pair-rule domains into two, forming 14 narrow stripes along the AP axis. Segment polarity genes expressed in neighboring cells interact between them and define the anterior and posterior compartments of each segment. When a segment polarity gene is mutated, one of the compartments (either the anterior or the posterior) disappears and is replaced by a mirror duplication of the remaining portion, as well seen on the larval cuticle. Transcription of segment polarity genes is initially regulated by a combination of positive and negative inputs from pair-rule genes. For example, the expression of *wingless* is activated by Odd-paired and repressed by Fushi-tarazu and Even-skipped. Then, reciprocal interactions between *engrailed* and *wingless* further establish and reinforce the boundary between segmental compartments.

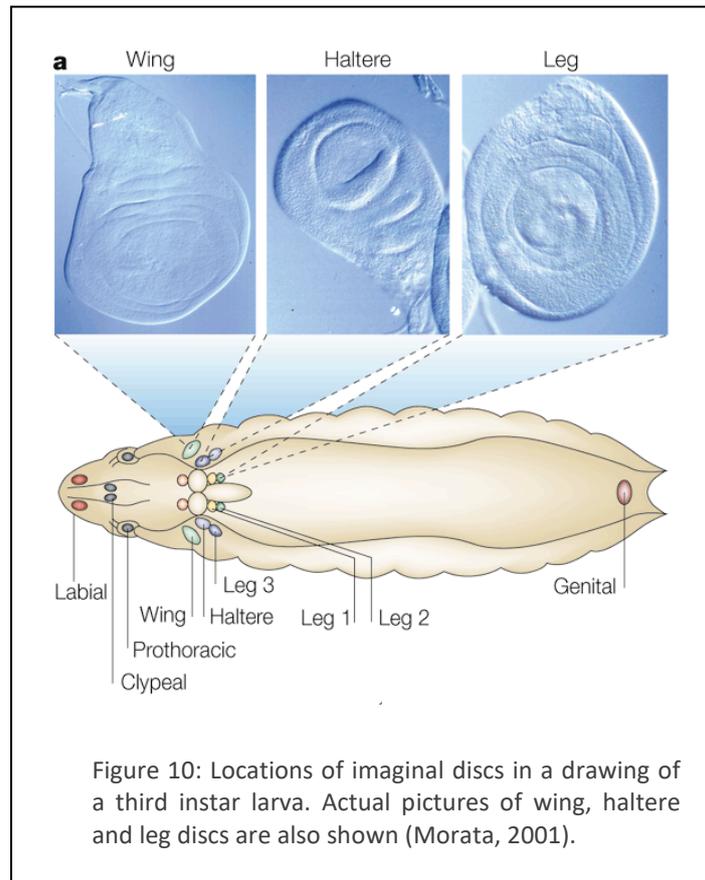
After the establishment of the segmental boundaries, homeotic genes that encode homeobox (Hox) TFs then act to specify the individual identity of each segment. The third chromosome of *Drosophila* contains two different regions gathering clusters of Hox genes. The Antennapedia complex that comprises the *labial*, *Antennapedia*, *sex comb reduced*, *deformed* and *proboscipedia* genes, specifies the identity of the head and thoracic segments. The Bithorax complex comprises three genes, *Ultrabithorax*, *abdominal A* and *B*, involved in the determination of the identity of the third thoracic segment and abdomen. *Hox* genes are expressed in adjoining segments, under the control, in a first step, of segmentation genes, then by functional interactions between homeotic genes. For example, the induction of *Antennapedia* expression is initiated by *fushi-tarazu* and *krüppel*, while *knirps* represses it. Later on, *Ultrabithorax* will repress *Antennapedia* to prevent its expression in the posterior thoracic and abdominal segments. Several studies have shown functional dominance among *Hox* genes, such as repression prevailing over activation to define posterior prevalence (Capovilla and Botas, 1998). The activity of *Hox* genes governs the execution of a broad range of developmental programs, giving rise to segment-specific patterns of cell differentiation across the different tissues contained in each segment. This is well illustrated by their effects on the larval cuticle development. The first abdominal segment (A1) that expresses *Ubx* differentiates a specific pattern of cuticular trichomes, as it will be further introduced in the later sections. In the absence of *Ubx*, these are transformed in a thoracic-like pattern of trichomes. Reciprocally, the ectopic expression of *Ubx* is sufficient to transform thoracic trichomes towards an A1 fate. It should be noticed, that the molecular mechanisms by which Hox factors impose segmental identities remain however not fully understood. Functional interactions between Hox factors also occurs during post-embryonic development, for example for the remodeling of the larval cardiac tube (Perrin et al., 2004), where steroid signaling temporally regulate the expression of *Ubx* and the function of *AbdA* (Monier et al., 2005).



▪ Larval development

After roughly 24h of development, the animal emerges from embryonic envelopes and starts the first larval stage. This small larva is highly mobile and able to feed autonomously. The

animal will then go through three larval stages and therefore undergo two molts under the control of the steroid hormone ecdysone, and by analogy with other insects, of juvenile hormone (Ashburner, 1980). During these stages, most larval cells proper do not divide but their size increases in very large proportions, almost 5 folds over the larval life (Nijhout et al., 2014). Most actively proliferating cells are found in 10 major pairs of groups of cells, called imaginal discs, which will later form adult structures during metamorphosis. There are three pairs of discs that will participate in the establishment of the head, three pairs for the formation of the thorax (including the wings and the halteres), three other pairs that give the legs, and finally a genital disc (fig. 10).



The first larval stage lasts about 24 hours at 25 ° C. The larva starts feeding upon its outbreak and will exploit the substrate surface. The second larval stage also lasts 24 hours; the larva begins to dig the medium in which it is located. The third larval stage lasts meanwhile about 48 hours; the larvae actively dig the medium. Approximately 110 hours after spawning, the third stage of larva stops eating and starts looking for a suitable site to accomplish its metamorphosis.

- **Metamorphosis**

Almost ten hours after it finishes eating and starting to look for a favorable place for metamorphosis, larva becomes less active (Robertson, 1936); the pupariation begins. The animal secretes glue, synthesized in the salivary glands that will allow it to firmly secure the substrate. The cuticle of the animal hardens to form the puparium (Zdarek and Slama, 1972).

Drosophila is then in the prepupal stage and will undergo very significant morphological changes.

Some larval organs yet undergo little change, including the gonads that continue to grow, but at a slower pace than in larval stages, the Malpighian tubules that ensure renal function, and the brain that continues to grow without undergoing fundamental changes during metamorphosis. In a striking contrast, the epidermis, salivary glands, gastrointestinal tract, the sense organs and muscles will be completely replaced by new structures formed from the imaginal discs (Robertson, 1936). About three hours after the start of prepupal stage, a gas bubble is formed in the posterior part of the pupa. This will move the animal to the back of the pupal case. These movements of the gas bubble, under the effect of muscle contractions, will ensure that the animal epidermis is released from the puparium (Bainbridge and Bownes, 1981).

Ten hours after the pre-pupal stage, legs begin to go out from bags of imaginal discs and wings become slowly visible, while the gas bubble disappears. Around 12 hours after pre-pupae, we pass to the pupal stage when the head imaginal bag goes out. Between 34 and 50 hours after the onset of metamorphosis, the transparent pupal cuticle separates from the epidermis of the animal; we speak of the adult stage pharate. During this period, the eyes start turning yellow and will gradually be redder. Sensory organs, including microchaetes and macrochaetes, will become more and more visible, taking a darker color. In the hours that precede the adult hatching, the wings will also become darker. Four and a half days after pupal stage, the cover of the pupal case opens to let out the animal. The newly hatched adult will gently unfold its wings and let harden in the air a few minutes before it can use them (Bainbridge and Bownes, 1981). The males start their sexual activity nine hours after hatching, while the females have at hatching period a vaginal plug that prohibits fertilization before seven hours.

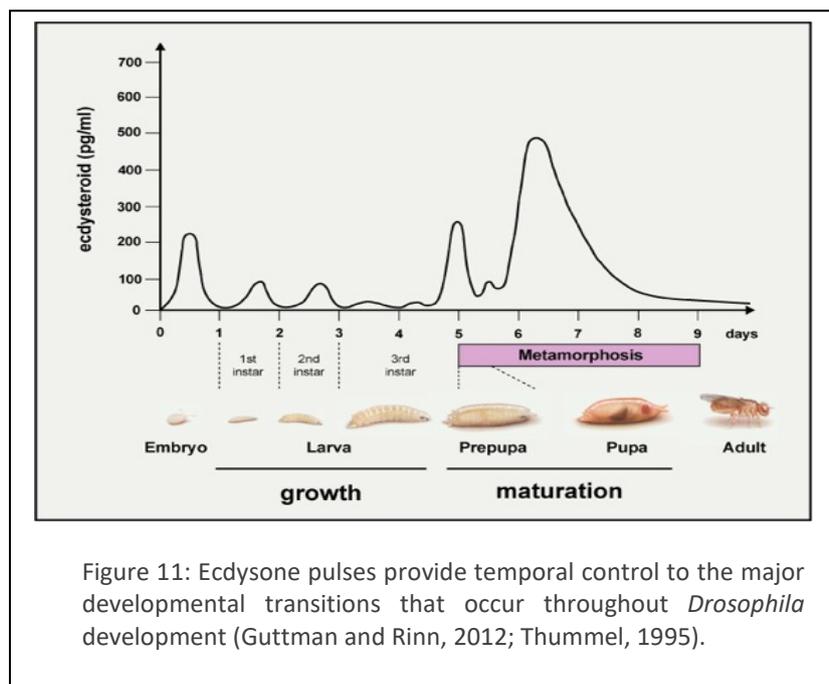
Reminding the multiple stages of *Drosophila* development, and the description of large changes in the animal organization and behavior that characterized each of these steps that successively occurs in a timed manner, highlight the importance of temporal control of development. The next issue we will discuss in the following is the importance of hormonal control for the developmental timing of *Drosophila* development.

4. The ecdysone steroid hormone times *Drosophila* development

Studies on the successive developmental stages featuring holometabolous insects have played a key role in elaborating the concept of developmental timing, as well as for first mechanistic insights into the action of hormones. Pioneering studies, starting with Kopec in 1917, have shown firstly that the metamorphosis is initiated by a hormone and, secondly, that this one is secreted by the prothoracic gland of insects (Beaulaton, 1968). In 1965, Huber and Hoppe were able to determine the precise formula of ecdysone: 2beta, 3beta, 14alpha, 22R, and 25-pentahydroxy-5beta-cholest-7-ene-6-one. In *Drosophila*, it is generally accepted that the 20-hydroxy-ecdysone (20-OH-ecdysone), not ecdysone, is the most active ecdysteroid. For the sake of simplification, we will use here, unless otherwise stated, the term 20E to designate the 20-OH-ecdysone.

▪ Ecdysone : making the signal

If we look closer at the life cycle of *Drosophila*, we realize that its growth is restricted to the three larval stages and that maturation occurs during metamorphosis. Each of these major developmental transitions, including the two molts and puparium formation, is triggered by peaks in the steroid hormone ecdysone (fig. 11).



Drosophila is not able to synthesize sterol compounds *de novo*, because it lacks the enzymes necessary for the sterol biosynthesis pathway that exist in vertebrates (Kurzchalia and Ward, 2003). Hence, *Drosophila* strictly depends on diet and thus must intake cholesterol from its food for steroid hormone biosynthesis (fig. 12). Cholesterol uptake occurs in the intestine, and then it is secreted into the hemolymph and finally assimilated by the prothoracic gland. The prothoracic gland is the center place for ecdysone biosynthesis from cholesterol, throughout all larval life. The secretion of ecdysone in the hemolymph is triggered by another hormone, the prothoracicotrope hormone or PTTH. When the larva reaches a certain size, PTTH is secreted by neurosecretory cells of the intercerebralis pars of the brain, then it is transferred via the axons into the *corpora cardiaca*, neurohumoral organs also associated with the brain, where it is stored before being secreted. In *Drosophila*, the prothoracic glands

are incorporated into an endocrine complex, the "ring gland" or Weismann ring, which, besides the two fused prothoracic glands includes the *corpora allata* involved in the secretion of juvenile hormone and *corpora cardiaca*. Once in the hemolymph, ecdysone will be assimilated by the fat body to be finally converted into 20E, through the 20-mono-oxygenase. In *Drosophila*, the prothoracic gland is destroyed during metamorphosis and thus absent from adults. However, ecdysteroids can be synthesized in the ovaries to be deposited in oocytes, to be used and eventually produced by the embryo.

Many studies have been conducted to understand the conversion of dietary cholesterol into 20E, showing that this mechanism is catalyzed by several enzymatic steps (Gilbert et al., 2002). Soon after uptake, a dehydrogenation of cholesterol leads to the formation of 7-dehydrocholesterol in the endoplasmic reticulum, which is then transported into mitochondria for oxidations (fig. 12).

The first step involves *neverland* (*nvd*), which is specifically expressed in prothoracic gland (PG) (a pair of endocrine glands that secrete ecdysteroids and regulate molting) and encodes an oxygenase-like protein indispensable for ecdysone production (Yoshiyama et al., 2006). At least eight other enzymes essential for 20E biosynthesis have been identified in *Drosophila* (Niwa et al., 2004; Niwa and Niwa, 2016; Warren et al., 2004). The so-called Halloween genes encode a family of cytochrome P450 mono-oxygenases (CYP450), including *phantom* (*phm*), *Disembodied* (*Dib*), *Shadow* (*Sad*), and *spook* (*spk*) (Namiki et al., 2005; Ono et al., 2006). A disability to synthesize 20E and thus to induce ecdysone-responsive genes has been demonstrated following mutation of any of these genes, which are expressed in the PG. Other studies have identified *shade* (*shd*) as a gene responsible for catalyzing the final conversion of ecdysone to 20E in peripheral tissues, and it too encodes a CYP450 (Niwa et al., 2004; Niwa and Niwa, 2014; Petryk et al., 2003; Warren et al., 2004). Four additional genes are further required for ecdysone biosynthesis: *Spookier* (*Spok*) (Ono et al., 2006), *CYP6T3* (Ou et al., 2011), *Non-molting glossy/Shroud* (*Nm-g/Sro*) (Niwa et al., 2010) and *spook* (*spk*) (Namiki et al., 2005; Ono et al., 2006). The corresponding enzymes have not yet been biochemically characterized and they are thus hypothesized to act in the so-called "Black Box" steps, which remain to be further elucidated. Interestingly, it has been realized that ecdysone-producing genes are not only specifically expressed in the PG, they are also temporally expressed in correlation with the ecdysone peaks during larval development (Niwa et al., 2004; Ono et al., 2006; Warren et al., 2004). Therefore, the regulation of ecdysone biosynthetic enzymes is likely to play a role in the proper temporal series of ecdysone pulses, for example contributing to a feed forward loop, in order to reach high levels of ecdysone titers in a narrow time window. Despite these recent progresses in understanding the ecdysone biosynthesis, it is clear that the multistep control of this pathway remains to be fully understood. For example, additional players are likely to regulate sterol metabolism and intracellular trafficking.

Having reached the target tissues, the next issue is to explain how ecdysone triggers developmental control. In other words, how ecdysone can modify the execution of genetic programs of differentiation. We will review some of these aspects in the following.

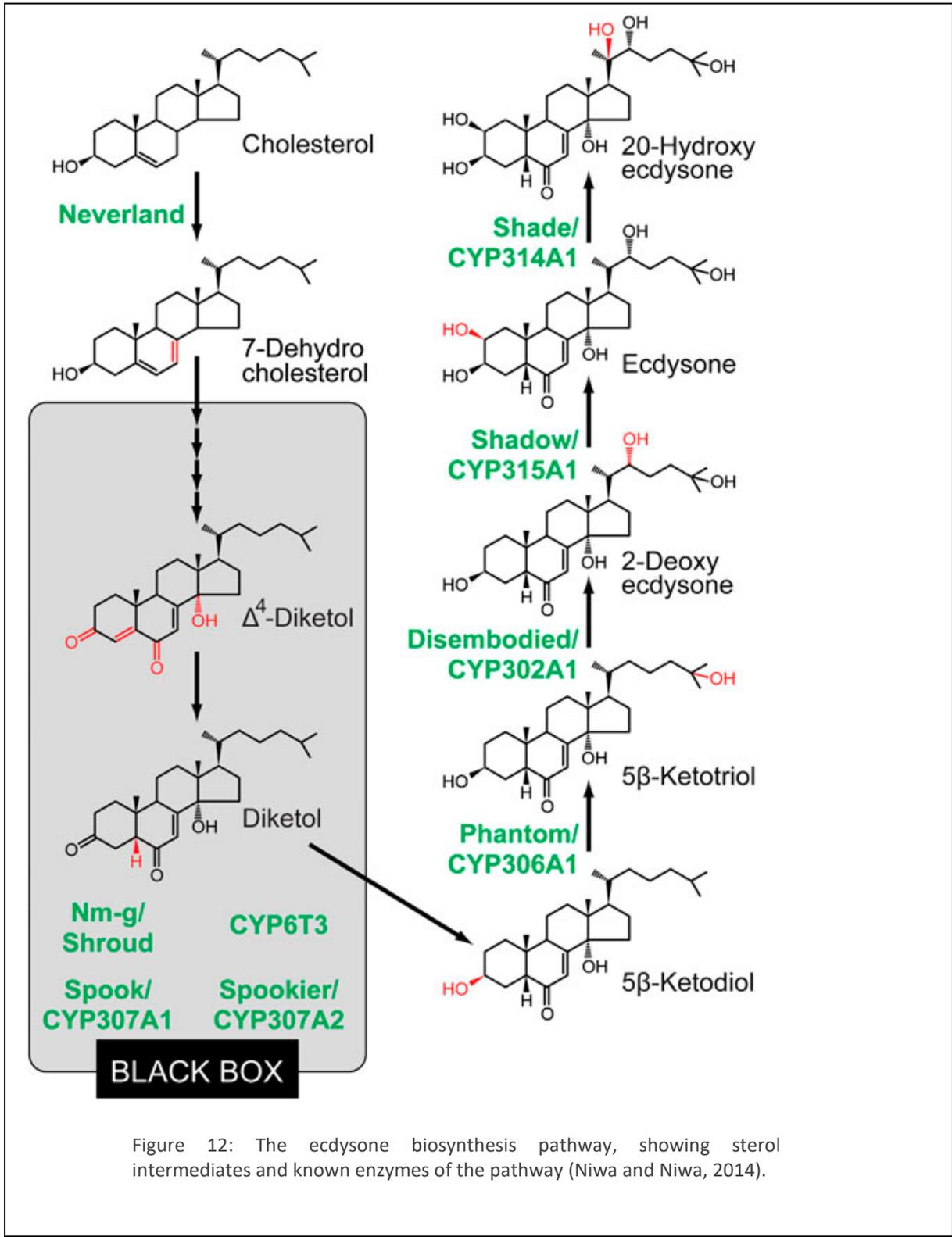


Figure 12: The ecdysone biosynthesis pathway, showing sterol intermediates and known enzymes of the pathway (Niwa and Niwa, 2014).

▪ The Ecdysone receptor (EcR)

The actual form in which ecdysone circulates in the hemolymph of *Drosophila* is not precisely known. Despite its lipophilic nature, it dissolves relatively well in aqueous solutions and may circulate freely in the hemolymph. Yet it is not excluded that it can bind to transport proteins that circulate with it in hemolymph. In the same way, how ecdysone penetrates into the cells is unclear, although it may passively diffuse through the membrane. Once in the cell, ecdysone binds its specific receptor: the heterodimer Ecdysone Receptor / Ultraspiracle (Yao et al., 1992). The Ecdysone Receptor (EcR) is a transcription factor of the nuclear receptors superfamily (Koelle et al., 1991) which as such presents five characteristic regions: the A / B domain that contains the activation domain AF1, C is the DNA binding domain that has two zinc fingers, the hinge domain D, the domain E capable of binding ecdysone and containing a second activation domain AF2, and finally domain F.

Ultraspiracle (Usp) is also a nuclear receptor (Henrich et al., 1990; Oro et al., 1990) that is homologous to the vertebrate RXR (Retinoid X Receptor), but to date no *bona fide* ligand could be associated with it, although crystallization experiments showed the presence of a phospholipid molecule into the ligand fixing pocket (Billas et al., 2001; Clayton et al., 2001). In the EcR / Usp heterodimer complex, ecdysone binds solely to EcR, whilst Usp is essential to this fixation. Once formed, the complex 20E / EcR / USP recognizes and binds to an extended DNA sequence (fig. 13), called the ecdysone response elements (ECRE) (Antoniewski et al., 1996; Cherbas et al., 1991; Riddihough and Pelham, 1987), and promotes the transcription of associated genes.

The EcR gene codes three different protein isoforms. The most distal promoter allows the formation of EcRA, while the proximal promoter produces both EcRB1 and B2 (fig. 13). The three proteins only differ in their A/B domains. Unlike EcR, the USP gene encodes a single transcript but it seems that the latter possesses at least two translation initiation sites (Henrich et al., 1994) (fig. 13). Thus, there is the possibility of forming at least three EcR/USP complexes that may provide a more specific regulation between different tissues, following the combination(s) of receptors expressed therein at any given time (Talbot et al., 1993).

EcR/Usp recruits coregulators, including chromatin remodelers such as the NURF complex (Kugler et al., 2011). In the presence of ecdysone, EcR/Usp interact with enzymes modifying histones to assist in activating target genes: the methyltransferase TRR (Carbonell et al., 2013), the acetyltransferases Taiman & p300/CBP (Lozano et al., 2014; Yoshida et al., 2005; Zhang et al., 2015), and the polyA polymerase PARP. In the absence of hormone, EcR interacts with the SMRTER corepressor (Heck et al., 2012; Tsai et al., 1999), which recruits histone deacetylases. Additional levels of control are to consider since both Usp and EcR also dimerize with alternative nuclear receptors (Baker et al., 2003) and more than half of family of nuclear receptors participate directly or indirectly in the ecdysone pathway (Ou and King-Jones, 2013).

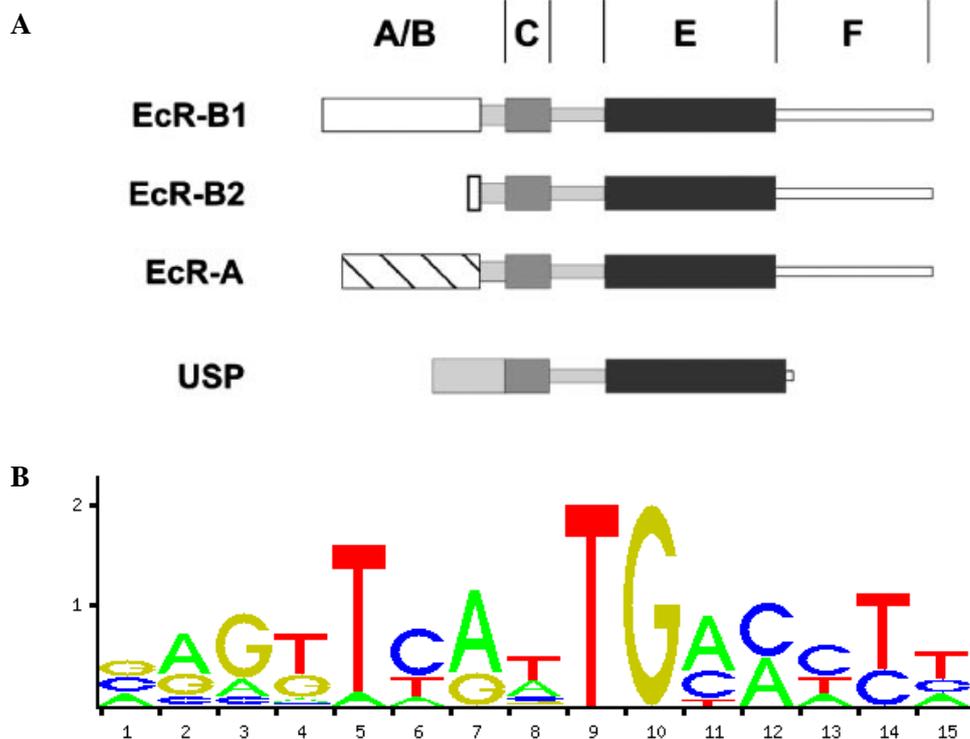


Figure 13: Functional organization of the EcR-USP heterodimer receptor. (A) the *EcR* gene encodes 3 isoforms : *EcRA*, *B1* et *B2*, and *usp* a single one. Region C is the DNA-binding domain (DBD); region E, the ligand-binding domain (LBD). The three EcR isoforms are identical in sequence except in the A/B regions that are unrelated (Cherbas et al., 2003; Hu et al., 2003). (B) The binding site for EcR-USP transcription factor heterodimer is shown as a Position Weight Matrix (From the Jaspard database).

- **Known targets of the ecdysone pathway: multitiered TFs**

The study of ecdysone response at the molecular level begins with the work of Ashburner on chromosomal "puffs", which are kind of swelling at polytene chromosomes that appear in a specific order between the middle of the third larval stage and late prepupal stage (Becker, 1959). Many studies on ecdysone-induced puffs have been carried out in the salivary glands, because their giant polytene chromosomes are easily observable. Ashburner and colleagues studied the behavior of puffs through *in vitro* cultures of salivary glands of a certain age, in presence or absence of ecdysone, and with or without various inhibitors. This work allowed

Ashburner to propose a model of hormonal response (Ashburner, 1974). Assuming that puffs actually reflect the transcriptional activity of certain genes they contain, Ashburner proposes that the ecdysone bound to its receptor induces the expression of a limited number of genes (early puffs), while repressing the expression of other genes (late puffs). The products of the early genes in turn allow expression of late genes and eventually repress their own expression, as schematized below.

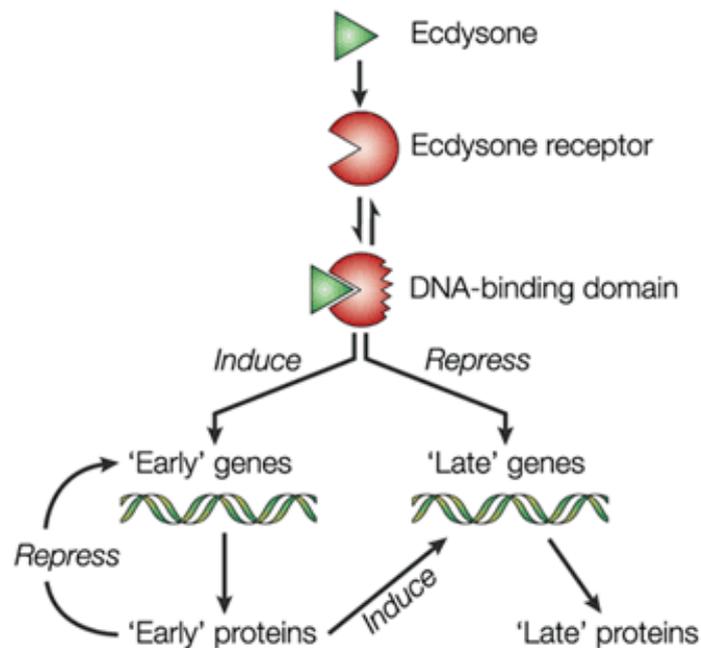


Figure 14: Model of the action of ecdysone receptor that activates the expression of early genes and represses late genes, as initially proposed by Ashburner. Figure taken from (Tata, 2002).

The Ashburner model also posits to the existence of genes positively regulated by 20E/EcR, but that additionally requires the function of early gene products to be expressed: these are early-late transcripts. There are also genes qualified as intermolt, since their expression occurs before or after molting, when the hormone titers are lowered back to basal levels.

Early transcripts

At the beginning of ecdysone response in the end of the third instar larvae, ecdysone induces six early puffs. One of these puffs contains the Broad Complex (BR-C), for which no less than 14 mRNAs -from three different promoters and alternative splicing- can be produced (DiBello et al., 1991). However, it seems that Br-C encodes “only” 4 different transcription factors, called BR-C Z1, Z2, Z3 and Z4 (Bayer et al., 1996). Other early puff transcripts are E74A and B, two isoforms of the E74 gene generated by utilization of two different promoters. These two proteins are relatively different between them, but they share a common region in their C-terminal part (Burtis et al., 1990). Similarly, the E75 gene encodes three transcripts E75A, E75B, and E75C (Feigl et al., 1989) and both E75A and E75C have two zinc fingers (features of nuclear receptor). E63-1 and E63-2 are genes which don't encode transcription factors; E63-1 being homologous to calcium binding proteins (Andres and Thummel, 1995; Vaskova et al., 2000) (fig. 15).

Early-late transcripts

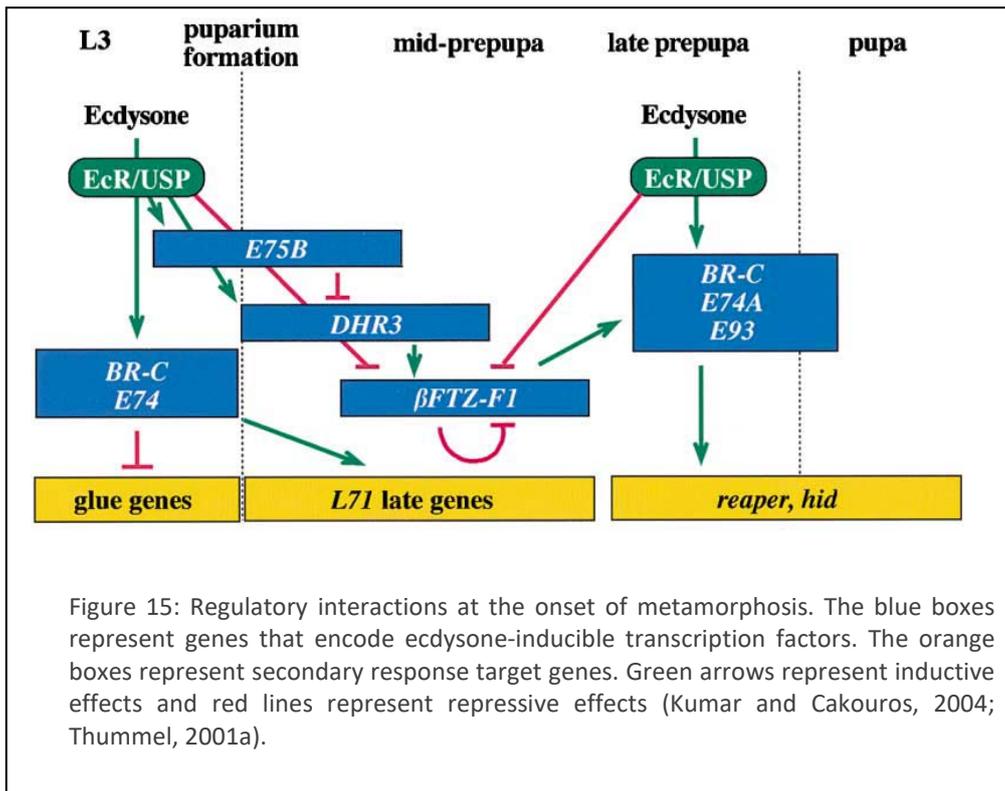
The gene E78 contained in the early-late puff encodes two isoforms, E78A and B (Stone and Thummel, 1993). Indeed, E78A, as E75A, is a homolog of the vertebrate nuclear receptor Rev-erb, while E78B has an incomplete DNA binding motif as E75B. However, only E78B is classified among the early-late transcripts. *DHR3* (*Drosophila hormone receptor 3*, aka *Hr46*) also encodes a nuclear receptor (Koelle et al., 1992). (Koelle et al., 1992). The ligand binding domain (LBD) of DHR3 is essential for metamorphosis, since growth defects were detected in DHR3 LBD mutant cells (Montagne et al., 2010). This gene is located in the early-late puff 46F and encodes at least three transcripts. The early-late gene *DHR39* was later identified on the basis of its strong homology with *Ftz-f1* (Ohno and Petkovich, 1993) (fig. 15).

The main late transcripts

There are over a hundred of late puffs and Ashburner proposed that these puffs result from the expression of transcripts from which the proteins have an effector role, rather than a regulatory function. Despite their large number, the molecular function of most late genes remains poorly understood. For example, *L82* is a late gene that encodes a protein necessary for development, but without known homologs that could shed light on its putative role (Stowers et al., 1999). *L63* also encodes several isoforms of uncharacterized function (Stowers et al., 2000).

The Inter-moult transcripts

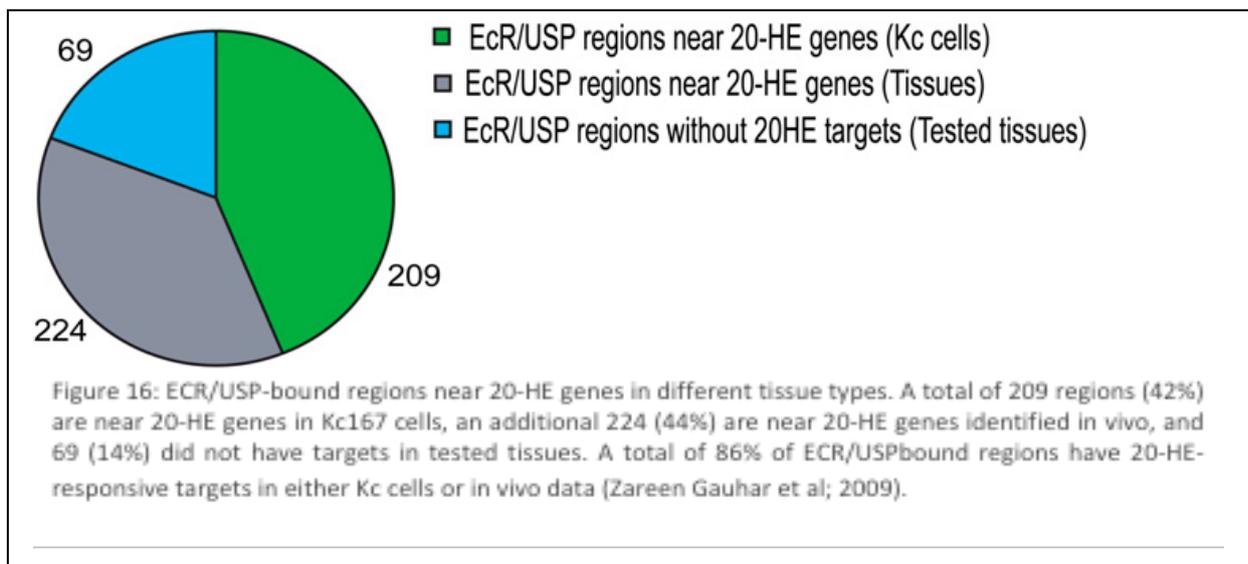
Inter-moult transcripts are expressed only when the hormone titer is down and increasing the levels of ecdysone leads to their repression. The intermolt puffs are present in mid-third instar larvae, and disappear when the 20E concentrations increase, later in the instar. One important gene in the mid-prepupal puffs is *Ftz-f1*, which is expressed between molts. *Ftz-f1* encodes two transcripts: α *Ftz-f1* and β *Ftz-f1* (Lavorgna et al., 1993; Lavorgna et al., 1991; Ueda and Hirose, 1990) encoding orphan nuclear receptors which share a common C-term region but have different N-termini. While α *Ftz-f1* is expressed in early stage embryos, β *Ftz-f1* appears later on, and shows periodic expression at the end of larval and prepupal stages, and finally in the late pupal stage. β *Ftz-f1* expression is repressed by EcR and its activation requires DHR3 and Hr4 activity. The inactivation of β *Ftz-f1* severely affects the ecdysone signaling pathway at the onset of metamorphosis and leads to lethality at the prepupal stage (Ou and King-Jones, 2013).



Later on during prepupal development, $\beta Ftz-f1$ is considered as a factor of competence, that's to say that its expression before the increasing of hormone is necessary to make the cells able to respond adequately to ecdysone. Thus when the ecdysone titer increases again and the hormonal response of the end of pupal stage begins, $\beta Ftz-f1$ allows the re-induction of *BR-C*, *E74A* and *E75A* (Woodard et al., 1994). $\beta Ftz-f1$ also directly induces the expression of *E93*, a gene essential for the destruction of salivary glands at the end of prepupal stage. Finally, as knowledge gradually increases on ecdysone hormonal response, it seems that these responses make a real network of regulations rather than a linear series of inductions and repressions.

Genome wide analysis defines different ecdysone responses amongst tissues.

Reaching a global view of genes that respond to ecdysone and those that require EcR for their regulation was a precious step that has been propelled by genome-wide approaches (Beckstead et al., 2005; Li and White, 2003; White et al., 1999). For example, high-density oligonucleotide microarrays has been used to profile EcR binding events in a cell line (Kc167) that maintains many characteristics of the original insect tissues such as differentiation in response to ecdysone (Gauhar et al., 2009). This work identified 502 regions of EcR/Usp binding throughout the genome, often located near ecdysone-regulated genes across various cell types (fig. 16). Moreover, three of the direct targets of EcR/USP (*hairy*, *vrlle* and *Hr4*) were found to be required for cellular differentiation in response to ecdysone. Hence, it was demonstrated, *in vivo*, the important role of *vrlle* for metamorphosis (Gauhar et al., 2009).



Other studies demonstrated that differences in the transcriptional response may in part be due to differences in the EcR isoforms present in different cell types since there are large differences between Kc cells and salivary glands concerning their genome-wide transcriptional response to 20E and since the level of induction of EcR isoforms differs between these two tissues (Gonsalves et al., 2011). Recently, a first genome-wide analysis of conditional mutants has been performed to model together the ecdysone and EcR gene expression regulation. This was done by integrating classical genetic mutant experiments with functional genomic techniques in *Drosophila*, where the ecdysone signaling response was investigated at the onset of metamorphosis. Thus traditional and novel ecdysone target genes were revealed. About 12% of the genome responds to the ecdysone signaling at the onset of metamorphosis and over half of these are independent of the receptor (Davis and Li, 2013). In a same vein, a recent study has profiled the genome-wide transcriptional response to ecdysone in a large panel of 41 different *Drosophila* cell lines, identifying a total of 1645 genes whose expression is significantly modified by ecdysone, in at least one cell line (Stoiber et al., 2016). Besides a small number of early genes (including *E75*, *Hr4*, *Hr46*, *bip1* or *CG44004*) that are induced in all 41 cell lines, most of ecdysone responsive genes appear cell-type specific. This specificity involves both the expression of different EcR isoforms, as well as the specific combination of a large number of TFs expressed in each cell line (Stoiber et al., 2016).

Ecdysone responsive TFs feed back on hormone production

The temporal expression of ecdysteroidogenic enzyme genes is correlated with periodic pulses of ecdysone titers, suggesting a tight transcriptional regulation. Several studies have

shown that downstream regulatory components of the ecdysone hierarchy also play important roles in the regulation of ecdysone production (reviewed in Niwa and Niwa, 2016; Ou and King-Jones, 2013). For example, the specific EcR-A isoform is expressed in the prothoracic gland where it may have a negative feedback role on ecdysone synthesis (Talbot et al., 1993). Also, mutation of E75A gives rise to a severe decrease in ecdysone levels (Bialecki et al., 2002). β -Ftz-f1, an homolog of Ad4BP/AS-1, the key regulator of steroid synthesis in mammals, is also required for proper levels of Phm and Dib proteins in the PG (Parvy et al., 2005). More recently, *broad* has been characterized as a gene involved in ecdysteroidogenesis by positively controlling the expression of *Npc1a* and thus coordinating cholesterol availability in the prothoracic gland (Xiang et al., 2010). *Npc1a* mRNA levels are strongly decreased in *broad* mutants, or following RNAi-mediated knocking down of *broad* function specifically in the ring gland. However, reexpression of *Npc1a* is not sufficient to compensate for *broad* loss of function, and the *broad* isoforms Br-Z1 and Br-Z4 bind directly to *phm* and *dib* cis-regulatory regions to regulate their expression (Moeller et al., 2013). Additional evidence have shown the involvement of other ecdysone-regulated transcription factors in the control of ecdysone synthesis, including EcR (Moeller et al., 2013; Parvy et al., 2014), Ultraspiracle (Koyama et al., 2014), DHR3 (Parvy et al., 2014) and DHR4 (Ou et al., 2011).

The two latter cases are of peculiar interest since they allow the integration of additional levels of regulation. *DHR4* integrates PTTH signaling in PG cells for the proper timing of ecdysone. The DHR4 protein shuttles between the nuclear and cytoplasmic compartments, with a cycle that closely matches the oscillating levels of PTTH mRNA (McBrayer et al., 2007). Following the ablation of PTTH neurons, DHR4 remains mostly nuclear, while PTTH overactivation keeps DHR4 in the cytoplasm. It has been reported that DHR4 might repress an ecdysogenic enzyme, called *Cypt6t3* (Ou et al., 2011). Moreover, Nitric oxide (NO) signaling regulates the interaction between E75 and DHR3. E75 contains a heme moiety that binds the NO messenger to promote the interaction with DHR3 (Reinking et al., 2005) and modulate the activation of β Ftz-f1 (Caceres et al., 2011). β Ftz-f1 also regulates the expression of *Snmp1* which is involved in lipid uptake in PG cells (Talamillo et al., 2013). It should be noticed that DHR3 also favors the ring gland growth in an autonomous fashion, integrating nutrient sensing and insulin signaling pathway (Montagne et al., 2010). Reciprocally, E75 and β Ftz-f1 counteract DHR3 to prevent precocious repression of ecdysone production (Caceres et al., 2011; Parvy et al., 2014). In addition to PTTH and 20E, ecdysteroidogenic gene expression is influenced by other pathways, such as insulin-like peptides-TOR (Colombani et al., 2005), TGF β /Activin-Smad (Gibbens et al., 2011) and many other cues are required for the proper timing of ecdysteroid biosynthesis during development (Niwa and Niwa, 2014; Niwa and Niwa, 2016). In sum, whereas these studies clearly establish the importance of both positive and negative feedbacks between ecdysone hierarchy factors and hormone synthesis, further large-scale studies are expected to provide novel insights into our comprehension of ecdysone hormone biosynthesis and regulation (Danielsen et al., 2016; Ou et al., 2016).

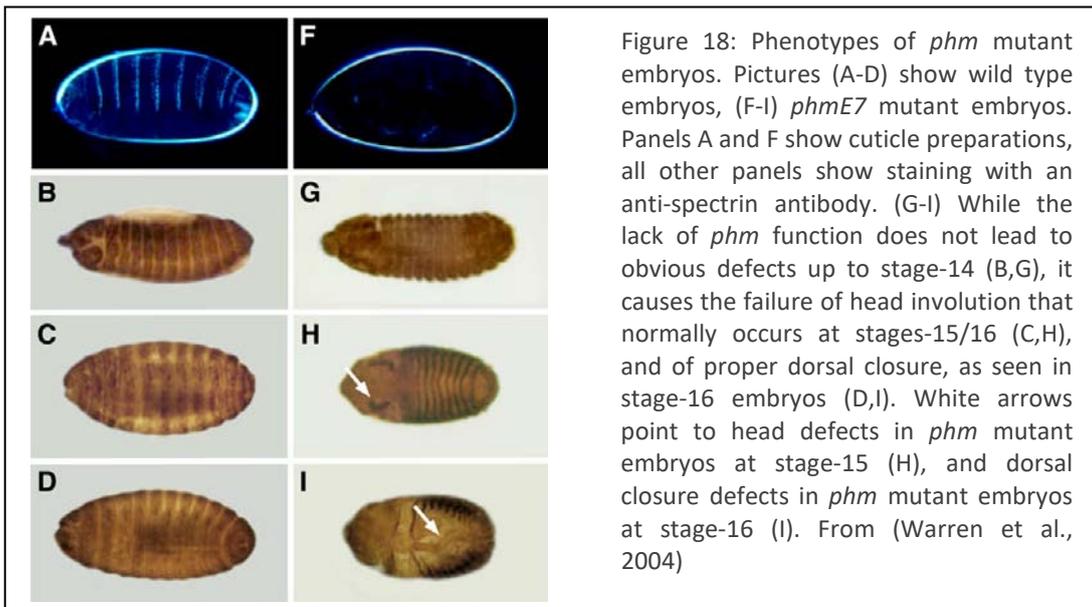
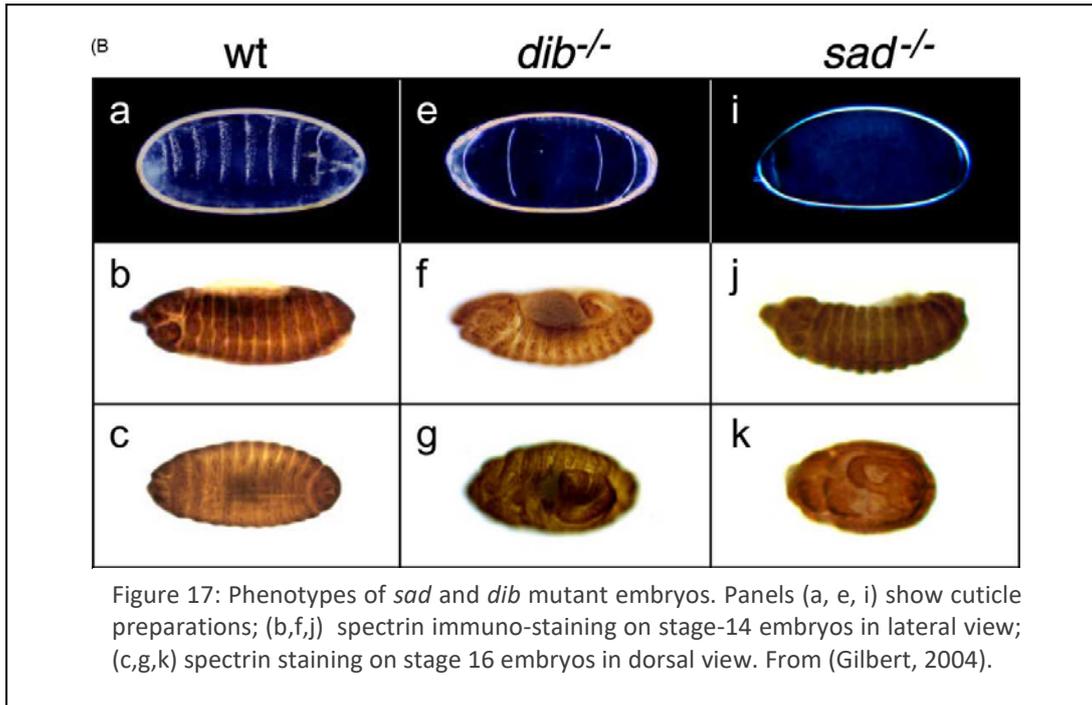
▪ A neglected role of ecdysone in embryonic development

As mentioned above, the ecdysone hormone is secreted by the PG throughout all larval stages. Formation of the PG occurs during late embryogenesis and therefore the significant levels of ecdysone detected in newly deposited eggs are likely of maternal origin. In addition, previous studies on the embryonic PG have noticed that it harbors a poorly differentiated appearance, without the well-developed smooth and rough endoplasmic reticulum that characterize the mature larval organ, suggesting that the PG is unlikely to assume efficient ecdysone synthesis in embryos (Dorn and Romer, 1976).

Using a transgenic reporter system, Kozlova and Thummel detected a strong activation of EcR-dependent gene expression in the amnioserosa during mid-embryogenesis (Kozlova and Thummel, 2003b). Interestingly, a hormone-dependent role of the amnioserosa may be consistent with several defects observed following EcR inactivation in the embryo, including aberrant germ band retraction and defects in head involution that both involve interactions with the amnioserosa (Bender et al., 1997; Kozlova and Thummel, 2003b).

Another set of studies showed that the TFs E74, E75, DHR3, and β Ftz-f1 mediating response to ecdysone at the onset of metamorphosis (Henrich et al., 1999; Thummel, 2001b), also display a similar pattern of sequential expression during mid-late embryogenesis (Sullivan and Thummel, 2003). In addition, it was demonstrated that there are similar regulatory interactions among these genes at both stages (Ruaud et al., 2010). Embryonic lethality was observed following the inactivation of *DHR3* or *Ftz-f1* (Bender et al., 1997; Yamada et al., 2000). Embryos carrying a null mutation of *DHR3* die at the end of embryogenesis (Carney et al., 1997; Ruaud et al., 2010), with weak alterations in the peripheral nervous system (Ruaud et al., 2010), but these defects are only partially penetrant and so are not likely responsible for the embryonic lethality (Kolodkin et al., 1993). *Ftz-f1* mutant embryos display poorly differentiated epidermal derivatives, including small and weakly pigmented denticles, but no other obvious strong alterations of the embryonic development (Carney et al., 1997; Ruaud et al., 2010; Yamada et al., 2000). Both *DHR3* and *Ftz-f1* mutations yet lead to a highly penetrant defect in air filling of the trachea (Ruaud et al., 2010). Since both mutants show normal muscle movements, it was suggested that only some developmental events are blocked during embryogenesis; it was also shown that DHR3 fulfills essential functions independently of β Ftz-f1 (Ruaud et al., 2010).

Additional evidence of the role of ecdysone in embryos came from several studies on the enzymes required for ecdysone production, using null mutations in the corresponding genes (Chavez et al., 2000; Ono et al., 2006; Ruaud et al., 2010). For example, the inactivation of *phantom* (*phm*), *disembodied* (*dib*) or *shadow* (*sad*) leads to strong morphogenetic defects in late embryos, which display a shorter size, defects in head involution and dorsal closure. In addition, all these mutants are characterized by the absence of a proper cuticle production that is very thin and almost transparent with no signs of differentiated denticles (fig. 17-18), revealing the existence of key functions for ecdysone in epidermal derivatives in late embryos.



All together, these data demonstrate that ecdysone production and signaling play key roles during embryogenesis. In particular they highlight that ecdysone is critical for the development of the epidermis, a topic that I will now introduce in more detail below.

5. Epidermis development

The adult insect is protected from the environment by a hard sclera exoskeleton, called cuticle. During embryonic and larval development, epidermal cells secrete a cuticle, thinner than at adult stage, thus facilitating the larvae movements. Immediately prior to cuticle deposition, a subset of epidermal cells undergo a spectacular rearrangement of the actin cytoskeleton, leading to apical cell extensions called ventral denticles, or dorsal hairs, and collectively referred to as trichomes. Thus the larval cuticle displays a stereotyped pattern of cuticle trichomes that alternate with smooth cells, which produce the so-called naked cuticle, along the whole body (fig. 19A). This trichome pattern is a major morphological feature of *Drosophila* larvae. It has been widely used by geneticists to study embryonic development (Nusslein-Volhard and Wieschaus, 1980), because the trichome pattern underlines the segmented organization and differentiation along both the anteroposterior and dorsoventral axes of the animal.

When looking at a closer detail in the ventral part of each abdominal segment, we distinguish 6 to 7 rows of cells producing denticles, with a characteristic hook shape, size or orientation and all strongly pigmented (fig. 19B). The trichomes that cover most of the dorsal segments are thinner, poorly pigmented, and without hook (fig. 19B) (Payre, 2004). There are also clear differences in the trichome pattern between segments, as well illustrated between thoracic and abdominal segments, both in the ventral and dorsal compartments. Even within a given segment, trichome rows display different identities as easily seen along the anteroposterior axis. All trichomes are nevertheless formed roughly at the same time, and result from the ordered assembly of the cuticle that retains the various shapes of underlying epidermal cells.

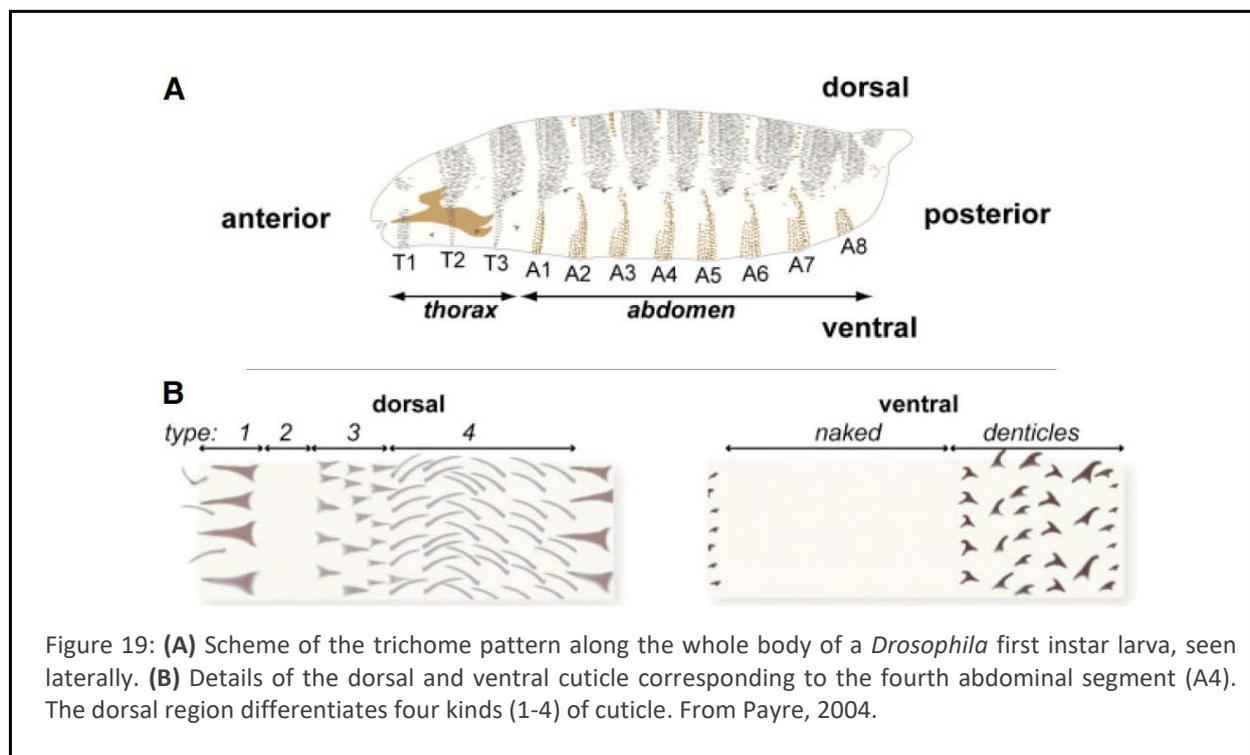


Figure 19: **(A)** Scheme of the trichome pattern along the whole body of a *Drosophila* first instar larva, seen laterally. **(B)** Details of the dorsal and ventral cuticle corresponding to the fourth abdominal segment (A4). The dorsal region differentiates four kinds (1-4) of cuticle. From Payre, 2004.

▪ **Cuticle composition and secretion**

Like in all arthropods, the structure and bio-chemical composition of the *Drosophila* cuticle is specific at each stage of the animal life. At larval hatch, the cuticle is formed by three layers, called envelope, epicuticle and procuticle. The lipid-rich envelope is in contact with the external milieu; it is mostly composed by lipoproteins and hydrocarbons and arranged into a fine trilamellar structure. Under, there is a thicker epicuticle which is made by a broad variety of proteins, contributing to the physical properties of the cuticle (rigid/hard, vs flexible/soft). Finally, the procuticle that is in direct contact with the plasma membrane is made of regularly arranged layers of chitin fibers. Chitin is a large polymer of N-acetylglucosamine residues, which represents the second most abundant polysaccharide on earth after cellulose. The complex assembly of cuticle begins twelve hours after fertilization, when relevant proteins are synthesized then secreted by epidermal cells. Secretion specifically occurs at the apical membrane, which forms a dense array of microvilli, and continues up to 15 hours after fertilization (Payre, 2004). Then the cuticle becomes harder and the microvillus on the apical membrane disappears (Ring and Martinez Arias, 1993). The procuticle further expands in stage 17, in the last couple of hours of embryonic development, and the three layers continue to thicken till the hatching of the first instar larva (Locke and Krishnan, 1971; Moussian, 2010). In addition, several enzymes of the catecholamine biosynthetic pathway produce active quinones, which contribute both to pigmentation and hardening (sclerotization) of the cuticle (Walter et al., 1991).

As mentioned above, the larval cuticle needs to be replaced to allow the large increase in size resulting from larval growth, as the animal will molt giving rise to the second and third instar larvae (Locke and Krishnan, 1971). Larval molts involve a complex series of physiological and behavioral changes and production of the new cuticle basically involves mechanisms similar to those described for embryogenesis. The main difference is that it begins by detachment of the old cuticle from the underlying epidermis. Epidermal cells also secrete a kind of gel that will later serve to digest the old cuticle, and the different steps of cuticle assembly start by epicuticle deposition, when ecdysone titers elevate to the highest levels. Following the rebuilding of a new cuticle, the old one is shed during ecdysis, when ecdysone is back to intermolt basal levels. Of note, cuticle assembly continues after ecdysis when the next instar larva has inflated, as manifested by deposition of chitin layers, increased sclerotization and pigmentation.

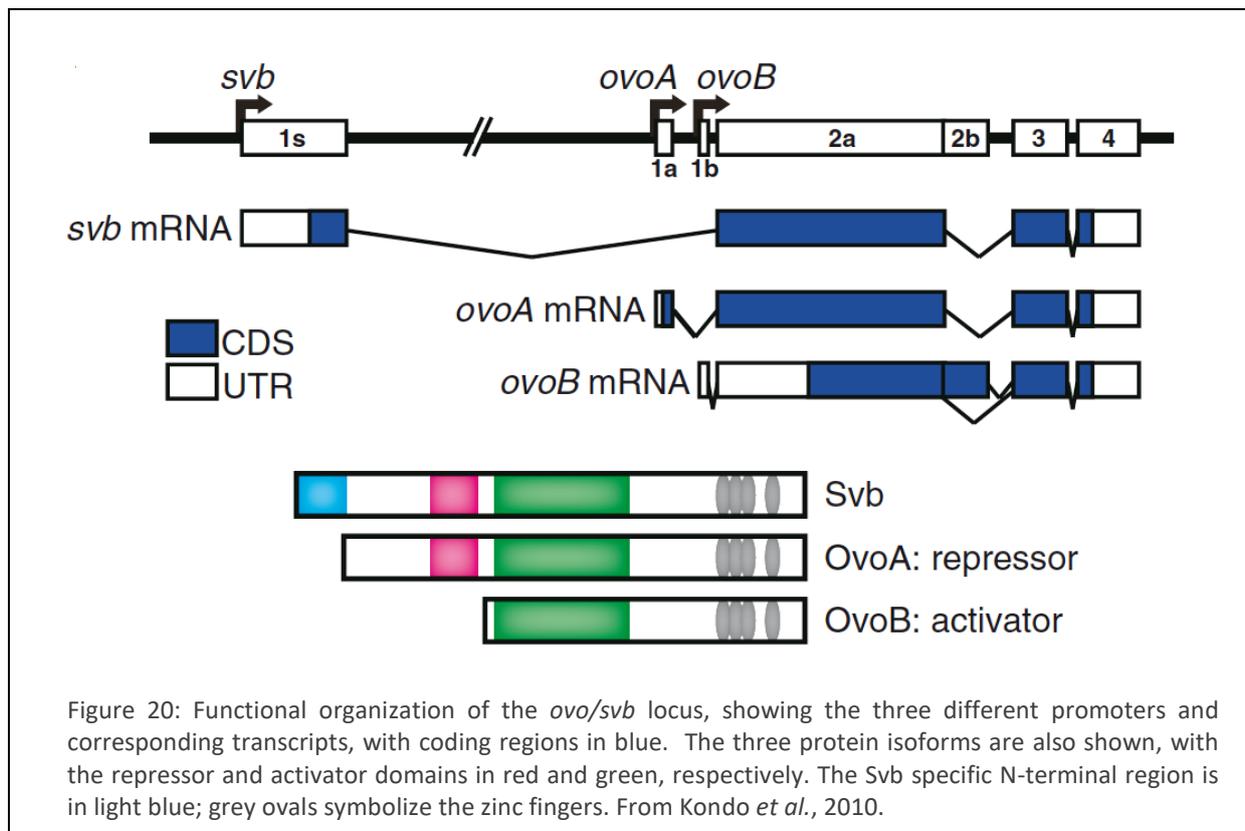
Major proteins required for cuticle formation and molts obviously include cuticle proteins, which broad range of spatiotemporal patterns likely contribute to both stage-specific and region-specific properties of the cuticle (reviewed in (Charles, 2010)). It also relies on the activity of the *Chitin Synthase-1* (*CS-1*) gene, called *krotzkopf verkehrt* (*kkv*), which inactivation affects procuticle formation and epicuticle stability, as well as cuticle pigmentation (Moussian et al., 2005) (fig. 14). Regulators of the intracellular trafficking are also of importance. For example, proper cuticle deposition depends on Syntaxin, a member of the SNARE family that mediates vesicle-fusion (Moussian et al., 2007). A few transcription factors have also been shown for their role in cuticle formation, including Grainy Head, a TF implicated in epidermal wound healing (Mace et al., 2005; Wang et al., 2009). The intermolt factor FtzF1, indirectly activated by ecdysone, plays as well key roles in the expression of cuticle proteins throughout *Drosophila* development (Charles, 2010; Chavoshi et al., 2010) (fig. 15).

- **Establishing the pattern of epidermal trichomes**

As we have seen, the larval cuticle is characterized by the segmental alternance of smooth/naked regions and cuticular trichomes, the latter being involved in larval motility. Hundreds of TFs impact on the trichome pattern and many segmentation genes have been identified, and classified, according to the defects observed in the trichome pattern following their inactivation. However, our lab has well established that the key player in trichome patterning and differentiation is a transcription factor called Ovo/Shavenbaby (Svb).

Molecular organization of ovo/shavenbaby locus.

Svb is expressed from the *ovo/svb* locus (fig. 20), which is indispensable for trichomes and female germ line development (Mével-Ninio et al., 1991). Two alternative promoters drive the expression of the germline-specific protein isoforms OvoA and OvoB. A third promoter is acting in somatic tissues and expresses an N-terminally extended isoform, Svb. The three isoforms share a common DNA binding domain and a region mediating transcriptional activation (Mével-Ninio et al., 1995). Consistently, OvoB, the shortest germline isoform acts as an activator (Andrews et al., 2000; Mével-Ninio et al., 1995). OvoA has an additional N-terminal region, carrying a repressor domain that dominates the transcriptional activity of this TF (Andrews et al., 2000). Svb basically correspond to a further N-term extension, encoded from the soma-specific exon 1S (Delon et al., 2003), and therefore contains all functional regions present in the OvoA repressor.



Svb governs the pattern and differentiation of epidermal trichomes

The first *svb* mutants were isolated by Nusslein-Volhard and Wieshaus in 1980. It was later demonstrated that *svb* encodes a zinc fingered transcription factor (Mével-Ninio et al., 1991) and that its epidermal expression is specific for trichome cells (Mével-Ninio et al., 1995). Further work showed that *svb* is both necessary and sufficient for trichome formation and has elucidated some aspects of its regulation, focusing on ventral denticle cells (Payre et al., 1999).

Following its role in embryonic segmentation, *wingless* (*wg*), a founding member of the Wnt cell signaling pathway, is determining the extent of naked cuticle. *wg* loss of function leads to a continuous lawn of denticles. Reciprocally, ectopic expression of the *wg* ligand or ectopic activation of the pathway completely represses denticles, leading to a bald ventral phenotype. These defects are indeed mediated by the action of *wg* to repress *svb* expression, which is respectively upregulated or downregulated throughout the ventral epidermis. In addition, the artificial re-expression of *svb* is sufficient to restore denticle formation in conditions of ectopic *wg* signaling. On the other hand, *svb* expression in trichome cells is activated by the EGF-r pathway. The normal expression domain of *svb* coincides with the location of active EGF signaling, as shown by patterns of the Erk kinase phosphorylation, or the expression of *sptiz*, the ligand that activates EGF-r in epidermal cells. As observed with Wingless, *svb* is epistatic to EGF activity, in other words the trichome defects observed following manipulation of EGF signaling are mediated by alterations of *svb* expression. For example, inhibiting the EGF-r pathway leads to the lack of trichomes, resulting from downregulated expression of *svb*. In sum, the reciprocal activities of Wg and EGF-r act together to finely control the expression domain of *svb* in epidermal cells, in turn determining the pattern of trichomes (Payre et al., 1999).

Additional data showed that various modifications in the trichome pattern observed following the inactivation of segmentation genes are prefigured by modifications of *svb* expression, strongly suggesting that Svb is the most downstream regulatory factor for the formation of trichomes. Independent support to this conclusion came from evolutionary studies focusing on the evolution of the trichome pattern across distant *Drosophila* species (Delon and Payre, 2004; Stern and Frankel, 2013). In all cases examined so far, the evolution of trichome patterns is due to changes in *svb* enhancers (Khila et al., 2003; Sucena et al., 2003), modifying the pattern of *svb* expression in epidermal cells (Arif et al., 2015; Frankel et al., 2011; McGregor et al., 2007; Stern, 2013; Stern and Frankel, 2013).

All together, these data show that the Shavenbaby transcription factor determines the formation of epidermal trichomes and that the pattern of *svb* expression defines the spatial pattern of embryonic epidermal cells forming trichomes (fig. 21).

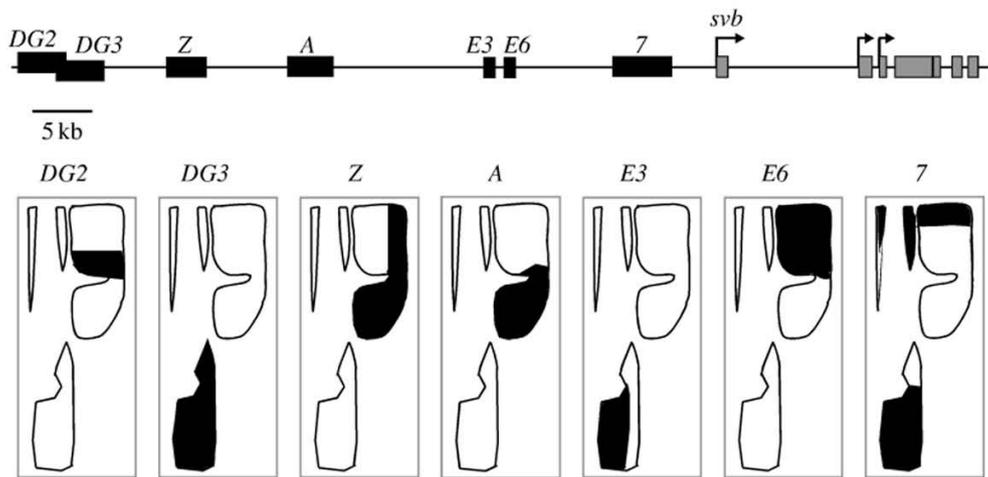
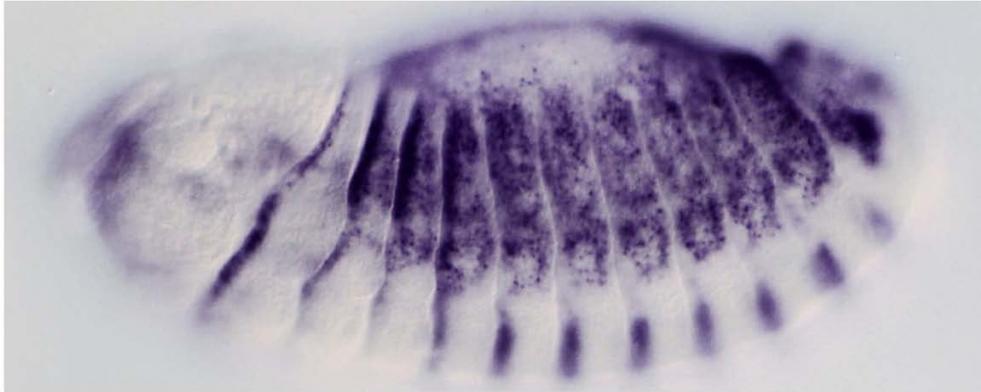


Figure 21: Expression of *svb* mRNA in a stage-14 embryo, and schematic representation of the different enhancers driving *svb* expression in overlapping subsets of epidermal cells. The drawings correspond to a lateral view of an abdominal segment, with the pattern of expression in dorsal (top) and ventral (bottom) cells driven by each of the *svb* epidermal enhancer. From Stern and Frankel (2013)

- **How *svb* controls the epidermal differentiation**

The key role of *svb* in establishing the trichome pattern reveals that it likely sits at the nexus between upstream regulatory cascades that progressively pattern the embryonic epidermis and the terminal program of differentiation that trigger epidermal cell remodeling for the formation of trichomes.

Indeed, *svb* is necessary and sufficient to trigger actin remodeling within epidermal cells and directs the whole sequence of transformations required for the formation of cuticle trichomes (Delon et al., 2003). Several studies done by our team aimed to understand how the Svb TF controls the reorganization of epidermal cell shape. This work has shown that Svb directly activates a large battery of cellular effectors, collectively responsible for the formation of epidermal trichomes (fig. 21). Svb downstream targets include genes encoding regulatory factors of the actin organization, such as Fascin (*singed*), Espin (*forked*) or Wasp; their individual inactivation leads to thin and broken trichomes (Chanut-Delalande et al., 2006). Likewise, Svb also activates the expression of genes encoding cuticle proteins and pigmentation enzymes (Andrew and Baker, 2008; Chanut-Delalande et al., 2006; Menoret et al., 2013) (fig. 22). More surprisingly, Svb also activates a whole family of extracellular matrix (ECM) components, which are required for trichome morphogenesis. Indeed, this includes eight genes encoding proteins of the Zona Pellucida family (Plaza et al., 2010), which collectively build an highly regulated apical extracellular scaffold that sustains the reorganization of epidermal cells for trichome differentiation (Fernandes et al., 2010).

Genome-wide molecular profiling, which combined transcriptomics and ChIP-seq of Svb-bound regions in epidermal cells, has further revealed that Svb directly activates the transcription of approximately 150 different targets (Menoret et al., 2013), encoding various effectors, or proteins of currently unknown function. This finding provides additional support to conclude that Svb is the most downstream TF required for trichome formation, thus acting as a “master gene”. In addition, the team has further delineated and dissected two dozens of enhancers driving effector gene expression in response to Svb. This work shows that Svb uses a small number (1-3) of TFBs to recognize its target enhancers. In addition, computational analyses coupled to *in vivo* mutational analyses of effector enhancers have disclosed the existence of additional motifs required for enhancer function, without any sign of a constrained grammar of *cis*-regulatory motifs (Menoret et al., 2013). Instead, the different functional elements comprising the Svb TFBs and other motifs display various combinations among enhancers driving very similar patterns, which indicate that these enhancers are based on a pretty flexible functional architecture (Slattery et al., 2014).

To summarize, a large body of evidence show that the *cis*-regulatory elements of *svb* integrate many inputs from early-acting regulatory cascades in order to define the specific subsets of epidermal cells that form trichomes. In turn, the Svb transcription factor directly activates the expression of many cellular effectors, which remodel distinct aspects of epidermal cell organization to produce epidermal trichomes. Furthermore, *svb* is also important in the adult epidermis for the morphogenesis of trichomes that cover the animal surface (Delon et al., 2003).

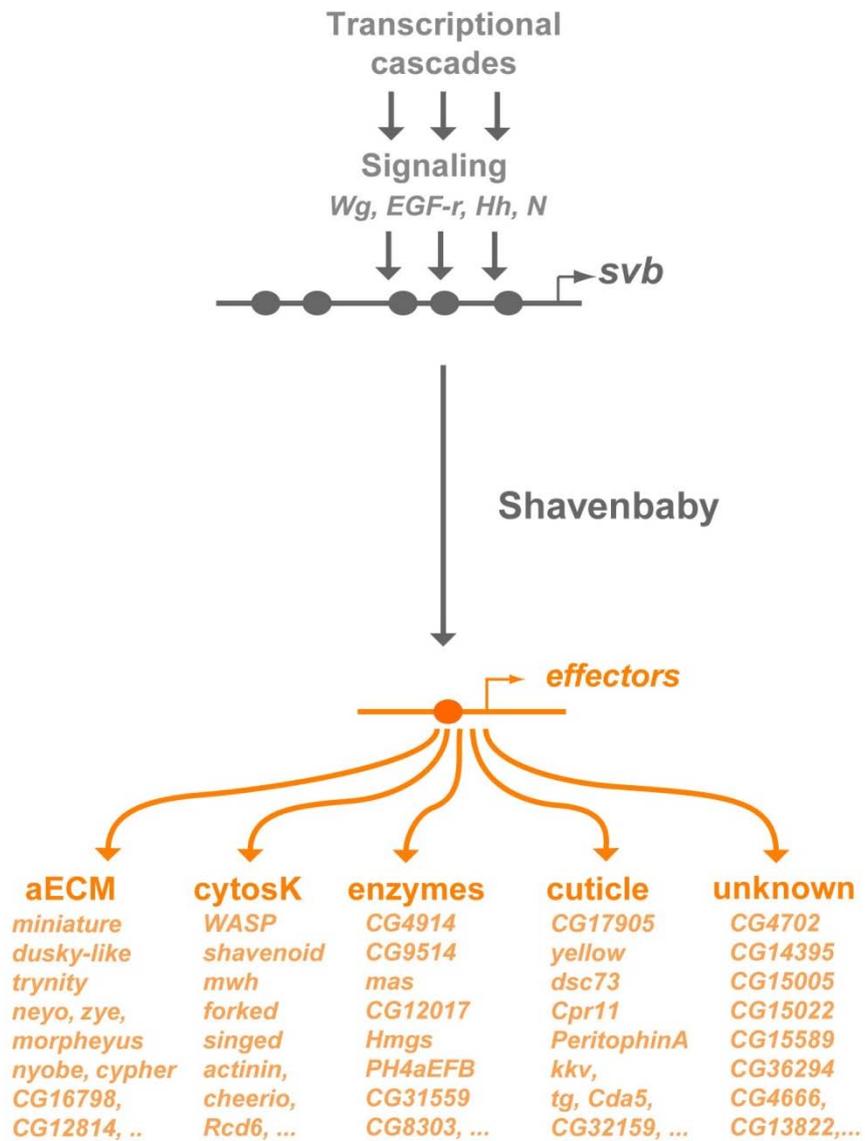


Figure 22: Genetic networks involved in the morphogenesis of trichome cells governed by Svb. Signals cues from Wg, EGF-R, Notch and Hedgehog (Hh) signaling pathways, are integrated by the regulatory regions of *svb*, causing expression specifically in trichome cells. In turns, Svb directly activates the transcription of different classes of trichome effectors.

▪ Pri peptides

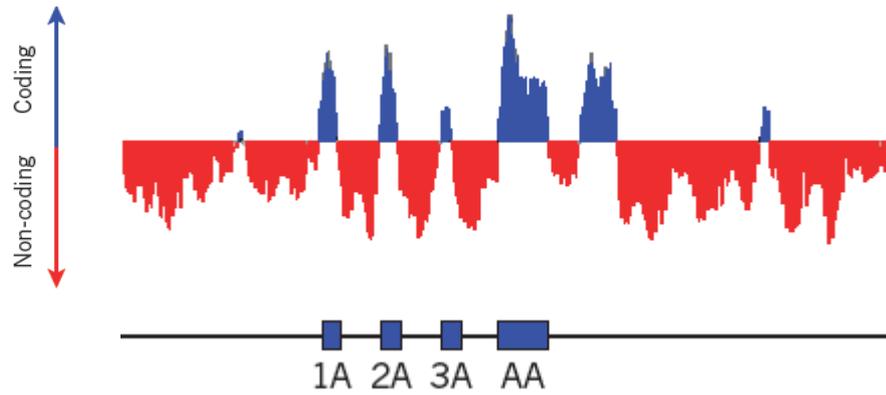
One apparent paradox raised by the above mentioned data was to understand how the Shavenbaby TF activates the expression of many genes for trichome morphogenesis in *Drosophila*. Indeed, how this TF that bears a repressor domain can nevertheless act as an activator? In this part I will talk about the discovery of unexpected additional key players of epidermis differentiation which thus allow filling the gap of this question mark.

The development of high-throughput profiling of cellular RNAs has revealed that a substantial proportion of them surprisingly do not encode proteins. For example, almost half of mammalian RNAs don't contain large Open Reading Frames (ORFs) and they are thus categorized as non-coding RNAs (ncRNAs) (Ota et al., 2004). While we now have a wealth of information to explain the activities of small ncRNAs (*i.e.*, miRNAs involved in gene regulation, siRNAs involved in the defense against viruses and transposon activity, etc...), the putative function of long ncRNAs remains poorly understood (Dogini et al., 2014). Recent studies converge on the conclusion that small ORFs (smORF) that are pervasive among large-sized RNAs often produce a wide diversity of small peptides (Albuquerque et al., 2015; Hashimoto et al., 2008; Kastenmayer et al., 2006). Therefore, various molecules annotated as long ncRNAs may act, at least in part, through the production of smORF-encoded peptides. One of them, called *polished rice*, *tarsal less* or *mille pattes (pri)* is required for epidermis differentiation in flies and has become a paradigm for studying the function of smORF peptides.

The Polished rice gene (pri) expresses a polycistronic RNA encoding smORF peptides

A long ncRNA (MRE29) was initially identified because of its evolutionary conservation and highly dynamic pattern of expression in developing tissues, throughout *Drosophila* embryogenesis (Inagaki et al., 2005). Independent studies have serendipitously identified its ortholog in *Tribolium*, named *mille pattes*, and demonstrated its key role in embryonic segmentation and leg formation (Savard et al., 2006). These authors also proposed that this putative ncRNA may instead represent a polycistronic transcript encoding four highly related smORF peptides (fig. 23).

This was later proven true, by the work of two laboratories focusing on different aspects of *Drosophila* development. As deduced from the analysis of an hypomorphic mutant allele, the same transcript (aka MRE29) was shown to be required for adult leg formation in flies and thus renamed *tarsal-less (tal)* (Galindo et al., 2007). In the meantime, another work has shown that the loss of function of this gene leads to embryonic lethality, with dramatic defects in the epidermis including the complete absence of trichomes, a phenotype at the origin of the name *polished-rice (pri)* (Kondo et al., 2007). Furthermore, the two studies provided compelling evidence that *pri/tal* actually produces 4 smORF peptides (11-32aa) (fig. 23), evolutionarily conserved throughout all arthropods, and most importantly are mediating the function of *pri* during development (Galindo et al., 2007; Kondo et al., 2007).



smORF1 : **MAAYLDPTGQY**
 smORF2 : **MAAYLDPTGQY**
 smORF3 : **MSHDLDPGTGY**
 smORF4 : **MLDPTGTYRRPRDTQDSRQKRRQDCLDPTGQY**

Figure 23: Representation of *pri* RNA, where blue boxes highlight the four small ORFs. The graph shows the distribution of a coding index, calculated from the pattern of evolutionary mutations that keep the coding potential (positive score, in blue) of introduce noncoding changes (negative scores, in red). Below are pictured the sequence of Pri peptides of 11 to 32 amino acids, encoded by each of the evolutionarily conserved *pri* smORF (adapted from Guttman and Rinn, 2012).

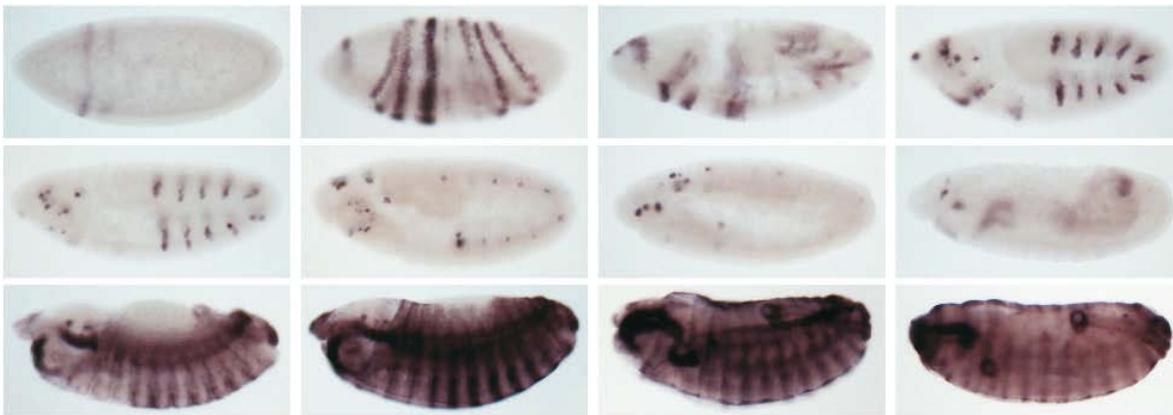
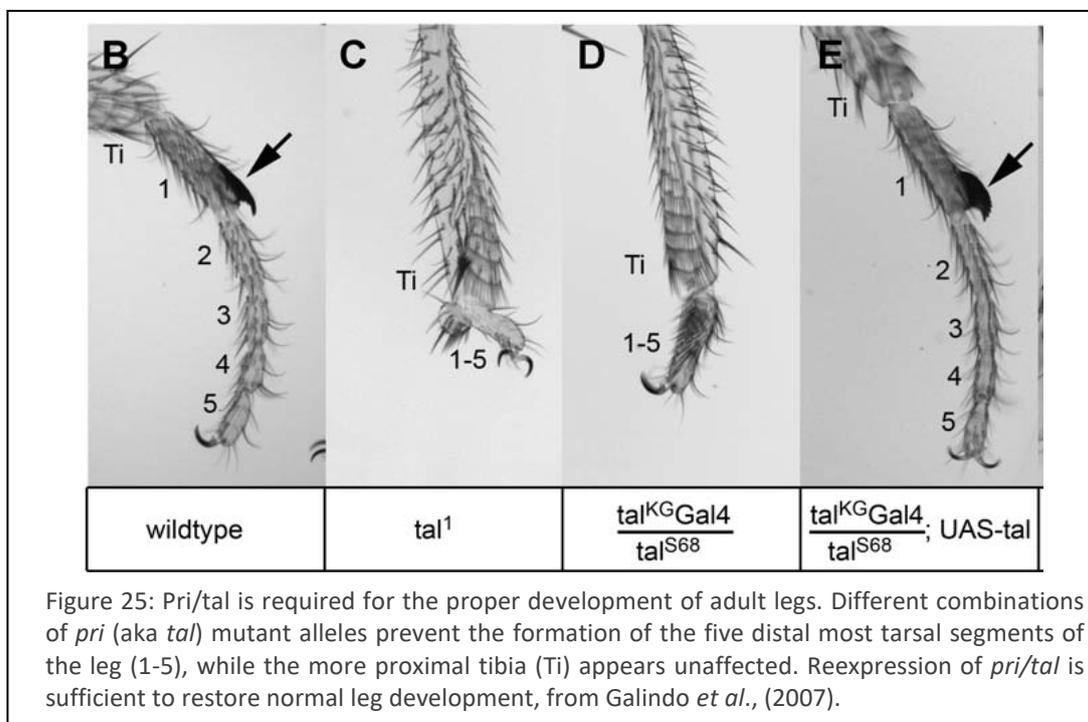


Figure 24: Dynamics of *pri* expression during embryogenesis, as revealed by *in situ* hybridization against the *pri* mRNA. Successive stages of embryonic development are shown from left to right. Taken from Kondo et al. (2007).

During embryogenesis, *pri* RNA is first expressed in 7 anteroposterior stripes at the blastoderm stage (fig. 24), then it displays fast evolving patterns of expression at different times, and in various tissues, including the spiracles, gut, trachea and epidermal cells (Kondo et al., 2007).

Pri mutant embryos display strongly affected trachea, the respiratory system of *Drosophila* made of a connected array of epithelial tubes mediating gas exchanges. There are early defects both in the shape and connection of tracheal branches, as well as later defects in the final differentiation of tracheal tubes, suggesting that *pri* is involved in multiple stages of trachea development (Kondo et al., 2007). Despite its early embryonic expression that evokes that of pair rule genes, the absence of *Pri* functions does not apparently impinge on embryonic segmentation. However, head structures seem also severely affected and ruling out a putative function of *Pri* in anterior most segmentation will require further work. The most prominent phenotype is nevertheless the one seen in the embryonic epidermis. Mutant embryos display a very thin and poorly differentiated cuticle, characterized by the absence of trichomes both in the dorsal and in the ventral regions. This phenotype is thus reminiscent of that observed in *svb* mutant embryos, even though the former appears even more severe. Importantly, experimental evidence has demonstrated that the re-expression of one or the other of the four *pri* smORF-encoding peptides is sufficient to rescue the denticle and tracheal phenotypes (Kondo et al., 2007).

Pri/tal is not only involved in embryogenesis, but its widespread function is also engaged in late larvae for the patterning of the leg imaginal disc (Galindo et al., 2007; Pueyo and Couso, 2008), and later for the proper development of adult legs during metamorphosis. Indeed, the inactivation of *pri* impairs proper leg formation and leads to adult legs lacking the whole tarsal region (Galindo et al., 2007) (Fig. 25).



The mechanism by which pri acts for epidermal development: a duo with Svb

As seen before, the Shavenbaby TF is the key regulator that directly activates the expression of cellular effectors underlying the development of embryonic trichomes (Chanut-Delalande et al., 2012). Because the phenotypes of *pri* and *svb* mutant embryos are strikingly similar, the two players were expected to functionally interact at some point. Therefore an important issue was to understand the molecular mode of action of these atypical Pri peptides.

To address this question, our team engaged a fruitful collaboration with the Kageyama laboratory in Japan. Despite the similarity of mutant phenotypes, the absence of *pri* does not impinge on *svb* expression in epidermal cells, showing that Pri peptides are not involved in the control of *svb* gene transcription. Reciprocally, the distribution of *pri* mRNA is not affected in *svb* mutant embryos, indicating that Pri peptides are not a novel *svb* target directly required for the remodeling of epidermal trichome cells. However, *pri* is critically required for the expression of Svb downstream targets. These results lead to the impairment of the *svb* transcriptional activation, since Svb-dependent enhancers become silent in the absence of *pri*, as observed both in cultured cell assays and *in vivo* (Kondo et al., 2010). This requirement for Pri peptides is yet alleviated when substituting Svb by the short germline isoform OvoB (see fig. 20), sufficient to activate effector-CRM expression, as well as trichome formation, with or without Pri peptides. Similarly, the activity of the intermediate-sized OvoA repressor is independent of *pri* function (Kondo et al., 2010). These findings therefore show that Pri peptides are specifically required for the function of the long Svb isoform, and that they should act in a way to control its transcriptional activity.

Indeed, biochemical characterization of the Svb transcription factor further showed that Pri peptides induce a postranslational cleavage of the Svb protein. Svb is translated as a long protein of 1351aa that behaves, like OvoA, as a transcriptional repressor. The expression of Pri peptides then triggers the production of a shorter product (907aa), removing the whole N-terminal region of Svb that contains the repressor domain, and thereby acts (like OvoB) as a transcriptional activator (Kondo et al., 2010). In early stages of epidermal development, the Svb repressor starts to accumulate in presumptive trichome cells. Later on, following the onset of *pri* expression in the epidermis, Pri peptides induce the maturation of Svb that allows the activation of the whole set of trichome effector genes. In *pri* mutant embryos, this maturation never occurs and Svb keeps accumulating in epidermal cells as the full-length repressor, thereby preventing the expression of Svb regulated cellular effectors and thus of trichome formation. Therefore, the main role of Pri peptides is to trigger an activating maturation of the Shavenbaby factor, switching its transcriptional activity from a large-sized repressor to an N-terminally truncated activator (fig. 26). These data therefore explain the key role of Pri in the regulation of epidermal trichome formation and provide one of the first cases that elucidate the function of smORF-encoded peptides in the control of animal development.

Additional work showed that that this functional interaction between Pri and Svb is not restricted to the embryonic epidermis, since Svb maturation is also involved at least in some functions of *pri* during adult leg morphogenesis (Pueyo and Couso, 2011). However, Pri peptides may also have Svb-independent functions, since *svb* mutants do not display the tracheal defects observed in the absence of *pri* function (Kondo et al., 2010).

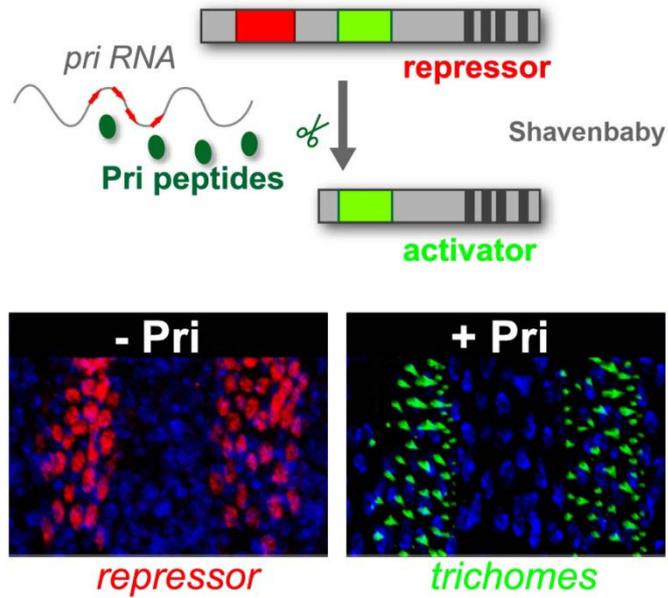


Figure 26: Pri smORF peptides switch the transcriptional activity of the Shavenbaby transcription factor. As observed in stage-15 embryos, the absence of *pri* leads to the accumulation of the large-sized Svb repressor (detected using an antibody specific for the N-terminal region, in red) that prevents the expression of Svb target effectors and thus of trichome formation. Nascent trichomes (in green) are revealed using an antibody directed against Dusky-like, a Zona Pellucida protein encoded by a downstream target gene of Svb. Nuclei are in blue. Adapted from Kondo *et al.* (2010).

At the beginning of my PhD work, the team had discovered the key role of Pri peptides in regulating the transcriptional activity of the Shavenbaby transcription factor (Kondo et al., 2010). While previous work has provided mechanistic insights into the molecular mode of action of Pri peptides (Kondo et al., 2010), as further deciphered in great details recently by our team (Zanet et al., 2015), the developmental function of this complex regulatory mechanism remained unknown. The rationale for synthesizing a transcription repressor, then expressing small peptides that elicit its processing into a shorter activator, and ultimately the expression of effectors of epidermal differentiation was unclear.

Because the timing of epidermal trichome differentiation is not primarily determined by the expression dynamics of the Shavenbaby TF, but instead relies on the onset of *pri* expression that turns ON Svb transcriptional activity, elucidating the control of *pri* expression might help to better understand the logics of this system. In addition, it might be also informative to unravel the wide range of *pri* functions across tissues and throughout the successive stages of the *Drosophila* lifecycle.

Therefore, my research program has focused on the functional definition of the cis-regulatory regions responsible for the control of *pri* expression during *Drosophila* development, with the aim of disclosing the mechanisms of their activity. This work has contributed to demonstrate the notion that *pri* is a direct target of the ecdysone pathway and, thereby, implements systemic hormonal control within genetically encoded programs for the temporal control of *Drosophila* development.

Main data are presented within two articles, the results of which will be briefly further discussed in the last part of my manuscript.

Results

6. Pri peptides are mediators of ecdysone for the temporal control of development

▪ Résumé

Le développement animal requiert un contrôle précis, à la fois dans l'espace et le temps, de l'expression du génome. Si nous disposons aujourd'hui de nombreuses informations concernant l'établissement des patrons spatiaux d'expression à travers différents tissus, les mécanismes assurant le contrôle temporel du développement restent mal connus. Dans ce travail, nous montrons que les peptides Pri, codés par quatre petits cadres ouverts de lecture, sont des médiateurs directs de l'hormone stéroïde ecdysone pour le timing de programmes de développement chez la drosophile. Nous identifions une nouvelle enzyme requise pour la biosynthèse de l'ecdysone, GstE14, et montrons que l'ecdysone déclenche l'expression de *pri* pour définir la temporalité d'exécution de la différenciation des trichomes épidermiques, en contrôlant l'activité transcriptionnelle du facteur de transcription Ovo/Shavenbaby. Nous montrons que la manipulation de l'expression de *pri* est suffisante soit pour mettre en pause, soit pour anticiper, la différenciation des trichomes épidermiques. De plus, le contrôle de l'expression de *pri* par l'ecdysone n'est pas restreint à l'épiderme embryonnaire et il est aussi important dans différents tissus et stades de développement. Ensemble, ces données élaborent un cadre conceptuel pour comprendre les mécanismes moléculaires par lesquels un signal hormonal systémique coordonne des programmes de différenciation spécifiques avec le contrôle temporel du développement.

▪ Article and supplemental information

Pri peptides are mediators of ecdysone for the temporal control of development

Hélène Chanut-Delalande^{1,2,8}, Yoshiko Hashimoto^{3,8}, Anne Pelissier-Monier^{1,2}, Rebecca Spokony⁴, Azza Dib^{1,2}, Takefumi Kondo^{3,9}, Jérôme Bohère^{1,2}, Kaori Niimi⁵, Yvan Latapie^{1,2,10}, Sachi Inagaki⁵, Laurence Dubois^{1,2}, Philippe Valenti^{1,2}, Cédric Polesello^{1,2}, Satoru Kobayashi³, Bernard Moussian⁶, Kevin P. White⁴, Serge Plaza^{1,2}, Yuji Kageyama^{5,7,11} and François Payre^{1,2,11}

Animal development fundamentally relies on the precise control, in space and time, of genome expression. Whereas we have a wealth of information about spatial patterning, the mechanisms underlying temporal control remain poorly understood. Here we show that Pri peptides, encoded by small open reading frames, are direct mediators of the steroid hormone ecdysone for the timing of developmental programs in *Drosophila*. We identify a previously uncharacterized enzyme of ecdysone biosynthesis, *GstE14*, and find that ecdysone triggers *pri* expression to define the onset of epidermal trichome development, through post-translational control of the Shavenbaby transcription factor. We show that manipulating *pri* expression is sufficient to either put on hold or induce premature differentiation of trichomes. Furthermore, we find that ecdysone-dependent regulation of *pri* is not restricted to epidermis and occurs over various tissues and times. Together, these findings provide a molecular framework to explain how systemic hormonal control coordinates specific programs of differentiation with developmental timing.

Recent studies have established that eukaryotic genomes express a wide variety of long non-coding RNAs (reviewed in refs 1,2). Small open reading frames (smORFs) are still pervasive among long non-coding RNAs, and a growing body of evidence^{3,4} indicates that at least some of them are actually translated into peptides⁵⁻⁷. However, the function and mechanistic roles of smORF-encoded peptides are largely unknown.

The *polished rice* (*pri*, also known as *mlpt* or *tal*) RNA encodes four smORF peptides (11–32 amino acids) that regulate various developmental steps across insect species⁸⁻¹¹. In *Drosophila* embryos, the absence of *pri* results in severe epidermal defects^{9,11}, preventing the formation of actin-rich cell protrusions, called trichomes¹². Previous work has shown that trichome patterning relies on a transcription factor, Shavenbaby (Svb, also known as Ovo), that specifies which subset of epidermal cells form trichomes¹³⁻¹⁷. Svb directly activates the expression of cell effectors including actin regulators^{18,19}, cuticle components¹⁸⁻²⁰ and zona pellucida extracellular proteins^{19,21}, collectively responsible for trichome formation. We previously found that Pri peptides are required for the post-translational maturation

of Svb, switching its activity from a repressor to an activator of transcription²². The developmental function of this sophisticated mechanism remained poorly understood.

Here, we report that *pri* defines when the Svb-driven trichome program is executed, at distinct developmental points, in response to ecdysone signalling. From a genetic screening, we isolated a previously uncharacterized gene, *Glutathione S transferase E14* (*GstE14*), indispensable for trichome formation. We find that *GstE14* is required for ecdysone biosynthesis from dietary cholesterol, and show that ecdysone controls the transcription of *pri*, across different tissues and developmental times. The ecdysone steroid hormone has classically been shown to provide a systemic control on developmental timing^{23,24}. Recent work further demonstrated that its production integrates various inputs from the internal and external milieu, including tissue growth²⁵, body size²⁶, nutritional uptake²⁷ and light²⁸. The ecdysone–Pri–Svb circuit thus represents a molecular elucidation of how a global signal implements local differentiation programs, at defined points, to synchronize development with the environment.

¹Centre de Biologie du Développement, Université de Toulouse, UPS, 31062 Toulouse cedex 9, France. ²CNRS, UMR5547, Centre de Biologie du Développement, Toulouse, 31062 cedex 9, France. ³Okazaki Institute for Integrative Bioscience, National institutes of Natural Sciences Okazaki, Aichi 444-8787, Japan. ⁴Institute for Genomics and Systems Biology and Department of Human Genetics, University of Chicago, Illinois 60637, USA. ⁵Research Center for Environmental Genomics, Organization of Advanced Science and Technology, Kobe University, Kobe 657-850, Japan. ⁶Animal Genetics, Interfaculty Institute for Cell Biology, University of Tübingen, 72076 Tübingen, Germany. ⁷Department of Biology, Graduate School of Science, Kobe University, Kobe 657-850, Japan. ⁸These authors contributed equally to this work. ⁹Present address: RIKEN Center for Developmental Biology, Kobe 650-0047, Japan. ¹⁰The authors would like to dedicate this article to the memory of Y. Latapie (1980–2014), whose life was taken by an avalanche in the Pyrenees.

¹¹Correspondence should be addressed to Y.K. or F.P. (e-mail: kageyama@ruby.kobe-u.ac.jp or francois.payre@univ-tlse3.fr)

Received 7 April 2014; accepted 15 September 2014; published online 26 October 2014; DOI: 10.1038/ncb3052

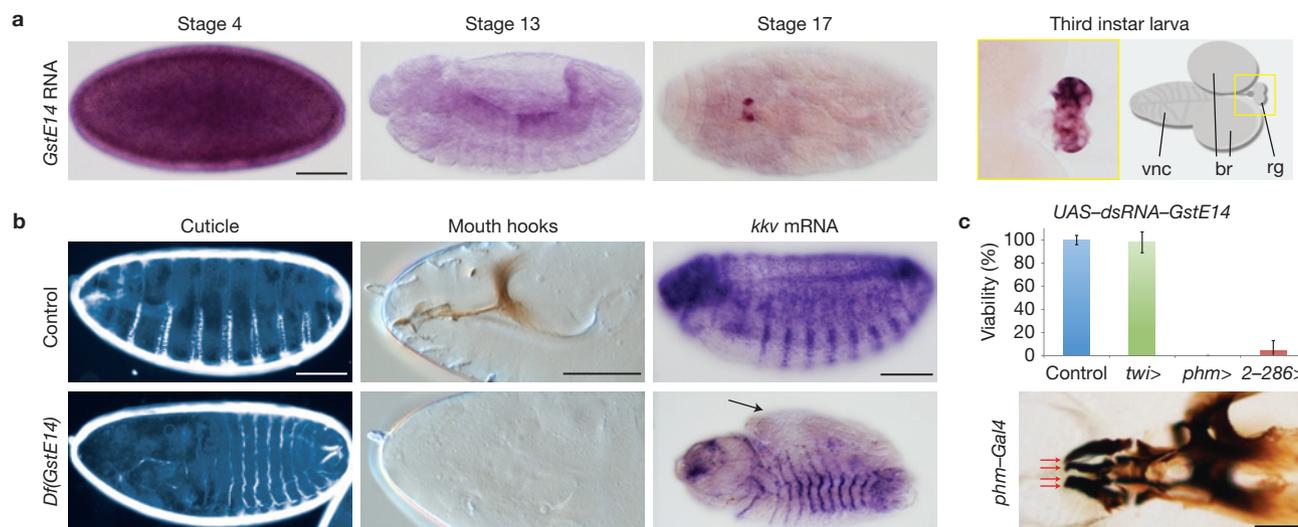


Figure 1 *GstE14* encodes a component of the ecdysone pathway. (a) *In situ* hybridization to *GstE14* mRNA shows ubiquitous expression in early cellularized embryos (st-4). Following weak signal throughout mid-embryogenesis (st-13), *GstE14* is specifically expressed in the ring gland at later stages (st-17). Dissected third instar larval brain shows *GstE14* expression in the ring gland, as previously noticed for other genes involved in ecdysone synthesis. vnc, ventral nerve chord; br, brain; rg, ring gland. Scale bar, 100 μ m. (b) *GstE14* mutant embryos show strongly affected embryonic development, with severe defects collectively reminiscent of the phenotype of Halloween mutants^{33,34,37,40,41}. These defects include poorly differentiated cuticle (left panels), defective head involution and mouth hooks (middle), and the abnormal expression pattern of *kkv* (right) encoding chitin synthase, a main player of cuticle formation. *GstE14* mutants also often show defects in dorsal closure (arrow). Scale bars, 100 μ m. (c) *GstE14* function in the larval ring gland is required for developmental transitions and adult viability. When compared with control (*phm* > *GFP*, green

fluorescent protein gene), silencing *GstE14* in the ring gland (*phm* > *UAS-dsRNA-GstE14*; *UAS*, upstream activating sequence) leads to developmental arrest at larval stages, with animals ultimately dying before pupation. *phm* > *GstE14* larvae are often blocked at the L2-L3 transition, showing characteristic double mouth hooks (four hooks instead of two, red arrows). Scale bar, 10 μ m. A similar, slightly weaker, phenotype is observed with the *2-286-Gal4* line also driving expression in the ring gland. In contrast, *GstE14* knockdown in mesodermal derivatives (*twi-Gal4*) does not affect embryonic and post-embryonic development. The graph plots percentage of viability in each condition, using two different RNA interference lines (*v101884* and *v40316*), with mean values representing three experiments. Error bars are s.d. The total numbers of counted individuals are the following: *phm-Gal4* > *UAS-GFP*, *n* > 1,000; *twi-Gal4* > *UAS-dsRNA-GstE14*, *n* > 1,000; *phm-Gal4* > *UAS-dsRNA-GstE14*, *n* = 624; *2-286-Gal4* > *UAS-dsRNA-GstE14*, *n* = 154 (see Supplementary Table 2 for further details).

RESULTS

GstE14 functions for ecdysone biosynthesis

To identify further regulators of epidermal differentiation, we conducted a genetic screen by systematically assaying a set of small deletions, each removing a molecularly defined genomic region and collectively representative of the whole second chromosome (approximately 30% of the genome; Supplementary Table 1). The Dusky-like (Dyl) Zona Pellucida protein provided a suitable readout, as it localizes in growing trichomes and is required for their formation²¹. Our screening identified a gene, *GstE14*, as an important player in trichome formation because its absence leads to a complete lack of Dyl staining (Supplementary Fig. 1). *GstE14* encodes an epsilon class glutathione *S*-transferase (Supplementary Fig. 2), an insect-specific enzyme that has not been studied yet in any species^{29,30}. We thus sought to analyse the developmental function of *GstE14* in more detail.

Unexpectedly, *GstE14* is not expressed in epidermal cells. Instead, *GstE14* messenger RNA accumulates in the ring gland from mid-embryogenesis, where it persists throughout larval stages (Fig. 1). The ring gland is an endocrine organ that produces ecdysone, the main steroid hormone in insects²⁴. Ecdysone is synthesized from cholesterol, through a series of enzymes including *Nvd* (refs 31,32), *Spo* (ref. 33), *Spok* (ref. 33), *Sro* (ref. 34), *Phm* (ref. 35), *Dib* (ref. 36) and *Sad* (ref. 37) (Fig. 2), all specifically expressed in the ring gland^{24,38}.

Inactivation of any of these genes leads to a characteristic phenotype, called Halloween because of poorly differentiated cuticle^{33,34,37,39-41}, as also manifest in embryos lacking *GstE14* (Figs 1b and 2a). These results therefore suggested that *GstE14* is involved in ecdysone synthesis, which we further investigated.

A main role for ecdysone is the timing of post-embryonic development, triggering larval moults and the larval-pupal transition²⁴. To investigate whether *GstE14* contributes to ecdysone function, we knocked down *GstE14* specifically in the ring gland (Supplementary Table 2). Reduced levels of *GstE14* lead to arrested development at larval stages (Fig. 1c). We observed larvae arrested at the transition from the second to the third instar (Fig. 1c), a phenotype characteristic of defective ecdysone signalling⁴². Finally, we found that treatment with ecdysone suppresses the embryonic lethality of *GstE14* mutants (Supplementary Fig. 3), therefore demonstrating that *GstE14* is required for ecdysone production.

Ecdysone triggers the differentiation of embryonic epidermal cells

Having established the role of *GstE14* in ecdysone synthesis, we next examined how compromised ecdysone production can explain the observed epidermal defects.

We found that the lack of *GstE14* prevents the expression of ecdysone-responsive genes in embryonic epidermal cells

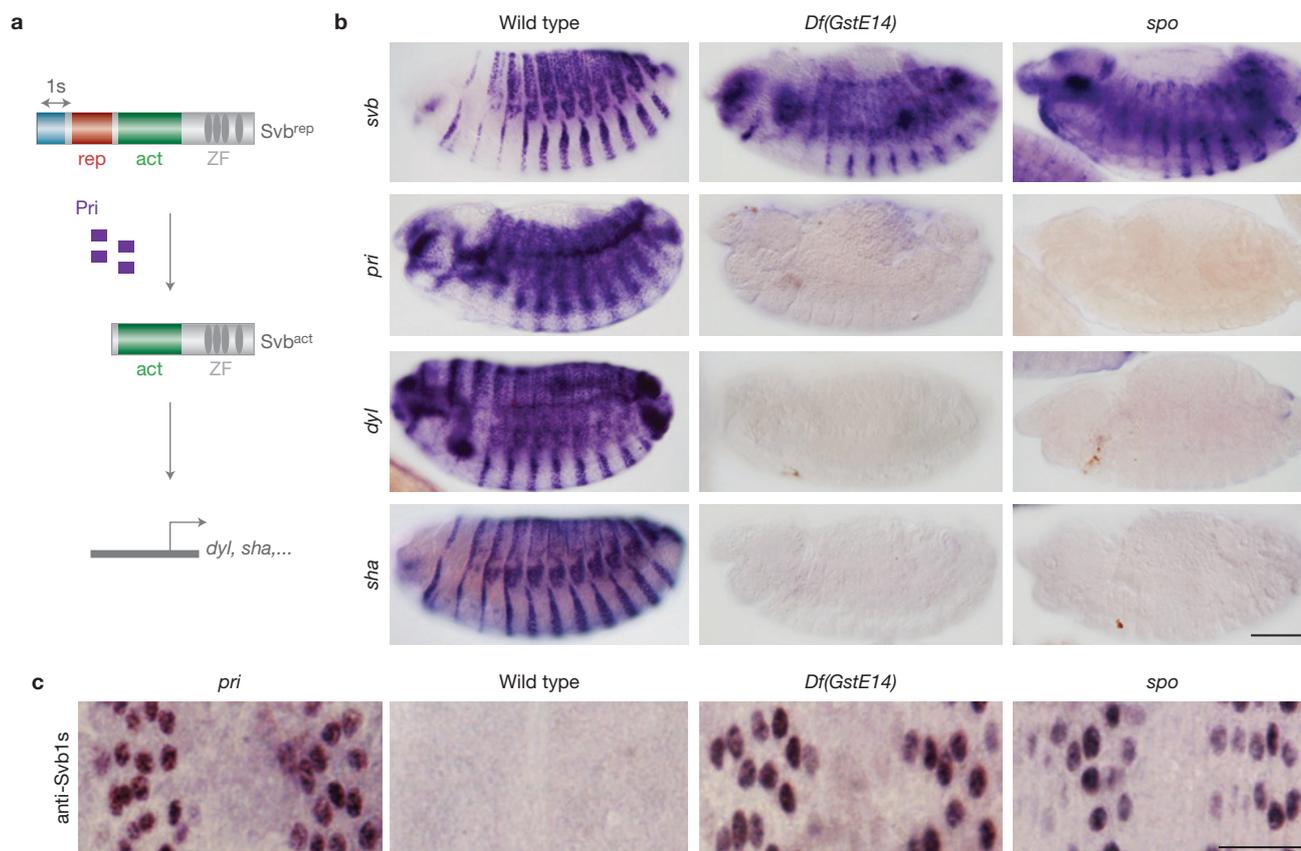


Figure 3 The ecdysone hormone is required for *pri* expression and Svb maturation in trichome cells. **(a)** *pri* encodes four small peptides that trigger a proteolytic maturation of the Svb transcription factor, switching its activity from a repressor to an activator. Activated Svb, in turn, triggers the expression of direct effectors of trichome formation, including *dyl* and *shavenoid* (*sha*). The red and green boxes schematize the repressor and activator protein regions, respectively. **(b)** Whole mount

in situ hybridization of *svb*, *pri*, *dyl* and *sha* mRNA in wild-type, *Df(GstE14)* and *spo* embryos (scale bar, 100 μ m). **(c)** Consistent with the decrease in *pri* mRNA levels that is observed in *Df(GstE14)* or *spo* mutant embryos, ecdysone is required for the proper maturation of Svb, as demonstrated by staining with anti-Svb1s, an antibody that specifically recognizes the long repressor form of Svb **(a)**. Scale bar, 15 μ m.

Svb needs to be activated by a proteolytic release of its amino-terminal region, triggered by Pri peptides²². Once matured, Svb activates the transcription of various cell effectors^{18–20}, including *dyl* and *shavenoid* (*sha*) (Fig. 3a). To define at which step(s) the ecdysone pathway was required, we evaluated the consequences of the lack of 20E on the different players of trichome formation.

In situ hybridization revealed a striking reduction in *dyl* mRNA levels, in both *spo* and *GstE14* mutant embryos (Fig. 3b), consistent with the absence of protein staining (Supplementary Fig. 1). Furthermore, the absence of 20E also resulted in the downregulation of other Svb targets. For example, whereas *sha* mRNA is normally high in trichome cells, it is strongly reduced in *GstE14* or *spo* mutants (Fig. 3b). This general failure in the expression of Svb target genes was suggestive of impaired *svb* function. Significant amounts of *svb* mRNA, however, were detected in mutant embryos (Fig. 3b). In contrast, we found a strong decrease in *pri* mRNA levels in *GstE14* embryos, as well as in *spo* and *phantom* (*phm*) mutants (Fig. 3b and Supplementary Fig. 4a). These results show that ecdysone controls the expression of *pri*, suggesting that the lack of trichomes observed in the absence of 20E results from impaired activation of

the Svb protein, which relies on *pri* activity²². Indeed, whereas Svb is fully matured in stage-15 wild-type embryos, the repressor form of Svb persists and prevents target gene expression in *pri* mutants²². Similarly, the repressor form of Svb accumulates in *GstE14* and *spo* mutants (Fig. 3c), confirming a lack of *pri* function in the absence of 20E.

Taken together, these results indicate that ecdysone is a requisite for *pri* expression in the embryonic epidermis and, thereby, for trichome formation.

Pri expression in the epidermis is sufficient to overcome ecdysone depletion

Ecdysone regulates gene expression following binding to, and activation of, the nuclear ecdysone receptor^{47–49} (EcR). The maternal contribution, however, hampers analysis of EcR zygotic function during embryogenesis⁴³, and the requirement of EcR for oogenesis prevents generation of embryos lacking EcR activity⁵⁰. Because of these genetic limitations, we undertook a combination of assays to investigate the role of EcR for *pri* expression.

We found that EcRDN, an ecdysone-blind EcR variant that acts as a constitutive repressor⁵¹, was sufficient to repress *pri* expression

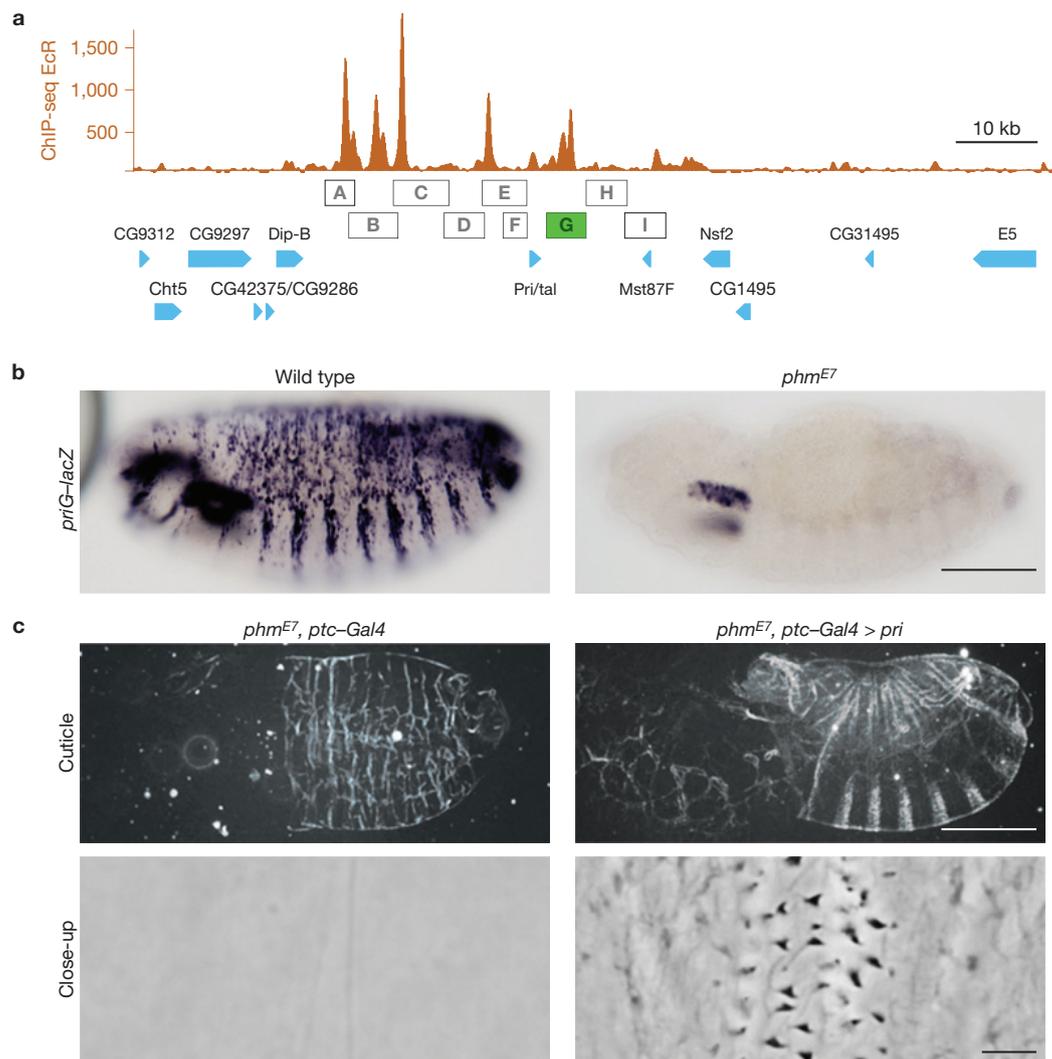


Figure 4 The ecdysone pathway drives epidermal remodelling through the regulation of *pri* transcription. **(a)** Representation of the *pri* genomic region, showing the intensity of ChIP-seq signal (arbitrary units, brown) for sequences bound by EcR. Providing internal control for specificity, EcR binding is restricted to regions flanking the *pri* transcriptional unit, when compared with neighbouring sequences. Similar EcR binding profiles are also seen for known direct targets of EcR, such as Hr46 and Blimp-1 (Supplementary Fig. 6a). Genes are drawn with blue arrows and boxes schematize genomic regions carried by transgenic *lacZ* reporter constructs. **(b)** The *pri-G-lacZ* construct

(green box in **a**) drives strong expression in the embryonic epidermis. Epidermal expression of *pri-G* is abolished in the absence of ecdysone, as observed in *phm^{E7}* mutant embryos. **(c)** Cuticle preparations showing the *phm* phenotype, characterized by a poorly differentiated cuticle and the absence of trichomes. Restoring *pri* expression in the epidermis, by means of *ptc-Gal4 > UAS-pri*, is sufficient for a significant rescue of trichome formation in the absence of ecdysone. Upper panels show lateral views of whole larvae and lower panels show ventral views of A4 segments. Scale bars are 100 μ m, except for cuticle close-ups, where it represents 15 μ m.

(Supplementary Fig. 5a). EcRDN provoked strong defects when expressed in the epidermis, including a thin cuticle and lack of trichomes. Similar defects were observed on expression of an enzyme that inactivates ecdysteroids⁵², indicating that both the hormone and its receptor are required in epidermal cells for trichome formation (Supplementary Fig. 5).

We then used genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) to identify the set of EcR binding sites. We observed strong ChIP peaks at the *pri* locus, showing that EcR binds *in vivo* to presumptive *pri* cis-regulatory sequences (Fig. 4a). ChIP peaks were also detected in known direct targets of EcR, but not in later ecdysone-responsive genes that are not directly regulated by EcR (Supplementary Fig. 6a).

To assess the transcriptional properties of *pri* genomic regions, we generated a systematic series of β -galactosidase (*lacZ*) reporters, including all EcR-binding regions (Fig. 4a). This analysis identified an enhancer, *pri-G*, driving a strong expression that mimics the endogenous *pri* pattern in the embryonic epidermis (Fig. 4b). Further evidence supported that *pri* is under direct control of ecdysone signalling. First, *pri-G* contains *in vivo* binding sites for EcR (Fig. 4a). Second, the activity of *pri-G* requires ecdysone, as its expression was abolished in *phm* mutants (Fig. 4b). Finally, ecdysone is also required for *pri* expression in cultured cells, probably in an EcR-dependent manner, as we deduced from recent genome-wide data⁵³.

Collectively, these data suggested that, on 20E binding, EcR directly activates *pri* transcription, thereby inducing *Svb* maturation

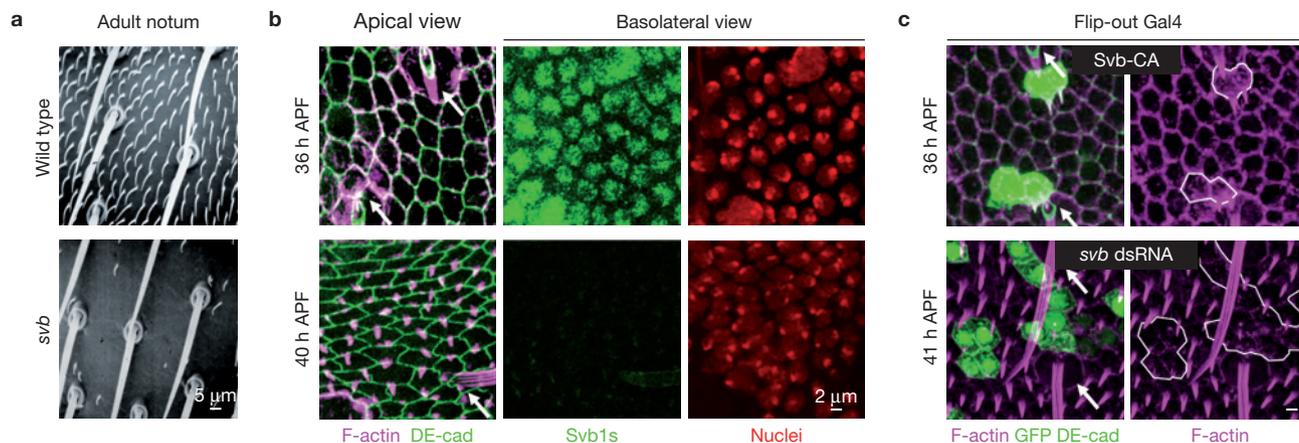


Figure 5 *Svb* specifies the differentiation of trichomes in the pupal notum. (a) Scanning electron micrographs of adult notum in wild type (top) and *svb* mutant (bottom). (b) Confocal microscopy pictures of presumptive adult epidermal cells at different times of differentiation APF. Left panels show apical views of pupal cells stained for F-actin (magenta) and DE-cadherin (green). Whereas at 36 h APF (top) microfilaments are mainly associated with cell junctions, growing trichomes are easily visible from 40 h APF, when F-actin bundles accumulate at the posterior vertex of each cell. In the meantime, basolateral optical sections show accumulation of the Svb repressor form, revealed by anti-Svb1s staining (green), in epidermal cell nuclei (red) at 36 h APF. Trichome formation coincides with the timing of

Svb maturation at 40 h APF (disappearance of anti-Svb1s signal). Sensory bristles are marked with white arrows. (c) Mosaic clones of cells labelled by GFP (green cytoplasm) co-stained for DE-cadherin (green cell contours) and F-actin (magenta). Upper panels show cell clones expressing Svb-CA at 36 h APF. In lower panels, clones of GFP-positive cells at 41 h APF express a dsRNA inactivating *Svb* function and thereby preventing trichome formation. White outlines in F-actin panels indicate the locations of manipulated cells. All pictures are shown with the anterior at the top, using the same magnification for each immunostaining panel. A typical experiment contains from five to ten notum samples. Each experiment has been reproduced at least three times.

in epidermal cells. This model predicts that restoring *pri* expression should compensate for impaired ecdysone signalling, a hypothesis we tested experimentally. Indeed, the re-expression of *pri* in *phm* mutant embryos was sufficient to restore cuticle differentiation and, notably, to rescue trichome formation (Fig. 4c).

These data thus establish that a main role of ecdysone signalling, in epidermal cells, is mediated by the control of *pri* expression.

Pri times the expression of trichome effectors

Given the key role of ecdysone in developmental timing²⁴, we next tested whether *Pri* peptides provide temporal control of the trichome program.

Whereas *Svb* starts accumulating as the long-form repressor in presumptive trichome cells at stage 11/12, the epidermal expression of *pri* is turned on later (stage 13/14), coinciding with *Svb* maturation and abutting the onset of effector expression (Supplementary Fig. 7). The dynamics of *pri* expression is thus consistent with a response to 20E (refs 49,54), as also supported by comparison with 20E-responsive genes (Supplementary Fig. 6b,c). If *pri* acted as a timing mediator, the trichome program should be paused without this signal, a prediction congruent with all alterations of the trichome gene network observed in the absence of *pri* (Fig. 3). Reciprocally, one would expect that the premature expression of *pri* should trigger a faster onset of the trichome program. To test this prediction, we forced early *pri* expression in dorsal cells and found that it is sufficient to induce premature expression of trichome effectors. This was probably due to precocious activation of *Svb*, as we observed similar results with a constitutively active form of *Svb* (*Svb*-CA) that behaves as a *pri*-independent activator (Supplementary Fig. 7).

Hence these results support a model whereby *pri* mediates 20E-dependent temporal control of trichome effector expression.

Pri is a temporal regulator of epidermal morphogenesis

To further test the notion that *pri* mediates the temporal control of epidermis development, we assayed its role in a later wave of epidermal differentiation that occurs for adult tissues. *Svb* is required for adult trichomes (Fig. 5a), and the inactivation of *Svb* embryonic targets also causes adult trichome defects (Supplementary Fig. 8). Focusing on the dorsal thorax (notum), we thus explored the timing of adult epidermal cell remodelling, which takes longer than embryogenesis and thus enables more detailed analyses of temporal hierarchies.

Whereas the differentiation of sensory organs is already seen at 30 h after puparium formation (APF), epidermal cells keep a smooth apical surface, with F-actin at cell junctions, as illustrated at 36 h APF (Fig. 5b). Trichome differentiation begins at 38–39 h APF, and actin-rich extensions are easily visible at 40 h APF (Fig. 5b). Interestingly, the formation of trichomes accurately coincides with the timing of *Svb* maturation. The uncleaved *Svb* repressor accumulated from early pupation to 36 h APF. The repressor form of *Svb* was no longer detected once cells developed apical extensions (Fig. 5b), whereas *svb* activity was cell-autonomously required for their formation (Fig. 5c).

These results argue that the trichome program is paused in notum cells until 38–39 h APF, through the repressor form of *Svb*. Hence, the expression of *pri* might provide a timing cue to turn on the trichome program in notum cells. We tested this hypothesis in a series of experiments. First, *in situ* hybridization revealed a strong but transient expression of *pri* throughout the notum at 40 h APF, whereas it was not detected at 36 h APF and was no longer seen at 44 h APF (Fig. 6a). It is worth noting that this time window matches the strongest peak of ecdysone throughout the life cycle^{23,55}. Second, young notum cells are competent for differentiating, as *Svb*-CA enabled the premature

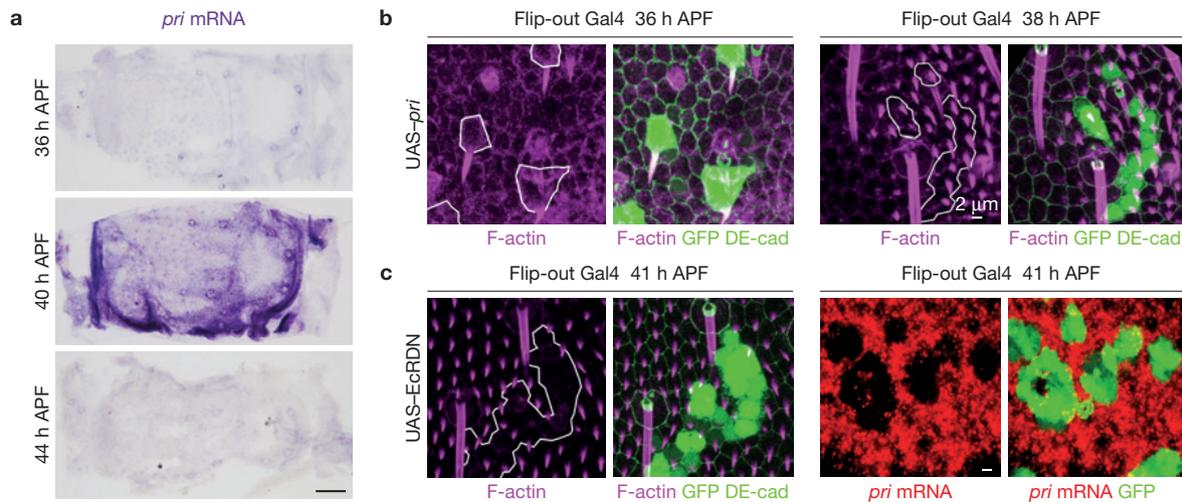


Figure 6 Pri mediates ecdysone-dependent temporal control of epidermal trichome differentiation. (a) *In situ* hybridization of whole notum dissected from wild-type pupae at different developmental times APF for *pri* mRNA (purple). Pri is transiently expressed in epidermal cells, in a time window that matches the timing of Svb maturation. Anterior is to the left; scale bar, 100 μ m. (b) Premature expression of *pri* is sufficient to trigger the formation of ectopic trichomes in differentiating epidermal cells. Mosaic clones (*flpGal4* > *UAS-pri*) are visualized by cytoplasmic GFP (green), F-actin is in magenta and the cell contour is outlined by DE-cadherin (green). Left images show

individual cell clones and right panels larger clones. (c) Mosaic clones of cells expressing EcRDN are unable to produce trichomes (left panels; F-actin is in magenta; GFP and DE-cadherin are in green). As revealed by fluorescent *in situ* hybridization, this results from the inhibition of *pri* mRNA expression in GFP-positive cells that express EcRDN (green, in right panels). All pictures in **b,c** panels are oriented with the anterior at the top, using the same magnification. White outlines highlight the location of clones in F-actin panels. All experiments have been reproduced at least three times, with five to ten dissected samples of the same genotype per experiment.

formation of trichomes at 36 h APF (Fig. 5c). Third, we found that precocious expression of *pri* can force premature execution of the trichome program (Fig. 6b). Indeed, single cells that artificially express *pri* produce long precocious trichomes. In larger clones, *pri* also induces premature trichomes in neighbouring cells (Fig. 6b), a non-cell-autonomous effect consistent with previous observations^{9,11}. Therefore, manipulating *pri* expression is sufficient to trigger untimely trichome differentiation.

Next, we tested whether the timing of *pri* expression in the notum depends on ecdysone signalling. We found that expressing EcRDN in notum cells prevents trichome formation (Fig. 6c). As observed in embryos, this is likely to be due to the inhibition of *pri* expression, because we observed a strong decrease in *pri* levels in EcRDN cells when compared with their wild-type neighbours (Fig. 6c).

We therefore conclude that Pri peptides act to provide, at successive steps of development, tight temporal regulation to the transcriptional program of trichomes.

Ecdysone-dependent control of *pri* expression at developmental transitions

An important question was to determine whether or not this regulatory mechanism is generalizable to other *pri*-dependent processes, that is whether Pri peptides play a broader role in ecdysone control of developmental timing. To address this question, we focused on the transition that occurs between larval stages and pupation, the paradigm of ecdysone action in insects²⁴.

We observed marked temporal changes in whole-body levels of *pri* mRNA, which matched well with ecdysone dynamics (Fig. 7a,b). There is a sharp pulse of *pri* at 2–4 h APF, that is following a major ecdysone peak, and then a second wave of expression at 10–12 h

(Fig. 7a,b). Periodic pulses of *pri* expression were also manifest within individual tissues, for example in the leg imaginal disc, where *pri* regulates morphogenesis of adult tarsal segments^{9,10}. Following a weak pattern at mid-third-instar, *pri* expression shuts down in late larval leg discs. The expression of *pri* then bursts across a broader region of the disc at pupariation, and fades again at later stages (Fig. 7c). As in the epidermis, we found that *pri* expression is regulated by ecdysone in the leg primordium. *In vitro* incubation of larval discs with 20E further showed that ecdysone is sufficient to induce *pri* expression (Fig. 7c). Finally, the expression of EcRDN in post-embryonic tissues blocks metamorphosis⁵¹, and the co-expression of *pri* in these conditions is sufficient to rescue pupal lethality (Fig. 7d and Supplementary Table 4).

Taken together, these data show that the expression and function of *pri*, across embryonic and postembryonic development, rely on its temporally specific direct regulation by ecdysone.

CONCLUSIONS

Periodic pulses of steroid hormones orchestrate the timing of organism-wide developmental transitions^{23,24}, as is well illustrated by moulting cycles in invertebrates. The main steroid hormone in insects, ecdysone (20E), is synthesized from cholesterol through the action of seven P450 enzymes^{33–37,41} and a Rieske protein³¹. This work identifies an unexpected extra enzyme, GstE14, which similarly to other members of the pathway is specifically expressed in the ring gland. GstE14 might participate in either early stages of 20E synthesis³⁴ or cholesterol homeostasis^{56,57}. Indeed, silencing GstE14 alters cholesterol levels (Supplementary Fig. 3), and a mutant (*noppera-bo*) that inactivates GstE14 shows defects in cholesterol transport and metabolism⁵⁸. Importantly, the absence of GstE14 can be compensated

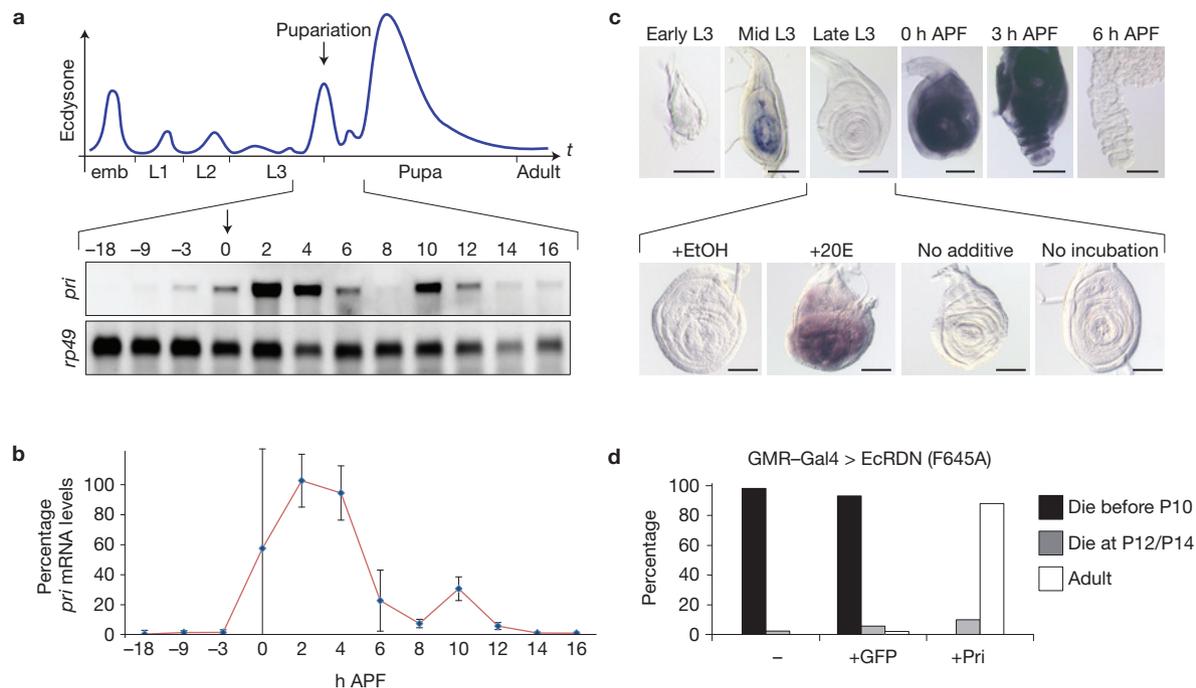


Figure 7 Ecdysone controls *pri* expression at the larval-pupal developmental transition. **(a)** Northern blot analysis of *pri* mRNA during prepupal stages in wild-type animals. A strong induction of *pri* expression is detected between 2 and 4 h APF, then a weaker peak appears at 10–12 h APF. *rp49*, encoding the ribosomal protein L32, is shown as a control. **(b)** Estimated relative levels of *pri* mRNA, as measured from three independent northern blot replicates. Error bars are s.d. **(c)** *In situ* hybridization shows dynamics of *pri* expression in leg discs at the onset of metamorphosis (upper panels). Lower panels show *in situ* hybridization of *pri* mRNA in late-third-instar larvae treated with 20E dissolved in ethanol. Discs with or without *in vitro* culture, or treated by ethanol only (EtOH), were used as controls. Scale bar, 100 μ m. **(d)** *pri* counteracts the lethality induced by EcRDN expression

during metamorphosis. UAS-EcRDN (a construct expressing EcR-B2 isoform with the F645A point mutation⁵¹) was driven in photoreceptor cells by glass multimer reporter (GMR)-Gal4, alone or in combination with UAS-GFP, for control, and in combination with UAS-Pri. Whereas EcRDN causes developmental arrest at prepupal-pupal transition, before stage 10 (P10), or later (stage P12–P14), the simultaneous expression of *pri* restores a large proportion of flies that reach adulthood. The total number of counted individuals are the following: *GMR-Gal4*, 3,035; *GMR-Gal4 > UAS-GFP*, 1,743; *GMR-Gal4 > UAS-pri*, 5,013. Similar results were obtained with each of two other EcR isoforms (EcR-A and EcR-B1) and dominant negative mutations (F645A and W650A). See Supplementary Table 4 for further information and data.

by feeding animals a high-cholesterol diet (Supplementary Fig. 3 and Table 2), showing the importance of the external milieu for the temporal regulation of development. Although decisive progress has been made on how ecdysone synchronizes whole-body development with varying environmental conditions^{24,27}, little is known about the mechanisms implementing this systemic control within developmental programs.

Our results unravel a scheme of interlocking molecular events explaining how ecdysone instructs the timing of trichome development, from reception of the hormone down to the terminal effectors of differentiation (Fig. 8). In response to ecdysone pulses, the EcR receptor directly activates the transcription of *pri*, providing a key temporal control on epidermis differentiation. Before the ecdysone signal, the whole transcriptional program that specifies trichome spatial patterning has been progressively assembled by hard-wired genetic interactions^{13,45}, but it is kept on hold through expression of the Svb repressor. Pri peptides thus time the conversion of Svb from a repressor to an activator²², which, in turn, triggers the onset of trichome effector expression¹⁸. The same mechanisms are used during embryogenesis and metamorphosis, showing how Pri peptides mediate temporal cues for the execution of a transcriptional program, at successive steps of development.

Interestingly, we find that *pri* can act at a distance, across several cells in the notum. It has been proposed that, probably owing to their tiny size (<1.3 kDa), Pri peptides can pass from one cell to another, to explain how clones of *pri* mutant cells are rescued by wild-type neighbours^{9,11}. Reciprocally, Pri peptides expressed at high levels within manipulated cells might pass into neighbouring cells, for example through gap junctions or cytoplasmic bridges^{59,60}. Although elucidating the underlying mechanism(s) will require further work, Pri diffusion could contribute to a robust temporal output, ensuring the synchronous differentiation of adjacent cells.

Beyond epidermal development, *pri* plays important roles in other tissues^{9–11,61,62}, for example for the formation of adult appendages^{8–10,61}. Our data show that *pri* expression in the leg primordium is also regulated in a timely manner by ecdysone signalling. On the organismic scale, there is a wide upregulation of *pri* levels at the larval-pupal transition, further extending the spectrum of *pri* in mediating response to ecdysone.

We propose that a major function of Pri peptides is to synchronize different genetic programs with the ecdysone-mediated systemic control of developmental timing. These data open new ways to explore the full range of developmental functions of Pri peptides,

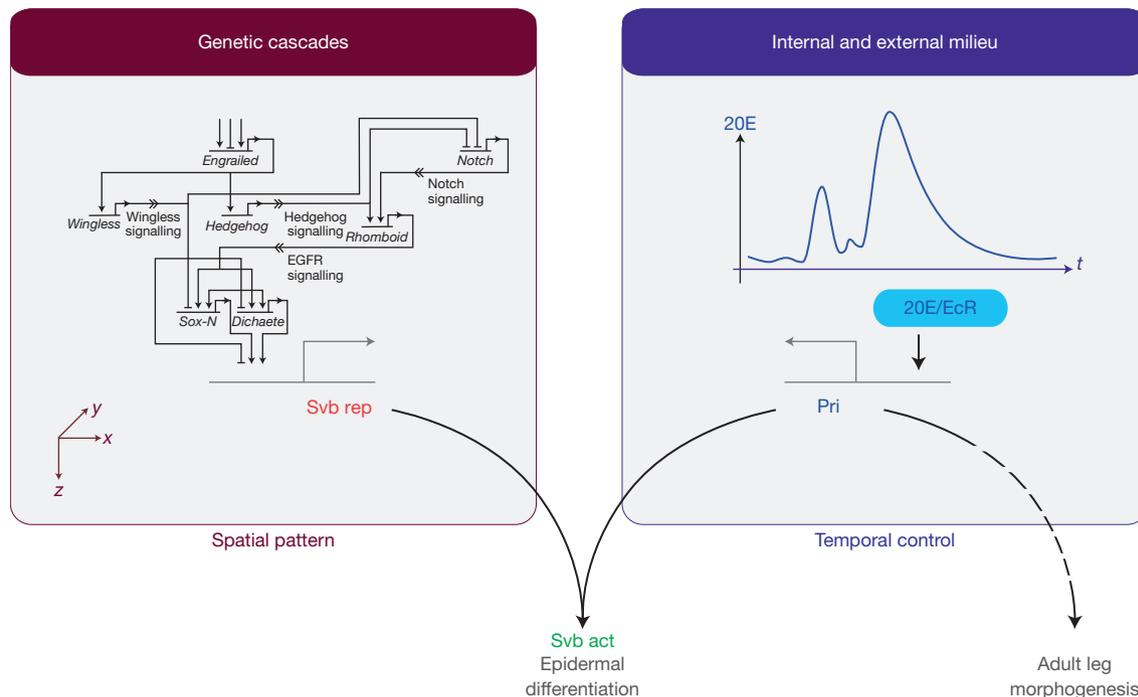


Figure 8 Role of *pri* in mediating the action of ecdysone for the temporal control of morphogenesis. *Svb* cis-regulatory regions integrate outputs from upstream transcriptional and cell signalling cascades to determine the subset of epidermal cells that have the potential to differentiate trichomes. The whole program of trichome formation is still paused by the accumulation of the *Svb* large-sized repressor. In response to both internal (whole-body development, tissue growth) and external inputs (nutrients, light), pulses in ecdysone levels promote EcR-dependent activation of *pri* expression. *Pri* peptides then induce the maturation of *Svb*, leading in turn to the direct transcriptional activation of *Svb* effectors and, thereby, to the morphological

differentiation of epidermal trichome cells. *Pri* thus constitutes a key mediator of ecdysone action for the temporal control of epidermal differentiation. In response to ecdysone, the expression of *pri* also pulses across extra tissues, at successive times throughout the larval–pupal transition. Temporal dynamics of *pri* mRNA includes a 20E-dependent strong burst within the adult leg primordium, where *pri* controls morphogenesis of tarsal segments. *Pri* peptides therefore provide a molecular framework to explain how systemic hormonal signalling implements the temporal execution of different hard-wired genetic programs, throughout embryonic and post-embryonic development.

as well as the mechanisms regulating the temporal control of development. □

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary Information is available in the online version of the paper

ACKNOWLEDGEMENTS

We are grateful to FlyBase and the Bloomington, Vienna and Kyoto stock centres, as well as R. Niwa, M. Kamimura and J. Colombani for providing flies, and H. Bellen for bacterial artificial chromosome constructs. We thank B. Ronsin (Toulouse RIO Imaging) for help with microscopy and O. Bohner for technical assistance. We also thank A. Khila, A. Vincent, P. Leopold and E. France for critical reading of the manuscript, and are indebted to R. Niwa for sharing unpublished results. This work was supported by ANR (smORFpeptides and Chrononet), Association pour la Recherche sur le Cancer (12011669), Azm & Saade Association, JST PRESTO program, MEXT KAKENHI (21115007) and Fondation RITC.

AUTHOR CONTRIBUTIONS

Y.K. and F.P. conceived and directed the project. Y.H. initiated the project and H.C.-D. carried out most experiments presented here. A.D., J.B., K.N., S.I., L.D., P.V. and C.P. conducted experiments and gave further helpful insights. H.C.-D., Y.H., A.P.-M., T.K., Y.L., R.S., B.M., S.K., K.P.W., S.P., Y.K. and F.P. designed the experiments, analysed data and contributed to data interpretation. H.C.-D., Y.H., Y.K. and F.P. prepared the figures and wrote the manuscript. All authors helped write and revise the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/doi/10.1038/ncb3052

Reprints and permissions information is available online at www.nature.com/reprints

- Pauli, A., Rinn, J. L. & Schier, A. F. Non-coding RNAs as regulators of embryogenesis. *Nat. Rev. Genet.* **12**, 136–149 (2011).
- Uliitsky, I. & Bartel, D. P. lincRNAs: genomics, evolution, and mechanisms. *Cell* **154**, 26–46 (2013).
- Aspden, J. L. *et al.* Extensive translation of small ORFs revealed by Poly-Ribo-Seq. *eLife* e03528 (2014).
- Ingolia, N. T., Lareau, L. F. & Weissman, J. S. Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. *Cell* **147**, 789–802 (2011).
- Hanada, K. *et al.* Small open reading frames associated with morphogenesis are hidden in plant genomes. *Proc. Natl Acad. Sci. USA* **110**, 2395–2400 (2013).
- Magny, E. G. *et al.* Conserved regulation of cardiac calcium uptake by peptides encoded in small open reading frames. *Science* **341**, 1116–1120 (2013).
- Slavoff, S. A. *et al.* Peptidomic discovery of short open reading frame-encoded peptides in human cells. *Nat. Chem. Biol.* **9**, 59–64 (2013).
- Savard, J., Marques-Souza, H., Aranda, M. & Tautz, D. A segmentation gene in *tribolium* produces a polycistronic mRNA that codes for multiple conserved peptides. *Cell* **126**, 559–569 (2006).
- Galindo, M. I., Pueyo, J. I., Fouix, S., Bishop, S. A. & Couso, J. P. Peptides encoded by short ORFs control development and define a new eukaryotic gene family. *PLoS Biol.* **5**, e106 (2007).
- Pueyo, J. I. & Couso, J. P. The 11-amino acid long Tarsal-less peptides trigger a cell signal in *Drosophila* leg development. *Dev. Biol.* **324**, 192–201 (2008).
- Kondo, T. *et al.* Small peptide regulators of actin-based cell morphogenesis encoded by a polycistronic mRNA. *Nat. Cell Biol.* **9**, 660–665 (2007).
- Chanut-Delalande, H., Ferrer, P., Payre, F. & Plaza, S. Effectors of tridimensional cell morphogenesis and their evolution. *Semin. Cell Dev. Biol.* **23**, 341–349 (2012).

13. Payre, F., Vincent, A. & Carreno, S. ovo/svb integrates Wingless and DER pathways to control epidermis differentiation. *Nature* **400**, 271–275 (1999).
14. Sucena, E., Delon, I., Jones, I., Payre, F. & Stern, D. L. Regulatory evolution of shavenbaby/ovo underlies multiple cases of morphological parallelism. *Nature* **424**, 935–938 (2003).
15. Delon, I. & Payre, F. Evolution of larval morphology in flies: get in shape with shavenbaby. *Trends Genet.* **20**, 305–313 (2004).
16. McGregor, A. P. *et al.* Morphological evolution through multiple cis-regulatory mutations at a single gene. *Nature* **448**, 587–590 (2007).
17. Frankel, N. *et al.* Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. *Nature* **474**, 598–603 (2011).
18. Menoret, D. *et al.* Genome-wide analyses of Shavenbaby target genes reveals distinct features of enhancer organization. *Gen. Biol.* **14**, R86 (2013).
19. Chanut-Delalande, H., Fernandes, I., Roch, F., Payre, F. & Plaza, S. Shavenbaby couples patterning to epidermal cell shape control. *PLoS Biol.* **4**, e290 (2006).
20. Andrew, D. J. & Baker, B. S. Expression of the Drosophila secreted cuticle protein 73 (dsc73) requires Shavenbaby. *Dev. Dyn.* **237**, 1198–1206 (2008).
21. Fernandes, I. *et al.* Zona pellucida domain proteins remodel the apical compartment for localized cell shape changes. *Dev. Cell* **18**, 64–76 (2010).
22. Kondo, T. *et al.* Small peptides switch the transcriptional activity of Shavenbaby during Drosophila embryogenesis. *Science* **329**, 336–339 (2010).
23. Thummel, C. S. Molecular mechanisms of developmental timing in C. elegans and Drosophila. *Dev. Cell* **1**, 453–465 (2001).
24. Yamanaka, N., Rewitz, K. F. & O'Connor, M. B. Ecdysone control of developmental transitions: lessons from Drosophila research. *Annu. Rev. Entomol.* **58**, 497–516 (2013).
25. Delanoue, R., Slaidina, M. & Leopold, P. The steroid hormone ecdysone controls systemic growth by repressing dMyc function in Drosophila fat cells. *Dev. Cell* **18**, 1012–1021 (2010).
26. Colombani, J. *et al.* Antagonistic actions of ecdysone and insulins determine final size in Drosophila. *Science* **310**, 667–670 (2005).
27. Andersen, D. S., Colombani, J. & Leopold, P. Coordination of organ growth: principles and outstanding questions from the world of insects. *Trends Cell Biol.* **23**, 336–344 (2013).
28. Yamanaka, N. *et al.* Neuroendocrine control of Drosophila larval light preference. *Science* **341**, 1113–1116 (2013).
29. Saisawang, C., Wongsantichon, J. & Ketterman, A. J. A preliminary characterization of the cytosolic glutathione transferase proteome from Drosophila melanogaster. *Biochem. J.* **442**, 181–190 (2012).
30. Board, P. G. & Menon, D. Glutathione transferases, regulators of cellular metabolism and physiology. *Biochim. Biophys. Acta* **1830**, 3267–3288 (2013).
31. Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H. & Niwa, R. Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. *Development* **133**, 2565–2574 (2006).
32. Yoshiyama-Yanagawa, T. *et al.* The conserved Rieske oxygenase DAF-36/ Neverland is a novel cholesterol-metabolizing enzyme. *J. Biol. Chem.* **286**, 25756–25762 (2011).
33. Ono, H. *et al.* Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. *Dev. Biol.* **298**, 555–570 (2006).
34. Niwa, R. *et al.* Non-molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the 'Black Box' of the ecdysteroid biosynthesis pathway. *Development* **137**, 1991–1999 (2010).
35. Warren, J. T. *et al.* Phantom encodes the 25-hydroxylase of Drosophila melanogaster and Bombyx mori: A P450 enzyme critical in ecdysone biosynthesis. *Insect Biochem. Mol. Biol.* **34**, 991–1010 (2004).
36. Chavez, V. M. *et al.* The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development* **127**, 4115–4126 (2000).
37. Petryk, A. *et al.* Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA* **100**, 13773–13778 (2003).
38. Huang, X., Warren, J. T. & Gilbert, L. I. New players in the regulation of ecdysone biosynthesis. *J. Genet. Genomics* **35**, 1–10 (2008).
39. Nusslein-Volhard, C., Wieschaus, E. & Kluding, H. Mutations affecting the pattern of larval cuticle in Drosophila melanogaster. I. zygotic loci on the second chromosome. *Roux Arch. Dev. Biol.* **193**, 267–282 (1984).
40. Rewitz, K. F., O'Connor, M. B. & Gilbert, L. I. Molecular evolution of the insect Halloween family of cytochrome P450s: phylogeny, gene organization and functional conservation. *Insect Biochem. Mol. Biol.* **37**, 741–753 (2007).
41. Warren, J. T. *et al.* Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of Drosophila melanogaster. *Proc. Natl Acad. Sci. USA* **99**, 11043–11048 (2002).
42. Talamillo, A. *et al.* Scavenger receptors mediate the role of SUMO and Ftz-f1 in Drosophila steroidogenesis. *PLoS Genet.* **9**, e1003473 (2013).
43. Chavoshi, T. H., Moussian, B. & Uv, A. Tissue-autonomous EcR functions are required for concurrent organ morphogenesis in the Drosophila embryo. *Mech. Dev.* **127**, 308–319 (2010).
44. Moussian, B. Recent advances in understanding mechanisms of insect cuticle differentiation. *Insect Biochem. Mol. Biol.* **40**, 363–375 (2010).
45. Payre, F. Genetic control of epidermis differentiation in Drosophila. *Int. J. Dev. Biol.* **48**, 207–215 (2004).
46. Frankel, N. *et al.* Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490–493 (2010).
47. Koelle, M. R. *et al.* The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59–77 (1991).
48. Yao, T. P. *et al.* Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature* **366**, 476–479 (1993).
49. Ruaud, A. F., Lam, G. & Thummel, C. S. The Drosophila nuclear receptors DHR3 and β FTZ-F1 control overlapping developmental responses in late embryos. *Development* **137**, 123–131 (2010).
50. Carney, G. E. & Bender, M. The Drosophila ecdysone receptor (EcR) gene is required maternally for normal oogenesis. *Genetics* **154**, 1203–1211 (2000).
51. Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E. & Cherbas, P. EcR isoforms in Drosophila: testing tissue-specific requirements by targeted blockade and rescue. *Development* **130**, 271–284 (2003).
52. Kamimura, M. *et al.* Fungal ecdysteroid-22-oxidase, a new tool for manipulating ecdysteroid signaling and insect development. *J. Biol. Chem.* **287**, 16488–16498 (2012).
53. Shlyueva, D. *et al.* Hormone-responsive enhancer-activity maps reveal predictive motifs, indirect repression, and targeting of closed chromatin. *Mol. Cell* **54**, 180–192 (2014).
54. Kozlova, T. & Thummel, C. S. Essential roles for ecdysone signaling during Drosophila mid-embryonic development. *Science* **301**, 1911–1914 (2003).
55. Riddiford, L. M. in *The Development of Drosophila melanogaster* (ed. Martinez-Arias, M. B.a.A.) 899–939 (Cold Spring Harbor Laboratory Press, 1993).
56. Fluegel, M. L., Parker, T. J. & Pallanck, L. J. Mutations of a Drosophila NPC1 gene confer sterol and ecdysone metabolic defects. *Genetics* **172**, 185–196 (2006).
57. Horner, M. A. *et al.* The Drosophila DHR96 nuclear receptor binds cholesterol and regulates cholesterol homeostasis. *Genes Dev.* **23**, 2711–2716 (2009).
58. Enya, S. *et al.* A Halloween gene *noppera-bo* encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in Drosophila. *Sci. Rep.* **4**, 6586 (2014).
59. Poodry, C. A. & Schneiderman, H. A. The ultrastructure of the developing leg of Drosophila melanogaster. *Roux Arch. Dev. Biol.* **166**, 1–44 (1970).
60. McLean, P. F. & Cooley, L. Protein equilibration through somatic ring canals in Drosophila. *Science* **340**, 1445–1447 (2013).
61. Pueyo, J. I. & Couso, J. P. Tarsal-less peptides control Notch signalling through the Shavenbaby transcription factor. *Dev. Biol.* **355**, 183–193 (2011).
62. Pi, H. *et al.* Identification of 11-amino acid peptides that disrupt Notch-mediated processes in Drosophila. *J. Biomed. Sci.* **18**, 42 (2011).

METHODS

Fly stocks and clonal analysis. Deficiency lines (Supplementary Table 1) were obtained from the Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu/>). Extra strains were *spo11/TM3,Dfd-YFP*, *phm[E7]/FM7a,Dfd-YFP* (YFP, yellow fluorescent protein), *UAS-EcRDN (EcR-A-F645A; EcR-A-W650A; EcR-B1-F645A; EcR-B1-W650A; EcR-B2-F645A; EcR-B2-W650A)* (ref. 51), *UAS-E22oxidase* (ref. 52), *Ptc-Gal4*, *pnr-Gal4*, *e22C-gal4*, *GMR-Gal4*, *UAS-svbCA* (ref. 63), *UAS-pri* (ref. 11) and *UAS-dsRNA-svb*, *UAS-dsRNA-GstE14 (v40316 and v101884*, Vienna Drosophila RNAi Center, <http://stockcenter.vdrc.at/>). To knock down *GstE14* in specific tissues, *UAS-dsRNA-GstE14* lines were crossed with *phm-Gal4*, *2-286-Gal4*, *P0206-Gal4* or *twi-Gal4* drivers. To ensure development in controlled conditions of larval density, 30 eggs were placed into food tubes, with yeast paste supplemented or not with 100 µg ml⁻¹ of cholesterol. For mosaic clones of Gal4-expressing cells in the thorax, *hs-flp; actin < y + < Gal4; UAS-GFP* flies were crossed with *UAS* lines and larvae heat shocked for 30 min at 37 °C, 8 h before puparium formation.

Screening procedure. Deficiency lines from the Bloomington Drosophila Stock Center were balanced over a *CyO-wg-LacZ* chromosome to genotype embryos. Eggs were collected overnight at 25 °C. To facilitate screening, embryos from 24 strains were treated simultaneously using the Fly Condo device (Flystuff). Embryos were dechorionated by bleach treatment, fixed in heptane saturated in formaldehyde for 40 min and subsequently devitellinized with heptane/methanol. Embryos were kept in methanol before immunostaining. Anti-Dyl immunostaining was processed in parallel for 22 samples at once, using a homemade device and standard procedures. Revelation was carried out with 3,3'-diaminobenzidine (Sigma), supplemented with nickel.

DNA constructs. The rescue construct for *GstE14* was obtained by PCR amplification of a 4.6-kilobase (kb) genomic DNA fragment, subsequently cloned into the pAttB transformation vector. LacZ reporter constructs were produced by cloning 5–6 kb DNA fragments of the *pri* genomic region into the pAttB-LacZ (ref. 18) reporter vector. All constructs were verified by sequencing. Transgenic lines, including bacterial artificial chromosome constructs (from P[acman] Resources, <http://www.pacmanfly.org/>), were generated using the PhiC31 system and inserted at the 86F position (BestGene). Further details are provided in Supplementary Table 3.

ChIP-seq experiments. EcR ChIP-seq data were generated by the modENCODE consortium and were extracted from the modMine pipeline (<http://intermine.modencode.org>). Although embryonic samples (7–10 h) gave low signal-noise ratio, we observed strong peaks for samples collected at pupal stages (5 h APF), well known for high ecdysone titres. Similar patterns of EcR binding were retrieved at later stages (33 h APF). The data have been submitted to the Gene Expression Omnibus. The accession number for combined ChIP-seq is GSM628268, and the input data are available at the accession numbers GSM628269 and GSM628270.

Embryo, larval and pupal staining. Homozygous embryos were identified by the lack of balancer chromosome (marked with GFP/YFP or LacZ). Sibling controls and mutant embryos were in all cases processed in the same batch; a typical collection includes more than 300 embryos in total. The staging of mutant embryos, subjected to *in situ* hybridization or immunohistochemistry, was determined according to the age of 2 h embryo collections. Staining was carried out as previously described²¹ using anti-Dyl (1:400), anti-Svb1s (1:3,000), anti-DE-cadherin (DCAD2, 1:100 from DSHB), AlexaFluor-488 or 555 secondary antibodies (1:1,000, Molecular Probes), biotinylated goat anti-rabbit (1:1,000, Vector Laboratories) and TRITC-phalloidin (Sigma). For anti-DE-cadherin staining, embryos were fixed for 5 min in 37% formaldehyde and devitellinized by hand. Digoxigenin (DIG)-labelled RNA antisense probes were synthesized *in vitro* from complementary DNA clones and processed for *in situ* hybridization as described¹⁹.

Staging of larvae was carried out as previously described⁶⁴. 20–30 adult flies were placed on *Drosophila* medium containing 0.05% bromophenol blue, and well-developed third instar larvae from their progeny were staged according to gut colour. White prepupae were collected and kept in humid vials until further processing for *in situ* hybridization or northern blot. Pupal thoraces were dissected in PBS-Tween 0.1%, fixed for 20 min in 4% paraformaldehyde and processed for immunostaining or *in situ* hybridization⁶⁵. Each mosaic animal showed clones of mutant cells surrounded by wild-type neighbouring cells, providing excellent internal controls. A typical experiment contains five to ten dissected samples, of proper stage and genotype. Data have been collected in at least three independent experiments. Samples were imaged with a Nikon 90i fluorescent microscope or a Zeiss 710 confocal microscope.

Ecdysone and cholesterol treatment on embryos and cuticle preparation. Embryos collected from deficiency lines or individual mutants, placed over *CyO-Wg-LacZ* or *TM3-Dfd-LacZ* balancers, were processed for 5-bromo-4-chloro-3-indolyl-β-D-galactoside staining for genotyping and cuticles were prepared in Hoyer's-lactic acid (1:1). For steroid rescuing assays, mutant chromosomes were balanced over *CyO-Dfd-YFP* or *TM3-Dfd-YFP*. Embryos were dechorionated and permeabilized with heptane for 5 min, then incubated with 25 mg ml⁻¹ of 20E, ecdysone or cholesterol (Sigma) diluted in Schneider's medium (Sigma) for 1 h, under mild agitation. Following incubation, embryos were covered with Voltalef oil and placed in a moisture chamber at 25 °C for further development. Mutant larvae were selected under a stereomicroscope equipped for epifluorescence and processed for cuticle preparation. Each rescue experiment has been carried out, independently, at least three times.

In vitro culture of leg imaginal discs and ecdysone treatment. Wandering mid-third-instar larvae were dissected in Schneider's medium and staged by salivary gland morphology as previously described⁶⁶. Dissected staged larvae without guts and ring glands were incubated at 22 °C for 17 h with rotation (198 r.p.m.), in 200 µl of Schneider's medium supplemented with 5 µM 20E (Sigma) dissolved in ethanol, or the corresponding amount of ethanol as a control. After incubation, samples were washed with Schneider's medium once and with PBS three times, fixed in 4% paraformaldehyde and subjected to *in situ* hybridization as described above.

Northern blot analysis. Whole-body RNA was isolated from staged animals using RNeasy (Qiagen) or Isogen (Nippon Gene). RNA was separated by formaldehyde-agarose gel electrophoresis and then transferred to a nylon membrane (Roche). Hybridization and wash procedures were carried out according to the DIG Application Manual (Roche). DIG-labelled *pri* probes¹¹ were reacted with an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and visualized with chemiluminescence using CSPD (Tropix) or CPD-Star (Roche), and LAS-1000 or LAS 4000mini (GE Healthcare). For quantification, northern blots of three independent collections (five animals were of each staged sample) were quantified with LAS 4000mini and normalized by the co-electrophoresed internal control (5 pg of dsDNA plasmid including the *pri* probe region). Expression levels are represented as percentages of the maximum intensity, with mean values and standard deviations being plotted in the graph.

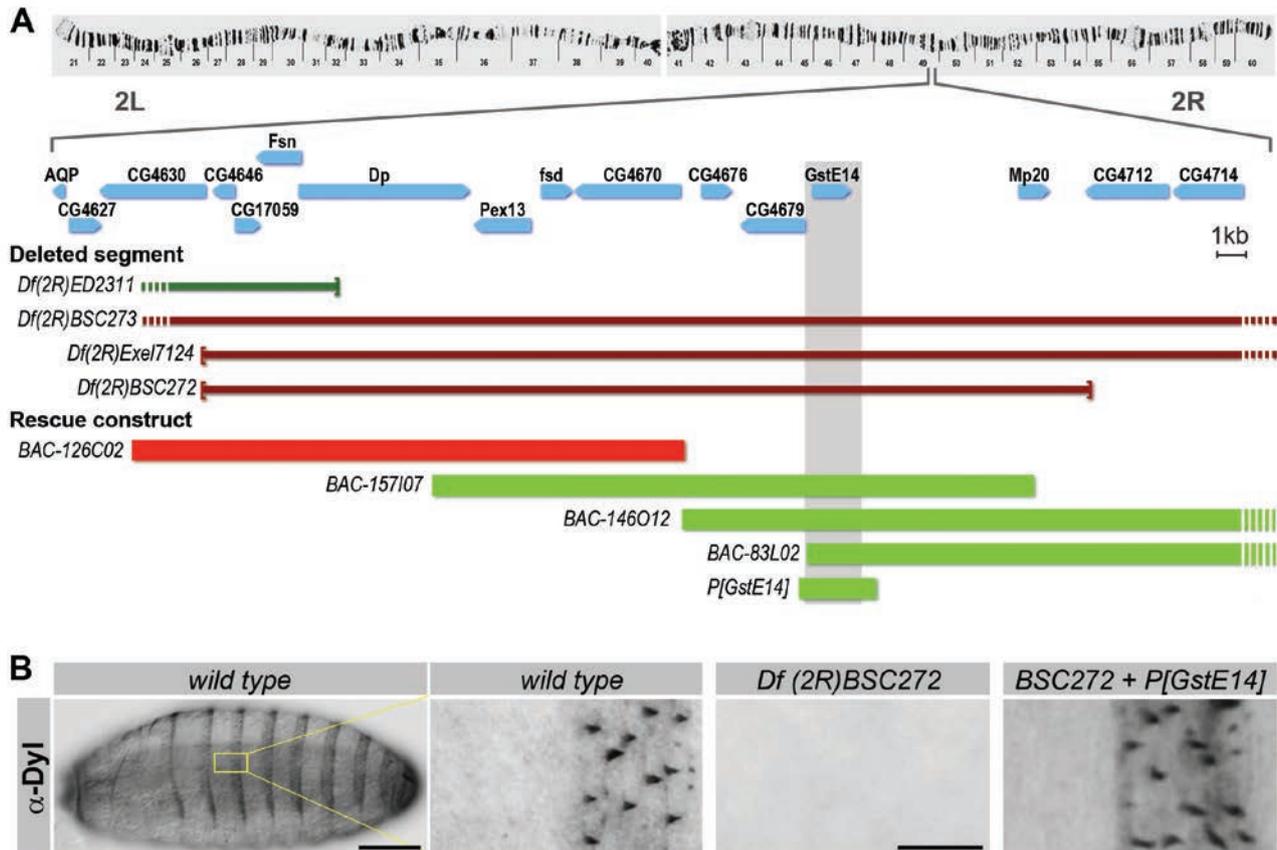
Transmission electron microscopy. Embryos were cryo-immobilized in a high-pressure freezer (Bal-Tec HPM 010, Balzers). Samples were transferred to 2% osmium tetroxide, 0.5% uranyl acetate and 0.5% glutaraldehyde in anhydrous acetone at -90 °C for 32 h at -60 °C and 4 h at -40 °C. After washing with acetone, samples were transferred into an acetone-EPON mixture at -30 °C (1:1 for 4 h, 1:2 for 12 h), warmed up to room temperature, infiltrated with EPON (three changes within 30 h) and polymerized at 60 °C for 48 h. Ultrathin sections (70 nm) were stained with 2% uranyl acetate in 70% methanol for 10 min, and in 0.4% lead citrate in 0.1 N NaOH for 2 min, and viewed in a Philips CM10 electron microscope at 60 kV.

Sterol quantification in embryos and larvae. Total cholesterol levels were determined using the Amplex Red Cholesterol Assay Kit (Invitrogen), as previously described^{36,57}. For each assay, we collected 100 embryos, either of wild-type or *Df(GstE14)* mutant genotype, the latter being selected by the lack of GFP-marked balancer. After washing, embryos were homogenized in 150 mM NaCl, 50 mM Tris at pH = 7.5, 2 mM EGTA, adjusted to a concentration of 100 mg ml⁻¹, and the homogenate was clarified by centrifugation at 2,700g for 5 min. Supernatant aliquots were used to assay sterol content according to the kit instructions and measured using a spectral fluorometer (Victor 3; Perkin Elmer), including a gradual series of cholesterol samples as internal standard. The same protocol was used for wild-type and *phm > UAS-dsRNA-GstE14* larvae, in this case with extracts prepared from 30 individuals aged for 4 days after egg laying. For each sample, sterol levels were expressed as percentages of matched wild-type levels. Experiments were repeated at least three times. Statistical analyses used nonparametric Mann-Whitney tests (two tailed).

Cholesterol diet assay. Parental rescue experiments were carried out using *Df(GstE14)/CyO-Dfd-YFP* adult flies fed for two days in tubes containing normal medium supplemented with 200 ng ml⁻¹ of cholesterol, as described³⁶. Then, fed flies were placed on agar plates to collect eggs. Homozygous individuals, selected from the absence of YFP, were transferred to culture tubes for the rest of development. *Df(GstE14)/CyO-Dfd-YFP* flies fed without cholesterol and *Spo/TM6b-Dfd-GFP* fed on high cholesterol were used as controls. The larval stages raised by rescued

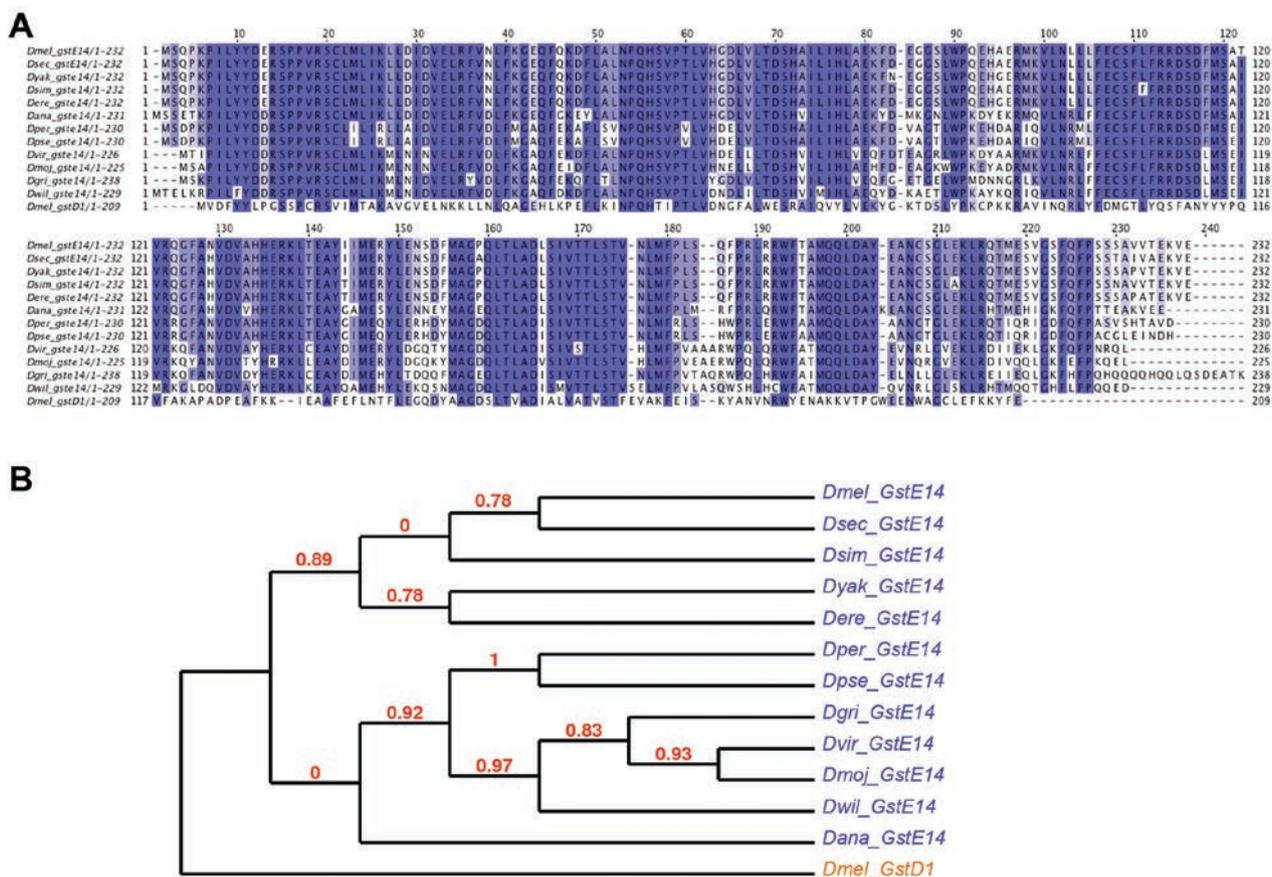
animals were determined according to the morphology of their mouth hooks. Experiments were repeated at least three times, and all data are reported as the mean with standard deviation. Statistical analyses used nonparametric Mann–Whitney tests (two tailed).

63. Delon, I., Chanut-Delalande, H. & Payre, F. The Ovo/Shavenbaby transcription factor specifies actin remodelling during epidermal differentiation in *Drosophila*. *Mech. Dev.* **120**, 747–758 (2003).
64. Andres, A. J. & Thummel, C. S. Methods for quantitative analysis of transcription in larvae and prepupae. *Methods Cell Biol.* **44**, 565–573 (1994).
65. Founounou, N., Loyer, N. & Le Borgne, R. Septins regulate the contractility of the actomyosin ring to enable adherens junction remodeling during cytokinesis of epithelial cells. *Dev. Cell* **24**, 242–255 (2013).
66. Dunne, J. C., Kondylis, V. & Rabouille, C. Ecdysone triggers the expression of Golgi genes in *Drosophila* imaginal discs via broad-complex. *Dev. Biol.* **245**, 172–186 (2002).



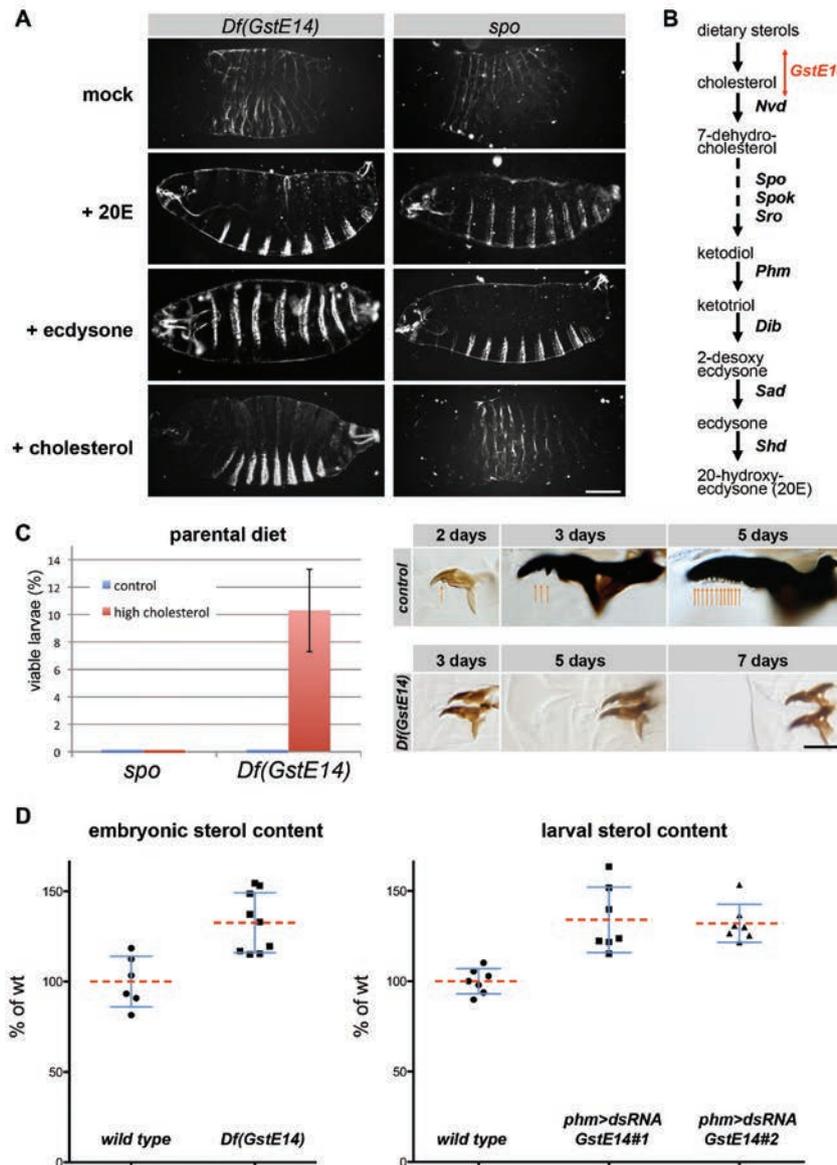
Supplementary Figure 1 *GstE14* is required for Dusky-like expression in trichome cells. **A.** Schematic representation of the second chromosome of *Drosophila melanogaster*, focusing on the cytogenetic position 49F10-F13 and associated genes (blue arrows). From all lines we tested in this screen (see Supplementary Table 1), we observed a complete absence of Dyl staining only in the two overlapping deletions *Df(2R)BSC273* and *Df(2R)Exel7124* (dark red). A neighbouring deletion with unaffected Dyl expression (*Df(2R)ED2311*) is in dark green. A secondary screening with a smaller deficiency, *Df(2R)BSC272*, restricted the genetic interval to 9 genes. To identify the responsible gene(s), we generated a series of transgenic lines carrying BAC genomic constructs (see Supplementary Table 3) and assayed their rescuing

activity when reintroduced in the *Df(2R)BSC272* background. While *BAC-126C02* (red box) did not restore Dyl staining, *BAC-157I07*, *-146O12* and *-83L02* (light green boxes) fully rescued Dyl expression in *Df(2R)BSC272* embryos. Since the three latter regions share a single gene, *GstE14*, we generated a construct narrowed down to a 4.6kb DNA fragment encompassing only this locus (*P[GstE14]*). **B.** As observed for rescuing BACs, *P[GstE14]* was sufficient to fully rescue Dyl expression within trichomes, as seen in stage-15 embryos (ventral views). Of note, *P[GstE14]* also suppressed the embryonic lethality observed for homozygous *Df(2R)BSC272* mutants. Rescuing assays have been performed in at least three independent experiments. Scale bars are 100 μ m (whole embryo) and 20 μ m for closeup pictures.



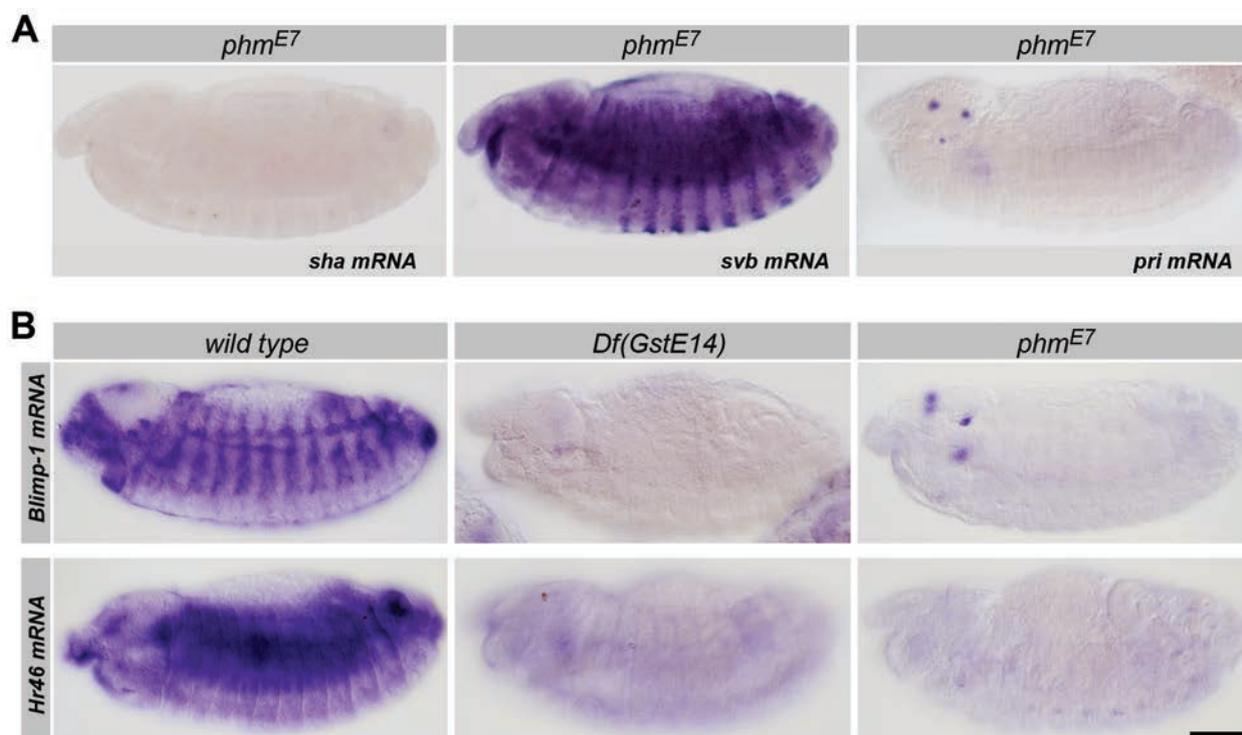
Supplementary Figure 2 *GstE14* encodes an insect-specific *Glutathione S transferase*. **A.** Alignment of GstE14 protein sequences across *Drosophila* species. *Dmel*, *Drosophila melanogaster*; *Dsec*, *Drosophila sechellia*; *Dyak*, *Drosophila yakuba*; *Dsim*, *Drosophila simulans*; *Dere*, *Drosophila erecta*; *Dana*, *Drosophila ananassae*; *Dper*, *Drosophila persimilis*; *Dpse*, *Drosophila pseudoobscura*; *Dvir*, *Drosophila virilis*; *Dmoj*, *Drosophila mojavensis*; *Dgrim*, *Drosophila grimshawi*; *Dwil*, *Drosophila willistoni*. **B.** Cladogram

showing the distribution of GstE14 sequences within *Drosophila* species. The GstD1 protein from *Drosophila melanogaster* was introduced as outgroup. Protein sequences were extracted from flybase (<http://flybase.org>), multiple alignment, curation, phylogenetic tree reconstruction and rendering were processed using ClustalW2 (<http://www.ebi.ac.uk>), and MUSCLE, Gblocks, PhyML, TreeDyn packages available at <http://www.phylogeny.fr>.



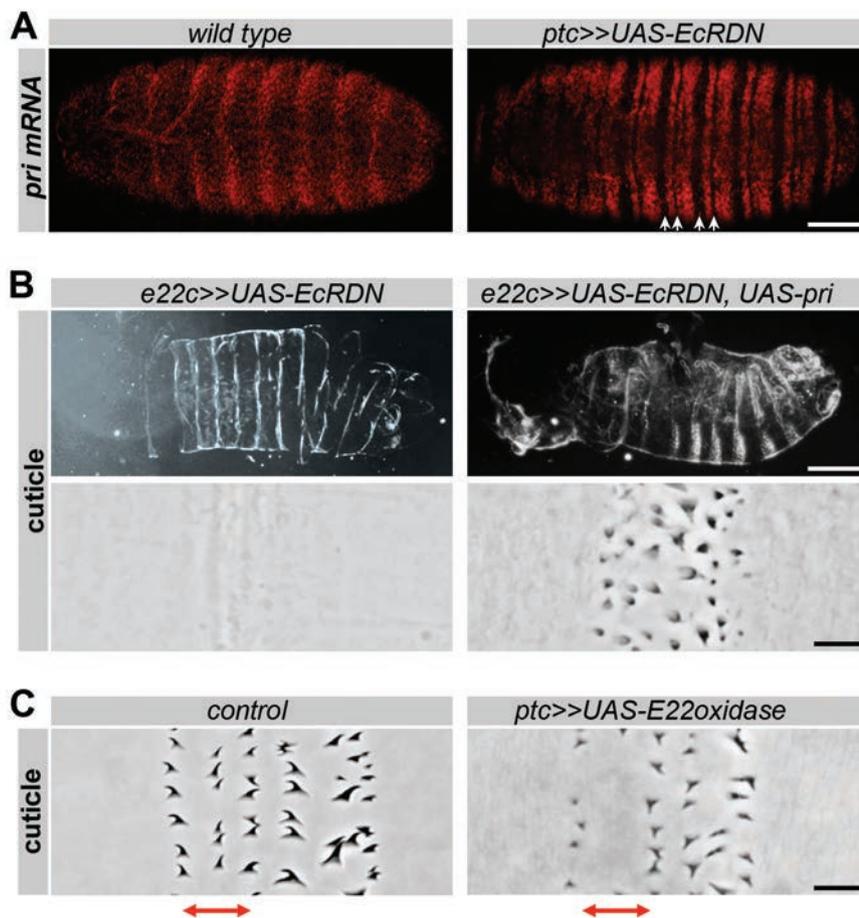
Supplementary Figure 3 *GstE14* functions in cholesterol metabolism. **A**. Cuticle preparation of *Df(GstE14)* and *spo* mutant embryos incubated in Schneider's medium supplemented with either 20E, ecdysone or cholesterol during mid-embryogenesis. Incubation with Schneider's medium alone (mock) was used as control. All three compounds significantly suppressed embryonic lethality, as well as rescued epidermal differentiation, *i.e.* cuticle differentiation and trichome formation, for *Df(GstE14)* mutants. In contrast, *spo* mutants were rescued by the exogenous addition only of 20E and ecdysone, but not by cholesterol, consistently with the documented requirement of *spo* activity for the transformation of 7-dehydro-cholesterol to ketodiol³³. Scale bar is 100 μ m. **B**. Schematic representation of the biosynthetic pathway leading to ecdysone production from dietary sterols. As deduced from rescuing experiments, *GstE14* activity is required for the very early stages of the pathway, since its lack can be rescued by cholesterol. **C**. High cholesterol diet of parental flies suppresses the embryonic lethality of *GstE14* mutants, allowing a dramatic increase in life span. *Df(GstE14)/CyoDfdYFP* and *spo/TM3DfdYFP* heterozygous flies were fed for two days with high cholesterol diet, or regular food medium for control, and transferred to egg collection devices. Parental high-cholesterol diet led to the survival of approx 10% of *Df(GstE14)* mutants, which hatched into viable L1 larvae. The experiments have been made four times independently. The total number of mutant embryos analyzed is 422

individuals for *GstE14* and >1000 for *spo*. Rescued larvae displayed no obvious morphological defects when compared to wild type larvae. Although these animals remained alive for several days (up to 7 days), they failed to proceed for pupariation, or even larval stage transitions, and instead remained long-lived L1 larvae as deduced from the examination of mouth hooks, a phenotypical marker of larval stages. Arrows highlight the number of mouth hook teeth in wild type, which displays a characteristic increase across larval stages. The chart plot means values, for three independent experiments. Errors bars are s.d., scale bar is 25 μ m. **D**. Inactivation of *GstE14* impinges on whole body cholesterol levels, both in embryos and in larvae. The sterol content of *Df(GstE14)* mutant embryos, and larvae driving *UAS-dsRNA-GstE14* (line #1: HMJ21555; line #2 v1018884) in the ring gland (*phm-Gal4*) was assessed using a commercial assay. When compared to wild type controls, *GstE14* embryos display higher levels of sterol (P value= 0.0028). The same was true for *phm>dsRNA-GstE14* larvae (P value = 0.0006), showing that *GstE14* activity in the ring gland is required for maintaining proper cholesterol levels. Extracts were made from hand-counted embryos or larvae, with 1 to 5 independent samples of the same genotype *per* experiment. All experiments have been repeated independently three times. The graph shows all data points. Statistical tests used two-tailed Mann Whitney tests, error bars are s.d. (blue), means are indicated by a red dotted line.



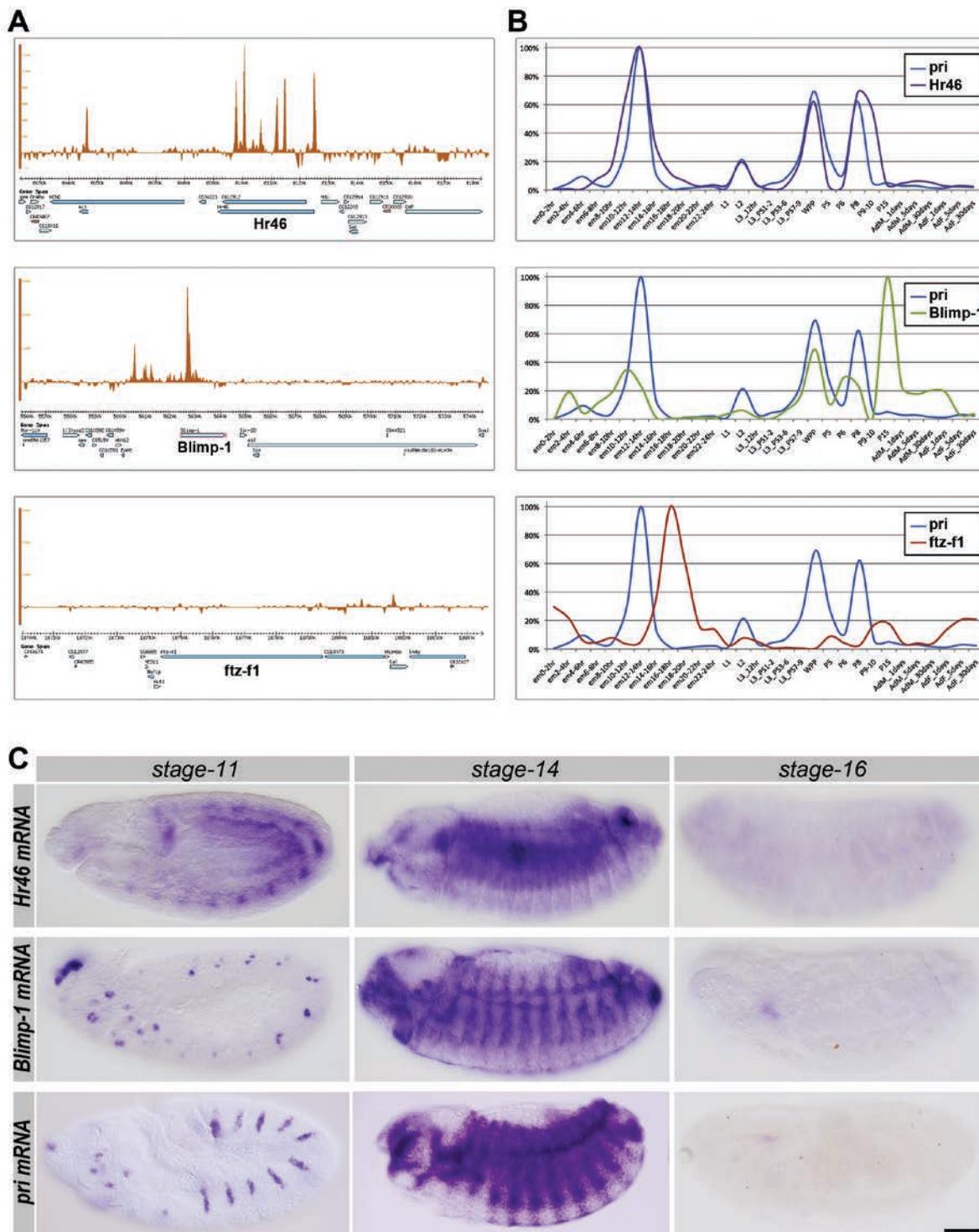
Supplementary Figure 4 Regulatory interactions within the ecdysone signalling pathway. **A.** *phm^{E7}* mutant embryos that are defective in 20E production (see Fig. 2B) show a strong down-regulation in the epidermal expression of *sha* and *pri* mRNAs. In contrast, *svb* mRNA remains expressed at normal-looking

levels in *phm^{E7}* mutants. **B.** *In situ* hybridization showing that *GstE14* activity is required for the embryonic expression of early ecdysone-responsive genes, such as *Blimp-1* and *Hr46*. These defects mimic the reduction of *Blimp-1* and *Hr46* expression observed in *phm^{E7}* mutant embryos. Scale bars are 100 μ m.



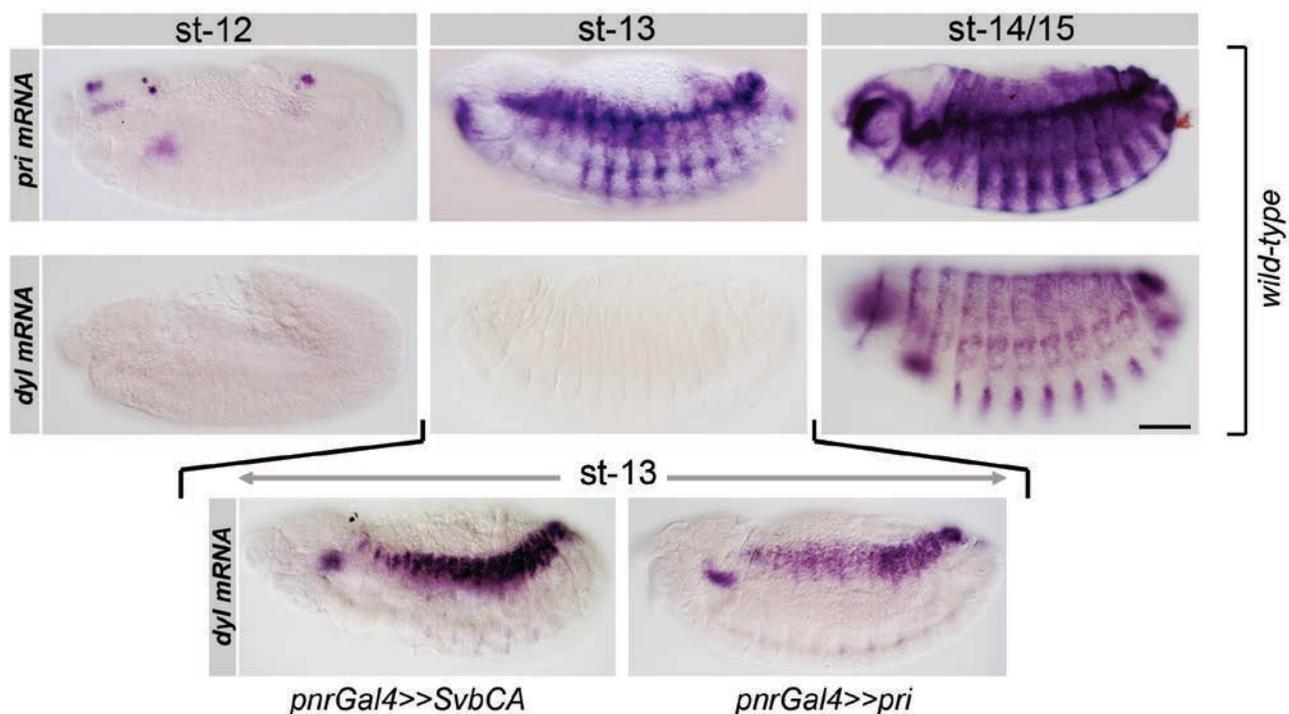
Supplementary Figure 5 Ecdysone signalling is required for trichome formation. **A.** Expression of EcRDN driven by *ptc-Gal4* in epidermal cells represses *pri* expression (right panel) compared to wild type embryos (left panel). White arrows highlight the reduction of *pri* expression in *ptc* cells. **B.** Cuticle of first instar larvae expressing EcRDN alone (left), or in combination with *pri* (right), throughout embryonic epidermal cells (using the *e22cGal4* driver). *Pri* over-expression allows a significant suppression

of EcRDN-induced epidermal defects, including the rescue of misshapen trichomes. Upper panels are lateral view of whole larvae, lower panels ventral views of A3-A4 segments. **C.** The enzymatic inactivation of ecdysone in epidermal cells, using *UAS-E22oxidase* driven by *ptc-Gal4*, prevents trichome formation in corresponding cells (red arrows). Scale bars are 100 μ m for pictures of whole embryos (A) and cuticles (B), and 10 μ m for higher magnification (B and C).



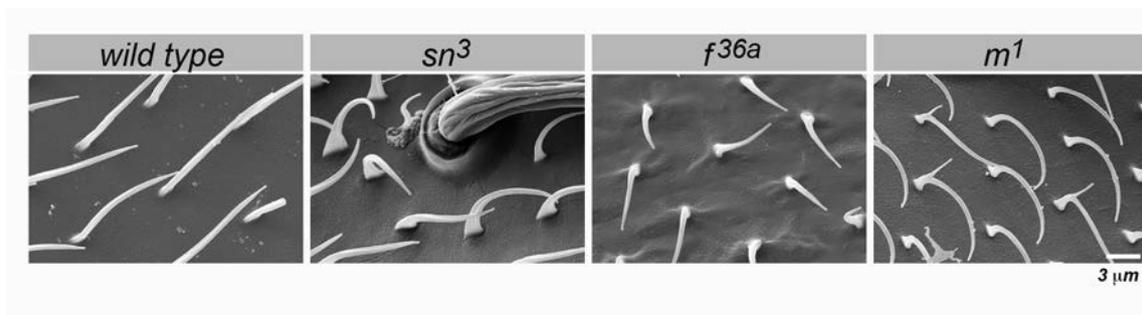
Supplementary Figure 6 *pri* is an early ecdysone-responsive gene. **A.** Snapshots of genomic regions encompassing the ecdysone-responsive genes *Hr46*, *Blimp-1* and *ftz-f1*, showing *in vivo* EcR binding events (4h APF) visualized by the intensity of ChIP-seq signal (brown). Genomic coordinates and gene position are indicated within an approx 150kb window. **B.** Dynamics of relative mRNA levels, extracted from modENCODE Temporal expression Data (mRNA-Seq). Throughout the *Drosophila* life cycle, *pri* displays temporal variations that strikingly parallels the ecdysone-responsive *Hr46* gene, and

correlates to a lesser extent to *Blimp-1*. In contrast, the temporal dynamics of *ftz-f1* mRNA levels appears clearly delayed, when compared to *pri* expression. **C.** *In situ* hybridization to *Hr46*, *Blimp-1* and *pri* mRNAs in wild type embryos, from stage-11 to stage-16. While their expression is restricted to a limited number of cell patches in early stages (stage-11), the three genes display a concomitant onset of their expression in embryonic epidermal cells at stage-14. Later on, the expression fades and only residual signal is detected at stage-16. All embryos are shown at the same magnification. Scale bar is 100 μ m.



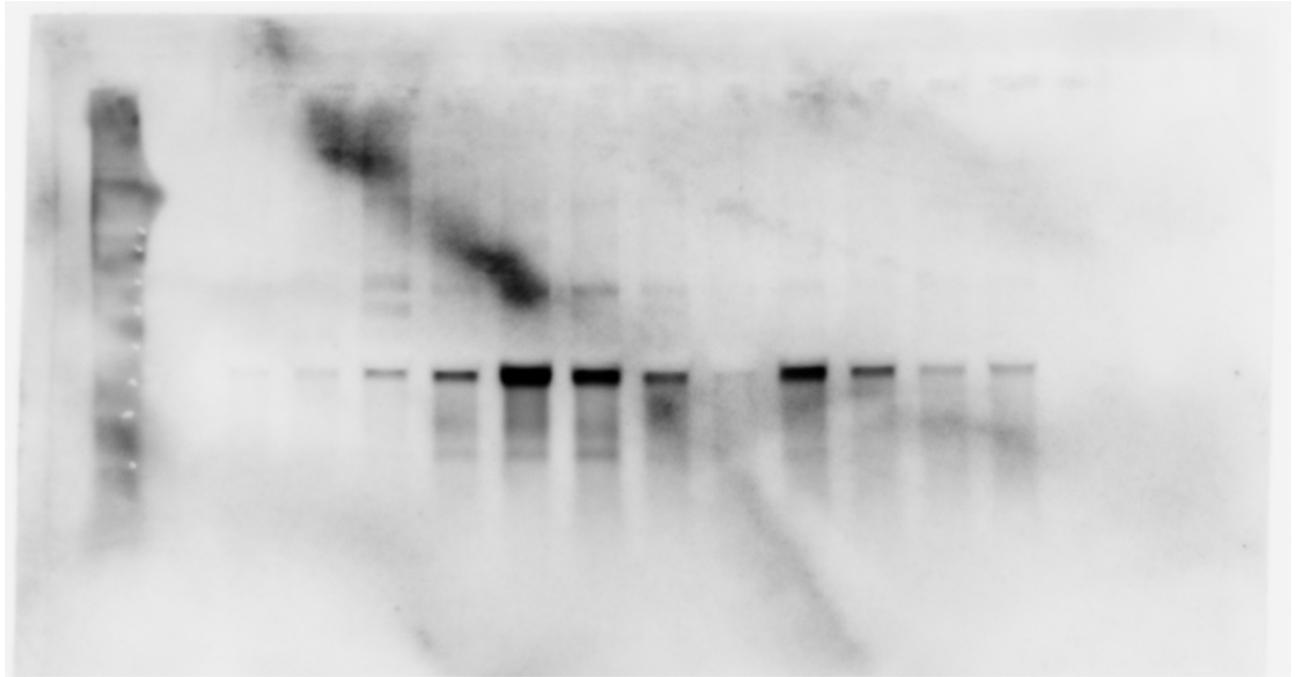
Supplementary Figure 7 Premature expression of trichome effectors during embryogenesis. *In situ* hybridization to *pri* and *dyl* mRNA show dynamics of their epidermal expression in wild type embryos, with an onset at stage-13 and stage-14/15, respectively. The precocious expression of *pri*, triggered by the early *pnr-Gal4* driver, induces premature *dyl* expression in *pnr* dorsal cells, showing that *pri* controls the temporal onset of trichome effectors in epidermal cells. Similar results were observed when driving a constitutively activated form of Svb (SvbCA), further demonstrating that *pri* expression normally times the onset of Svb activation, and thereby, the whole program of trichome formation. Of note, this artificial advance in the onset of trichome effector expression was nevertheless not sufficient

to induce premature trichomes, indicating that embryonic epidermal cells at stage-13 are yet not competent to engage morphological differentiation. Therefore, while Svb defines the spatial pattern and *pri* the temporal onset of epidermal trichomes, their formation can occur only once epidermal cells have reached a competent stage, likely relying on independent factors involved in the general differentiation of the embryonic epidermis. Such general factors known for their role in epidermal differentiation can include transcription factors (e.g., Grh, Vri, Ribbon, Ttk, and/or Gata factors)⁴⁴, as well as regulators of apico-basal polarity, cell junctions, vesicle trafficking or secretion (reviewed in^{44,45}). All pictures are at the same magnification. Scale bar is 100 μ m.

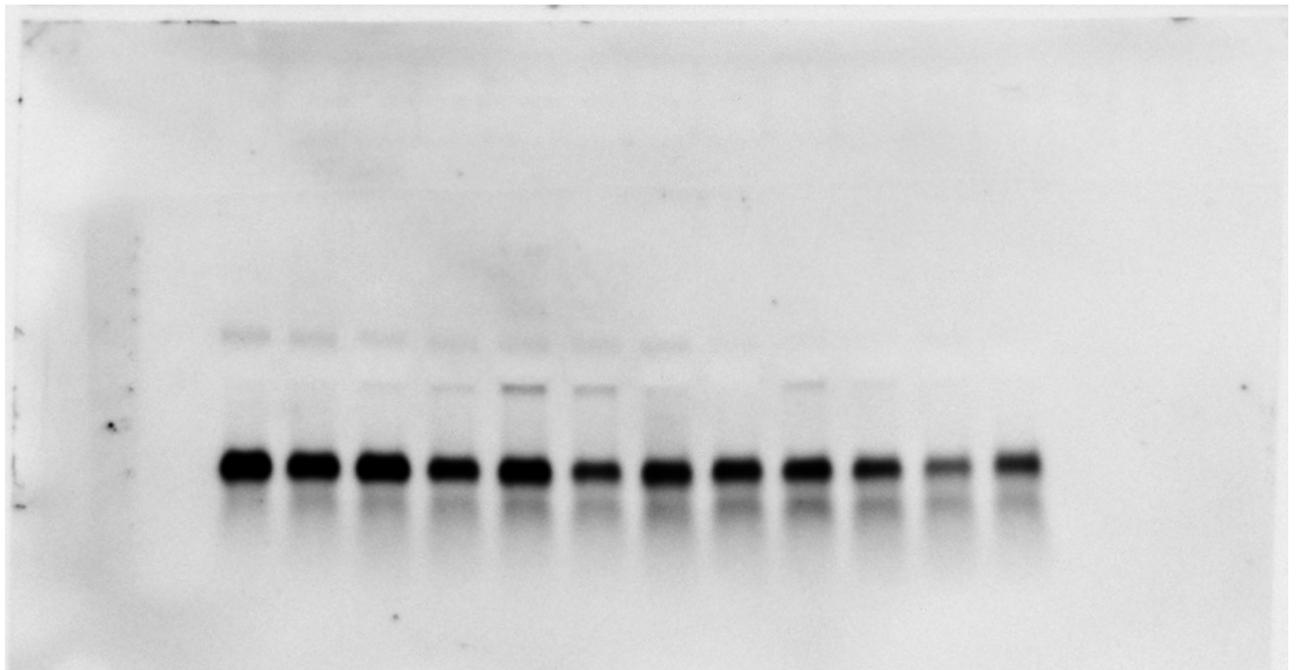


Supplementary Figure 8 Effectors of embryonic trichome formation are required for the differentiation of adult trichomes in the notum. Scanning Electron Micrographs of trichomes in the adult notum, showing consequences of the inactivation of three genes: *singed* (*sn*), *forked* (*f*) and

miniature (*m*), which are direct targets of the Svb transcription factor during embryonic epidermal differentiation^{19, 21}. When compared to wild type, the notum trichomes of *sn*³, *f*^{36A} and *m*¹ mutants display characteristic alterations of their shape and improper organization. Scale bars are 3 μm.



pri mRNA



rp49 mRNA

-18h -9h -3h 0 2h 4h 6h 8h 10h 12h 14h 16h

Supplementary Figure 9 Full scans.

Supplementary Table Legends

Supplementary Table 1 List of tested deficiencies. For each line carrying a small deletion on chromosome II, the table indicates the stock ID number at the Bloomington Drosophila Stock Center at Indiana University (<http://flystocks.bio.indiana.edu>), as well as detailed genotype, cytolocation and molecular breakpoints when available. Additional information for each line is available at Flybase (<http://flybase.org>).

Supplementary Table 2 *GstE14* is required in the ring gland for survival and developmental transitions. Gal4 drivers were used to target expression of *UAS-dsRNA-GstE14* to specific tissues/cells. Tissue specificity is indicated for each driver line. The ring gland endocrine centre is functionally subdivided into the Corpus Cardiacum, Corpus Allatum, and Prothoracic Gland, the latter being responsible for ecdysone secretion. *UAS-dsRNA* lines used in these experiments are indicated. The percentage of animals arrested at pupal stages, or eclosing in adults is calculated by dividing the number of rescued animals with the number of sibling animals that do not express RNAi. Each experiment has been independently repeated at least three times. All data has been pooled for each genotype, and values are expressed as percentage of the expected population (total number). N.D., not determined.

Supplementary Table 3 Transgenic and Bac constructs. Location of the genomic regions carried by transgenic reporters and BAC constructs, according to the fly genome (release FB2014_01, January 17th, 2014).

Supplementary Table 4 *pri* expression counteracts the lethality induced by EcR-DN expression during metamorphosis. GMR-Gal4 driven expression of each of the EcR isoform (EcB-A, EcR-B1 and EcR-B2), carrying one or the other Dominant Negative point mutation (F645A or W650A), induces lethality during pupariation and prevents the emergence of adults. The simultaneous expression of wild-type *pri* mRNA (*pri*), or of an artificial construct that expresses only the small-ORF1 of *pri* (ORF1) strongly suppresses pupal lethality. In contrast, a construct encoding *pri* mRNA with four point mutations frameshifting each of the four *pri* small ORF (1-4FS) is devoid of rescuing activity, as also observed when driving GFP (GFP) as a negative control. Raw data, percentage and a summary graph are presented in the table, including the data presented in Figure 7D. The table also contains data source for *pri* mRNA levels shown in Figure 7B.

7. Multiple enhancers drive the spatio-temporal expression of *polished rice*, involving direct control by the ecdysone receptor

▪ Résumé

Le développement de la drosophile passe par des stades embryonnaires et post-embryonnaires successifs, dont la temporalité dépend de pics périodiques de l'hormone stéroïde ecdysone. Comment ce signal systémique est intégré dans différents tissus pour générer des réponses variées et spécifiques reste cependant mal connu. Nous avons montré récemment que le gène *polished rice (pri)*, qui code quatre petits peptides, agit comme un médiateur de l'ecdysone pour le contrôle temporel de la différenciation épidermique. Dans ce présent travail, nous identifions fonctionnellement différentes régions *cis-régulatrices* (ou enhancers), qui dirigent l'expression de *pri* à travers différents tissus et au cours des stades progressifs du développement. Si ces enhancers possèdent des activités transcriptionnelles indépendantes, ils sont cependant individuellement régulés par l'ecdysone. Se concentrant sur un enhancer dirigeant l'expression de *pri* dans l'épiderme embryonnaire, nous montrons que la mutation de deux sites de fixation au complexe nucléaire de réponse à l'ecdysone est suffisante pour abolir son activité. Ces données montrent que différents enhancers du gène *pri* intègrent probablement directement la signalisation ecdysone, suggérant que ce type d'architecture *cis-régulatrice* pourrait contribuer à spécifier différents profils de réponse à l'ecdysone au cours du développement.

▪ Manuscript and supplemental information

Multiple enhancers drive the spatio-temporal expression of *polished rice*, involving direct control by the ecdysone receptor

Azza DIB^{1,2}, H el ene CHANUT-DELALANDE^{1,2}, Yoshiko HASHIMOTO³, Takefumi KONDO³, Philippe VALENTI^{1,2}, Serge PLAZA^{1,2}, Yuji KAGEYAMA³ and Fran ois PAYRE^{1,2}

¹ Universit e de Toulouse, UPS, Centre de Biologie du D veloppement, Bat 4R3, 118 route de Narbonne, F-31062 Toulouse, France.

² CNRS, UMR5547, Centre de Biologie du D veloppement, F-31062 Toulouse, France.

³ Department of Biology, Graduate School of Science, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

Correspondence should be addressed to H el ene Chanut-Delalande or Fran ois Payre

Summary

Drosophila development progresses through successive embryonic and post-embryonic stages, the timing of which relies on periodic pulses of the steroid hormone ecdysone. How this systemic hormonal signal is integrated to generate various temporal and tissue-specific responses remains yet not fully understood. We have recently found that the *polished-rice* gene (*pri*), encoding four small Open Reading Frame (smORF) peptides, acts as a mediator of ecdysone for the temporal control of epidermal differentiation. Here we report the functional identification of a large array of separate enhancers, driving specific subsets of *pri* expression across tissues and developmental stages. Although these enhancers display distinct spatiotemporal activities, they are nevertheless individually responsive to ecdysone. Focusing on a main enhancer driving *pri* expression in the embryonic epidermis, we provide evidence that the mutation of ecdysone receptor binding sites is sufficient to abolish its transcriptional activity. Furthermore, we identify an ecdysone-dependent function of *pri* during eye development. All together, these data show that different enhancers capture ecdysone signaling for the regulation of *pri* expression, further suggesting that this *cis*-regulatory architecture may contribute to specifying distinct patterns of ecdysone response throughout development.

Introduction

Animal development requires a precise coordination, across the whole organism, of genetic programs underlying the formation of the different tissues and organs. In many species, major morphological changes highlight this synchronization, as well illustrated by the transition of immature juveniles into reproductive adults. Steroid hormones are playing a key role in the temporal control of such developmental transitions. These diffusible molecules act as systemic signals, timely released in response to various inputs from both the internal milieu and the environment.

The *Drosophila* life cycle comprises successive developmental stages, from the egg, followed by three larval stages separated by molts, and ultimately metamorphosis, a stunning remodeling process where most larval structures are destroyed and replaced by adult tissues. Periodic pulses of ecdysteroid hormones orchestrate these developmental transitions and provide timing cues for larval molting and metamorphosis (Riddiford, 1993; Thummel, 1995). The major form of ecdysteroids is ecdysone, also known as the molting hormone, synthesized from dietary cholesterol in the endocrine prothoracic gland. The hormone is released in the haemolymph to target peripheral tissues, where it is converted to its active form 20-hydroxyecdysone (20E), often referred to as ecdysone. The small lipophilic 20E acts as a ligand, which directly binds to and activates the Ecdysone Receptor (EcR), a transcription factor of the nuclear receptor superfamily. EcR generally hetero-dimerizes with Ultraspiracle (Usp), an orphan nuclear receptor homolog to mammalian RXR (Bender et al., 1997; Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1992). The mechanisms of ecdysone response have been extensively studied at the onset of metamorphosis and the hormone triggers the expression of a temporal series of transcription factors (Ou and King-Jones, 2013; Thummel, 2001). Bound to 20E, the activated receptor directly induces the expression of early response genes (Ashburner, 1974), which encode transcription factors, including *Broad-complex (Br-C)*, *E74* and *E75*. This cascade goes on with the activation of late genes, while inhibitory feedback loops repress early genes to ensure tight temporal regulation (Ou and King-Jones, 2013; Thummel, 2001). Ecdysone signaling is also critical during early development; mutations inactivating EcR are embryonic lethal (Bender et al., 1997; Kozlova and Thummel, 2003) and ecdysone responsive transcription factors are expressed in late embryos, following a similar temporal regulation (Ruaud et al., 2010). Genome-wide

profiling has further uncovered hundreds of genes whose expression is influenced directly or indirectly by ecdysone (Gauhar et al., 2009; Li and White, 2003). Besides a few number of early genes, the sets of genes regulated in response to ecdysone are however strikingly different between cell types (Li and White, 2003; Stoiber et al., 2016). How the systemic ecdysone signal can provide this broad range of tissue-specific responses is poorly understood. In addition, little remains known about the mechanisms physically implementing ecdysone signaling within a specific developmental program.

A prominent target of ecdysone is the epidermis that secretes a new cuticle exoskeleton at each developmental transition. The cuticle produced by epidermal cells displays different composition and organization across developmental stages (Moussian, 2010). This also holds true along the animal body that is decorated by a stereotyped pattern of cuticle extensions, called denticles or hairs, and collectively referred to as trichomes (Payre, 2004). We recently identified a novel target of ecdysone, the atypical gene *polished rice (pri)*, which mediates its action for timing the onset of trichome differentiation (Chanut-Delalande et al., 2014).

Pri, also known as *tarsal less* or *mille pattes*, was initially identified as a long noncoding RNA (Inagaki et al., 2005; Tupy et al., 2005), and is involved in various developmental processes in insects (Galindo et al., 2007; Kondo et al., 2007; Savard et al., 2006). Mounting evidence suggests that apparently noncoding RNAs may often encode smORF peptides (Andrews and Rothnagel, 2014; Pauli et al., 2015; Pueyo et al., 2016) and *pri* represents a good paradigm of this emerging field (Andrews and Rothnagel, 2014; Zanet et al., 2016). Further work has demonstrated that *pri* acts through the production of evolutionarily conserved peptides, encoded from small Open-Reading-Frames (smORFs). In *Drosophila*, *pri* encodes four smORF peptides (from 11 to 32 aa), all bearing the conserved LDPTGLY motif. Hypomorphic mutations have revealed a role of *pri/tarsal less* in the formation of adult appendages, mutant animals displaying atrophic legs with missing/fused distal segments (Galindo et al., 2007; Pueyo and Couso, 2008). *pri* displays highly dynamic expression during embryogenesis, and loss-of-function mutations are embryonic lethal. *pri* mutants display altered differentiation of the tracheal respiratory system (Kondo et al., 2007; Kondo et al., 2010; Ozturk-Colak et al., 2016), whilst the most striking phenotype is the complete absence of epidermal trichomes (Galindo et

al., 2007; Kondo et al., 2007). Previous work has established the pivotal role of a transcription factor, Ovo/Shavenbaby (Svb), whose expression integrates regulatory inputs to define the trichome pattern (Crocker et al., 2015; Delon et al., 2003; Payre et al., 1999; Stern and Frankel, 2013). Svb directly activates the expression of genes encoding cellular effectors (Menoret et al., 2013), regulating the cytoskeleton and the extracellular matrix (Fernandes et al., 2010), as well as cuticle organization (Andrew and Baker, 2008) and pigmentation (Chanut-Delalande et al., 2006; Chanut-Delalande et al., 2012). We showed that Pri peptides are essential for trichomes, since they trigger a postranslational conversion of Svb from a large-sized repressor to a shorter activator, which triggers the expression of trichome effectors (Kondo et al., 2010). Recent findings have further identified the mode of *pri* action. Through a direct interaction, Pri peptides activate an E3 ubiquitin-ligase, Ubr3, allowing its binding and subsequent ubiquitination of the Svb N-terminal region, which is degraded by the proteasome to release the Svb activator (Zanet et al., 2015).

From an unbiased genetic screen, we found that ecdysone signaling regulates the expression of *pri*, at different stages throughout *Drosophila* development (Chanut-Delalande et al., 2014) and *pri* thus acts as a mediator of ecdysone to control the timing of epidermal trichome differentiation, both in the embryo and in pupae.

Here, we describe a detailed analysis of the transcriptional regulation of *pri* expression during development. We characterized the functional genomic region of *pri* necessary for epidermal differentiation and adult development. We identified *cis*-regulatory sequences driving *pri* expression during embryogenesis, two in epidermis and one in the tracheal system. These enhancers are, at least partially, controlled by ecdysone. The dissection of epidermal enhancers led to the definition of the minimal regions driving epidermal expression and we identified for one of them two EcR binding sites essential for enhancer activity. We extended this analysis to the larval/prepupal transition and identified an additional ecdysone-dependent enhancer driving *pri* expression in the adult leg and eye/antenna primordia. Furthermore, we find that *pri* function is required in the eye and that its re-expression is sufficient to suppress the defects resulting from compromised ecdysone signaling in this tissue. All together, these data provide insights into the mechanisms regulating *pri* expression, to better understand how this novel mediator of ecdysone signaling may be involved in the temporal control of various developmental programs.

Results

Defining the functional unit of the pri gene

The *pri* gene is transcribed as an intron-less polyadenylated RNA of 1.5kb in length and is separated from neighboring genes by large upstream and downstream intergenic regions (Fig. 1A). To investigate the transcriptional control of *pri* expression, we first sought to define the extent of the genomic locus required for its functions throughout development.

We generated a series of transgenic lines bearing overlapping genomic constructs (selected from available libraries of Bacterial Artificial Chromosomes (BAC)(Venken et al., 2009)) and assayed their rescuing activity when reintroduced in a genetic background lacking *pri* function. We used a combination of two null *pri* alleles placed in trans (*pri*¹/*pri*³) to avoid the possible influence of additional mutations that may exist on each individual mutant chromosome. We tested two parameters: the differentiation of embryonic trichomes and the restoration of full development up to adult viability. All BAC constructs, which largely covered the transcribed region of *pri*, were able to significantly restore the formation of cuticular trichomes (Fig. 1 A). This result suggested that the genomic region required for *pri* function in trichome formation in the embryonic epidermis was included in the sequence shared by all rescue constructs, which is 8,7 kb long (bright yellow region, Fig. 1A) although we could not rule out the existence of separate *cis*-regulatory regions of similar functions in the different rescuing BACs. None of the two smaller BAC constructs (176K10 and 150C8, of 20,4kb and 21,9kb, respectively) was sufficient to restore animal viability. In contrast, the large 51O1 construct (98,7kb) fully rescued the emergence of viable and fertile adults and trichomes when introduced in a *pri* null genetic context. Similar rescuing activity was also observed for BAC 08H01 (Fig. 1B), restricting the minimal genetic interval for *pri* function to a 52,6kb region (light yellow, Fig. 1A). These data show that, despite a compact transcribed region, the developmental functions of *pri* relies on the activity of large upstream and downstream genomic sequences, contained within a 50kb region.

Activity of pri enhancers during embryogenesis

Having defined the functional genomic unit of *pri* for *Drosophila* development, we next aimed to delineate the *cis*-regulatory sequences controlling its expression. We used a series of transgenic LacZ reporter lines (Chanut-Delalande et al., 2014) to systematically examine the entire region.

Genomic DNA fragments were approximately 5kb long and displayed overlapping regions to ensure coverage of the entire *pri* locus (Fig. 2A). We performed LacZ staining to define the spatial and temporal patterns of expression driven by each region during embryogenesis. Three DNA sequences drove an embryonic expression that reproduced part of the endogenous pattern of *pri* mRNA. As previously described (Chanut-Delalande et al., 2014), *priG-LacZ* is expressed in the epidermis from stage-13 mostly in ventral cells, and its activity extends to the dorsal region in stage 15/16 (Fig. 2B). A second remote enhancer, *priA*, also exhibited epidermal expression in the dorsal and ventral regions of embryos. Following a faint onset of epidermal expression detected at stage 13, the *priA-LacZ* construct drove strong expression in the embryonic epidermis, which peaked at stage 15/16 (Fig. 2B). While *priA* and *priG* enhancers drove distinct patterns in the embryonic epidermis, they both displayed stronger expression in anteroposterior stripes (as best seen in the ventral region), consistently with the expression of *pri* mRNA that is reinforced in broad epidermal stripes overlapping the pattern of trichome cells (Kondo et al., 2007; Kondo et al., 2010). Finally, we identified the *priB* element that was strongly activated in the embryonic tracheal system from stage 12 to 16 (Fig. 2B). This activity was yet slightly delayed when compared to the endogenous expression of *pri* in the tracheal system, the onset of which being already visible at stage 11. All additional DNA regions we tested, including those comprising the proximal promoter of *pri*, either displayed barely detectable activity (*priC,I,J*), or expression in other tissues (*pri D,E,F,H*, posterior gut, groups of mesodermal cells, see Fig. S1) that might reveal additional aspects of *pri* expression, but which currently lack functional evidence.

All together, these results identify three main embryonic enhancers of *pri*. Two separate *cis*-regulatory regions, *priA* and *priG*, drive *pri* expression in the epidermis and a third one, *priB*, in the tracheal system.

Ecdysone regulates embryonic pri enhancers

Consistently with the requirement of ecdysone for the expression of *pri* (Chanut-Delalande et al., 2014), *in vivo* ChIP-seq shows strong peaks of EcR binding at the *pri* locus at pupal stages and their specificity is well illustrated when compared to a large window of neighboring regions (Fig. 3A). The three embryonic enhancers *priA*, *B*, *G* contain major EcR peaks (highlighted in blue in Fig. 3A). In addition, profiling of EcR binding in cultured S2 cells (Shlyueva et al., 2014) also detected a strong EcR peak in the *priA* sequence, as well as weak but significant binding to *priB* and *priG*, since the signal was only seen in presence of ecdysone (Fig. 3A). The same study has used high throughput profiling of enhancers active in cultured cells, and the *priA* region contains the strongest ecdysone responsive enhancer of the third chromosome in S2 cells (Shlyueva et al., 2014). A weaker activity was also found in the *priG* region in S2 cells, while it matches the major ecdysone responsive enhancer (Fig. 3A) in this region in another cell type (OSCs, (Shlyueva et al., 2014)) highlighting the cell type specific response to ecdysone.

The regulation of *pri* expression by ecdysone is further demonstrated during embryogenesis since the endogenous expression of *pri* mRNA detected in the tracheal system, epidermis, gut and pharynx is abolished in *phantom* (*phm*) mutant embryos (Fig. 3B), which are unable to synthesize ecdysone (Warren et al., 2004). We next tested whether the lack of ecdysone also influenced the activity of the *pri* embryonic enhancers. In embryos deprived of ecdysone, the activity of the *priG* enhancer was strongly impaired (Chanut-Delalande et al., 2014), preventing the detection of any epidermal expression (Fig. 3B). For additional validation purposes we drove a dominant negative form of EcR using *ptc-gal4*, leading to a strong reduction of *priG* epidermal expression in corresponding cells, as also observed for endogenous *pri* mRNA (Fig. S2). The activity of the second epidermal enhancer, *priA*, was also dramatically decreased in absence of ecdysone (Fig. 3B). Finally, although not entirely abolished, expression of the *priB-LacZ* enhancer was strongly reduced in the tracheal system (Fig. 3B). Taken together, these data indicate that ecdysone is required for the activity of *priA* and *priG* epidermal enhancers in the embryo, and to a lesser extent of the tracheal enhancer *priB*.

Molecular dissection of embryonic epidermal pri enhancers

Although the 20E/EcR complex directly regulates the expression of many genes, experimental evidence on the role of its binding sites for *in vivo* expression of ecdysone responsive enhancers remains limited. We aimed to get a deeper understanding of *pri* regulation in epidermis by ecdysone and the *priA* and *priG* enhancers could constitute good examples for direct regulation by EcR.

We carried out systematic dissection of these two enhancers to identify the minimal cis-regulatory regions, sufficient to drive expression in embryonic epidermis. The *priA* element contains a sequence of 288bp, previously shown to drive ecdysone-dependent expression in S2 cells and its activity was abolished following the mutation of two EcR binding sites (Shlyueva et al., 2014) (see Fig. 4A-C). These two sites are evolutionarily conserved across *Drosophila* species (Fig. 4C) and a 3' deletion of *priA* that lacks this region, *priAb1*, was devoid of activity (not shown). Reciprocally, the *priAb2* region displayed epidermal expression, although significantly decreased when compared to the full *priA* enhancer (Fig. 4B). However, the enhancer active in S2 cells (*priAs*) was not sufficient to drive *in vivo* expression in the embryonic epidermis. Instead, we observed ectopic expression in scattered cells that likely represent blood cells. The same result was also observed with an overlapping region extended to 421bp, *priAse* (Fig. 4B). These data indicate that the *in vivo* activity of *priA* requires cis-regulatory elements present in the 5' region of the *priA* sequence, which may include other EcR binding sites. Accordingly, mutations of one or both EcR binding sites in the backbone of the entire *priA* enhancer were not sufficient to significantly impact on its epidermal expression (Fig. 4C).

The *priG* enhancer was further dissected in a series of overlapping DNA fragments (*priG1-5*, see Fig. 4D). Corresponding transgenic lines revealed no epidermal activity for *priG1* and *priG4*, while *priG3* and *priG5* only displayed weak expression (Fig. 4E). In contrast, *priG2* exhibited a strong epidermal activity comparable to *priG*. We searched in this region for predicted EcR/Usp binding sites using the JASPAR database that contains an updated list of experimentally defined transcription factor binding sites (Mathelier et al., 2014). We found two putative juxtaposed EcR sites, which are also well evolutionarily conserved across *Drosophila* species (Fig. 4F). We inactivated the EcR binding sites in the *priG2* construct by site-directed mutagenesis and the disruption of both sites, *priG2-EcRmut*, abolished the activity of the enhancer

(Fig. 4F). Hence, we identified a 1kb minimal regulatory region, *priG2*, sufficient to drive expression in epidermal cells. Its epidermal activity is dependent on ecdysone signaling, likely through a direct action of EcR since the mutation of EcR binding sites is sufficient to inactivate this enhancer.

Analysis of pri regulation in the larval/pupal leg

The complex regulation of *pri* expression through tissue specific enhancers, highlighted above, led us to study the regulatory mode of *pri* in other tissues where *pri* is required. *pri* is essential for leg morphogenesis (Galindo et al., 2007; Pi et al., 2011; Pueyo and Couso, 2008, 2011) and we previously showed that endogenous *pri* expression in the larval leg disc is activated by ecdysone (Chanut-Delalande et al., 2014). Using our comprehensive set of LacZ reporter lines, we searched for putative genomic regions driving *pri* expression during leg morphogenesis. LacZ staining revealed no expression for *priC, D, E, F, H, J* and a faint activity for *priA, B and G*. In contrast, we observed a very strong activity for *priI* in the leg primordium from the 3rd instar larval stage to 6h APF (Fig. 5A). Previous work has described a *P-lacZ* insertion in 5' of *pri* coding region (*tal-LacZ*) that is expressed in the leg primordium (Galindo et al., 2007). When compared to *tal-LacZ* staining (Fig. 5B), we observed that *priI* drove a comparable pattern, but with far stronger activity (Fig. 5) suggesting that *priI* efficiently captures the expression of *pri* during leg morphogenesis.

We studied further the *priI* enhancer by testing whether its activity in the leg is linked to ecdysone. For this, we expressed EcR-DN in the posterior compartment of the leg disc using the *en-Gal4* driver and performed Engrailed staining to label the posterior region of the disc. This led to a clear reduction in *priI* expression, specifically in the posterior region, when compared to the non-manipulated anterior compartment (Fig. 5C). Therefore, the *priI* enhancer contributes to the expression of *pri* in the adult leg primordium, under control of the ecdysone pathway. Consistent with this conclusion, we found that targeting EcRDN in the distal region of the leg disc (using the *distal-less (dll)-Gal4* driver) led to tarsal fusion (Fig. 6C), a phenotype reminiscent of interfering with *pri* function either by genetic inactivation (see Fig. S3) or by expressing an RNAi construct (Fig. 7C).

Functional characterization of pri during eye development

The global levels of *pri* mRNA vary at the larval/prepupal transition following periodic pulses of ecdysone (Chanut-Delalande et al., 2014). Given the important expression of *pri* at this developmental stage, we undertook the characterization of *pri* expression during morphogenesis of other adult tissues. *In situ* hybridization revealed a strong expression of *pri* in the eye-antenna imaginal disc. Once again, the pattern of *pri* expression was highly dynamic, both spatially and temporally (Fig. 6A). In third instar larvae, *pri* started to be expressed in the morphogenetic furrow, where the differentiation of photoreceptors occurs. Then, from 0 to 3 hours APF, a strong burst of expression was detected across the entire imaginal tissue.

We went further in the examination of *pri* expression during eye formation by the identification of *pri* regulatory regions active in this tissue. Using the same strategy as above, we performed LacZ staining on eye discs expressing *pri-LacZ* reporters during eye morphogenesis. The *pri* sequence was the sole enhancer that drove expression in the eye disc from third instar larvae to 6 hours APF (Fig. 6B). To test whether the activity of *pri* was also controlled by ecdysone in the eye disc, we expressed EcR-DN in the morphogenetic furrow using the *GMR-Gal4* driver (Fig. 6C), and observed a great decrease in *pri* activity.

Finally, we aimed to study the function of *pri* during eye formation by producing mosaic clones of *pri* mutant cells. We generated new mutant alleles for *pri* by imprecise transposon excision, *pri*⁴ and *pri*⁵; both of them are embryonic lethal. We produced null mutant clones using mitotic recombination, in a background context aimed to favor the *pri* mutant cells to develop. While mutant cells in small clones did not exhibit obvious defects, larger clones led to roughened eyes with necrotic melanized patches (Fig. 7A-C). This phenotype was occasionally observed in the center of large clones, consistent with the non-cell autonomous function of *pri* (Galindo et al., 2007; Kondo et al., 2007). Histochemical experiments revealed large defects in eye retinas of *pri* mutant cells (Fig. 7C).

Ecdysone signaling regulates eye development in *Drosophila* (Cherbas et al., 2003) and the expression of EcR-DN in the eye induces strong eye deformation and

necrotic melanized patches in adult escapers (Fig. 7B), since it also causes lethality at the pupal stages. We used this condition of impaired ecdysone signaling in the eye to test whether the expression of *pri* was sufficient to restore the defects due to EcR-DN expression. Co-expression of *pri* and EcR-DN rescued adult lethality and reduced eye defects (Fig. 7B). These rescuing effects were also manifest with the coexpression of the *pri* ORF1 region, coding only for the first 11aa smORF peptide. In contrast, the *1-4FS* mutant of *pri* mRNA that introduces four point mutations frame shifting the sequence of Pri peptides (Kondo et al., 2007), was devoid of any rescuing activity of EcR-DN-induced defects. These data highlight an additional function of *pri* for the proper development of adult eyes. In addition, it shows that the regulation of *pri* expression is an important target for the function of ecdysone during eye formation.

Discussion

We recently found that *polished rice (pri)* smORF peptides play a key role in epidermal differentiation in flies, as a novel target of ecdysone signaling. *pri* induces the maturing activation of Svb (Kondo et al., 2010; Zanet et al., 2015), a transcription factor that governs the formation of epidermal trichomes (Chanut-Delalande et al., 2012; Stern and Frankel, 2013), and thereby *pri* mediates the action of ecdysone for the temporal control of trichome development (Chanut-Delalande et al., 2014). *Pri* function extends to additional developmental programs (Galindo et al., 2007; Pueyo and Couso, 2008, 2011), also in a *svb* independent manner (Kondo et al., 2007; Kondo et al., 2010). Here, we report further analyses on the regulation of *pri* developmental expression. We find that the *pri* locus involves a large genomic region and we delineate several enhancers driving expression across various tissues and developmental stages. A complementary set of evidence supports that the separate *pri* enhancers capture direct inputs from ecdysone signaling, providing a framework to explain how *pri* can contribute to mediate tissue- and stage-specific regulation of developmental processes.

***pri* as a direct target of ecdysone signaling**

The steroid hormone ecdysone is well established to regulate major developmental transitions, as well as morphogenetic changes in many developing tissues, at given time points, from the embryonic to the adult stages (for review (Ou and King-Jones, 2013; Yamanaka et al., 2013)). While we now have a wealth of information on the regulation of ecdysone production (Andersen et al., 2013; Niwa and Niwa, 2014; Ou and King-Jones, 2013; Yamanaka et al., 2013), how ecdysone signaling is integrated within terminal differentiation programs remains to be fully elucidated.

Recent unraveling of *Pri* peptide function provides a molecular connection between the intimate mechanisms of epidermal differentiation and ecdysone signaling. Several arguments show that *pri* behaves as a primary response gene of ecdysone. First, the embryonic expression of *pri* is abrogated in mutant embryos deficient for ecdysone production. Of note, the inactivation of any enzyme of the ecdysone biosynthetic pathway causes a similar phenotype, referred to as Halloween (Chanut-

Delalande et al., 2014; Enya et al., 2014; Gilbert, 2004; Namiki et al., 2005; Niwa et al., 2004; Niwa et al., 2010; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2002; Warren et al., 2004), since mutant embryos produce –as also manifest in *pri* mutants- a poorly differentiated cuticle and the complete lack of trichomes. Furthermore, the re-expression of *pri* is sufficient to restore trichomes in the absence of ecdysone, showing that *pri* is an important mediator of ecdysone action in the epidermis (Chanut-Delalande et al., 2014). In the same vein, the expression of functional Pri peptides is sufficient to counteract the defects resulting from targeted expression of EcRDN (Cherbas et al., 2003) in developing adult eyes, restoring both the viability and the proper differentiation of ommatidia. Second, genome-wide profiling identified prominent peaks of EcR binding to *pri* genomic regions *in vivo* (Chanut-Delalande et al., 2014), as well as in different cells lines following ecdysone treatment that induces a rapid burst of *pri* transcription (Shlyueva et al., 2014; Skalska et al., 2015). We show that, when assayed *in vivo*, several of these regions act as tissue-specific enhancers responsive to ecdysone during development. Indeed the activity of *priA*, *priB* and *priG* embryonic enhancers is compromised in embryos lacking ecdysone. The targeted expression of EcR-DN represses the expression of *priG* and *priI* enhancers in corresponding cells, respectively in embryos and larval/pupal tissues. Finally, for one short epidermal enhancer, *priG2*, we show that mutation of two motifs matching the EcR/USP binding site (Antoniewski et al., 1994; Gauhar et al., 2009; Shlyueva et al., 2014) is sufficient to knockout its activity in the embryonic epidermis. Therefore, we interpret these data to imply that the transcription of the *pri* gene involves a direct control by the activated 20E/EcR nuclear receptor complex.

***pri* expression is controlled by a wide cis-regulatory landscape**

Contrasting with the compact size of its transcribed region, *pri* functions throughout development involve an unexpectedly large genomic locus, as defined by rescuing assays. The smallest DNA fragment that allows full rescue of *pri* activity spans over 50kb, strongly suggesting that *pri* expression relies on a large array of cis-regulatory regions scattered over this region, which may underlie the highly dynamic expression of *pri* across tissues and developmental stages (Galindo et al., 2007; Kondo et al.,

2007; Savard et al., 2006). Consistently with this conclusion, we identify remote enhancers located as far as 20kb upstream and 10kb downstream of the *pri* transcriptional unit. The systematic scanning of 50 kb *pri* genomic region showed that two separate enhancers *priG* and *priA* are active in the embryonic epidermis. Although *priA* and *priG* expressions overlap, each enhancer displays a distinct pattern according to cell rows suggesting both complementary and redundant functions of these enhancers to mediate ecdysone action in embryonic epidermal cells. Later on, during leg morphogenesis, the spatiotemporal regulation of *pri* is required for both patterning and leg joint formation (Galindo et al., 2007; Pueyo and Couso, 2008, 2011). In a same way, whereas the *priI* enhancer sustains most expression in the leg disc, additional regions may also contribute to a lesser extent to the regulation of *pri* expression in this tissue. Some important developmental genes contain apparently redundant or shadow enhancers that ensure robust spatio-temporal expression, in particular when development proceeds under non optimal conditions or in slightly compromised genetic backgrounds (Frankel et al., 2010; Lagha et al., 2012; Perry et al., 2010). It is therefore tempting to speculate that the multiple enhancers we identified could collectively provide robustness against genetic and/or environmental variations to ensure the proper regulatory activities of *pri*, throughout embryonic and post-embryonic development.

The attribution of cis-regulatory elements located within large intergenic regions to their respective target genes is a common difficulty in functional genomics. For example, the *priI* enhancer overlaps with *Mst87F*, a male specific transcript only expressed from late pupal stages onwards and specific of the male germline (Kuhn et al., 1991). Despite this peculiar arrangement, the remote *priI* enhancer captures the ecdysone responsive expression of *pri* in the developing larval/prepupa leg disc. Also, the strong EcR binding peak contained in the *priA* enhancer was previously attributed to *Dip-B* (Shlyueva et al., 2014), which is located closer than *pri*. Several pieces of evidence are however consistent with its role in the regulation of *pri* expression. First, *priA* drives a stage-specific embryonic expression in epidermal cells like *pri* mRNA, while *Dip-B* is mostly expressed in the hindgut and malpighian tubules. Second, *priAb2* that exhibits epidermal expression is contained in the smaller *pri* rescuing BAC. Third, levels of *pri* mRNA induction triggered as soon as 1 to 3 hours of ecdysone treatment is several times higher than for *Dip-B*, as seen in

Kc cells (Skalska et al., 2015), well in line with the strength of this ecdysone responsive element (Shlyueva et al., 2014). It remains nevertheless possible that this enhancer contributes, at least in part, to the control of *Dip-B* expression, or might influence both *Dip-B* and *pri*. The *priA* region also represents an interesting case to compare the functions of a given enhancer between cultured cells and *in vivo* assays. While *ex vivo* assays have demonstrated the strong activity of a short region and its requirement for direct EcR binding (Shlyueva et al., 2014), neither this sequence nor an extended version of it is sufficient to faithfully drive *pri* (or *Dip-B*) expression in the embryo. Instead, it leads to ectopic expression in blood cells. It is interesting to note that its activity in S2 cells also requires putative binding sites for GATA factors (Shlyueva et al., 2014), and the blood cell lineage is also controlled by the expression of the GATA factor Serpent (Muratoglu et al., 2007; Rehorn et al., 1996). Therefore, these data show the interest of studies using cultured cells for high-throughput discovery of enhancers and associated transcription factors, even though *in vivo* transgenic assays are further required to validate these data, well illustrating the complementarities of these approaches.

Conclusion

To explain the tissue-specific expression of a primary gene, a simple model could involve a single ecdysone-responsive element gating the temporal activity (Potier et al., 2014) of additional enhancers, for example in controlling their communication with the proximal promoter (Spitz, 2016). In contrast, our findings show that *pri* expression involves several independent enhancers, each of them integrating both spatial regulatory cues and ecdysone timing. This complex *cis*-regulatory architecture appears more adapted to generate various spatiotemporal patterns of expression in the response to ecdysone, and suggests that hormonal signaling has evolved at the level of individual enhancers rather than at the whole gene level. Future work will be necessary to further test these alternative models and their respective prevalence for the hormonal control of animal development.

Materials and Methods

Fly stocks

Most mutants were obtained from the Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu/>). The strains we used were: *spo[1]/TM3,Dfd-YFP*, *p hm[E7]/FM7a,Dfd-YFP*; *UAS-EcRDN* (*EcR-A-F645A*; *EcR-A-W650A*; *EcR-B1-F645A*; *EcR-B1-W650A*; *EcR-B2-F645A*; *EcR-B2-W650A*)(Cherbas et al., 2003), *Ptc-Gal4*, *en-Gal4*, *wg-Gal4*, *GMR-Gal4*, *Dll-Gal4*, *UAS-pri* (Kondo et al., 2007). For larval studies, to ensure development in controlled conditions of larval density, 30 eggs were placed into food tubes, supplemented with fresh yeast paste.

Isolation of novel *pri* alleles

To obtain new *pri* alleles, we remobilized the two insertions P{XP}d01244 and P{XP}d06750 (Thibault et al., 2004) by crossing to the $\Delta(2-3)99B$ jumpstarter strain (Robertson et al., 1988). The white-eyed imprecise excision strains were established, which failed to complement both *pri1* (Kondo et al., 2007) and P{XP}d01244, and did not develop into adult flies. Genomic DNA of these strains was extracted and subjected to PCR, followed by sequencing for molecular definition: *pri4* and *pri5* contain a 1,096 base deletion (9,638,623 – 9,639,719) with a 28-base insertion at 9,638,739 and a 3,104-base deletion (9,637,336 - 9,640,440, respectively (Fig. S3). *pri4* and *pri5* lack the upstream region, and all four functional smORFs of the *pri* gene, indicating that they are amorphic mutations. Alleles were balanced with a TM6B,Tb chromosome bearing Ubi-GFP to distinguish mutants.

Clonal analysis

Since previously reported null alleles (*pri*¹, *pri*², and *pri*³) (Kondo et al., 2007) contain an FRT sequence at the *pri* locus that may interfere with the production of mutant clones (Parks et al., 2004), we used newly isolated amorphic, *pri*⁴ and *pri*⁵ alleles (see above). To generate *pri* mutant clones, *pri*⁴ and *pri*⁵ alleles were recombined

into a $P\{ry+7.2, hs-neo, FRT\}82B$ ($FRT82B$) chromosome (Xu and Rubin, 1993). To generate *pri* mutant clones specifically in the eye we generated $y w ey-FLP;FRT82B pri^4/w+ FRT82B w+ 90E$ flies, as well as $ey-FLP;FRT82B pri^4/FRT82B w+ l(3)cl-R3$ to increase the size of mutant clones. Alternatively, $w;ey-GAL4 UAS-FLP;FRT82B pri^4/FRT82B GMR-hid l(3)$ flies were also used to obtain eyes consisting of only mutant cells. In these flies, *hid*-induced apoptosis completely removed wild type ommatidia, resulting in expanded necrotic patch; as pri^4 and pri^5 showed the same phenotypes in these clonal analyses.

DNA constructs

lacZ reporter constructs were produced by cloning 5-6 kb DNA fragments of the *pri* genomic region into the pAttB-LacZ (Menoret et al., 2013) reporter vector. All constructs were verified by sequencing. Transgenic lines, including BAC constructs (from P[acman] Resources, <http://www.pacmanfly.org/>), were generated using the PhiC31 system and inserted at the 86F position (BestGene). BAC transgenic lines were recombined with pri^1 mutants. w^+ (BAC) recombinants were first screened for lethality (*pri* mutant). Candidates were verified for the presence of pri^1 deficiency by PCR amplification using a forward primer located in the promoter region of *pri* and a reverse primer in the residual P element present in the deficiency. Additional details are provided in Supplementary Table 4.

ChIP Seq and related data

EcR ChIP_seq data were generated by the modENCODE consortium, and were extracted from the ModMine pipeline (<http://intermine.modencode.org>) (Chanut-Delalande et al., 2014). The data is accessible in GEO; the accession number for combined ChIP-seq is GSM628268, and for the input are GSM628269 and GSM628270. Other genome-wide data were from Shlyueva et al (2014).

Embryo, larval and pupal staining

Homozygous mutant embryos were identified by the lack of balancer chromosome

(marked with GFP/YFP or LacZ). Sibling controls and mutant embryos were in all cases processed in the same batch; a typical collection includes >300 embryos in total. The staging of mutant embryos, subjected to *in situ* hybridization or immunohistochemistry, was determined according to the age of 2h embryo collections. Embryos were dechorionated by bleach treatment, fixed in heptane saturated in 4% formaldehyde for 20 min and subsequently devitellinized with heptane/methanol. Embryos were kept in methanol before immunostaining. Staining was performed as previously described (Fernandes et al., 2010) using: anti- β -gal (1/400), biotinylated goat anti-rabbit or anti-mouse (1/1000, Vector Laboratories) preincubated with streptavidin-HRP (Vector Laboratories) and revelation was performed with DAB (3, 3'-diaminobenzidine) (Sigma). For fluorescence immuno-staining, embryos were devitellinized by hand and treated with 80% ethanol to preserve actin organization. We used AlexaFluor-488 secondary antibodies (1/1000, Molecular Probes) and TRITC-phalloidin (Sigma). DIG-labeled RNA antisense probes were synthesized *in vitro* from cDNA clones, processed for *in situ* hybridization and reacted with an alkaline phosphatase-conjugated anti-DIG antibody (Roche) as described (Chanut-Delalande et al., 2006).

Staging of larvae was performed as previously described (Andres and Thummel, 1994). 20-30 adult flies were placed on *Drosophila* medium containing 0.05 % bromophenol blue, and well-developed third instar larvae from their progeny were staged according to gut colour. White prepupae were collected and kept in humid vials until further processing for dissection and staining. Data have been collected in at least three independent experiments. Samples were imaged with a Nikon 90i fluorescent microscope or a Zeiss710 confocal microscope.

Cuticle preparation

Embryos collected from individual mutants, placed over TM3-*Dfd-LacZ* balancers, were processed for X-Gal staining for genotyping and cuticles were prepared in Hoyers/lactic acid (1/1). Each rescuing experiment has been performed, independently, at least three times.

Histology

For the histological analysis of eyes with melanized patches in *pri* mosaic animals, fly heads were prefixed with 2% glutaraldehyde, postfixed with 1% OsO₄ for one hour, washed with PBS (1% Tween20), dehydrated in an ethanol series, embedded in EPON (TAAB), and polymerized for 48 hours at 60°C according to standard routines. The EPON-embedded samples were sectioned at 1-μm thickness and stained with boracic Toluidine Blue.

Acknowledgments

We are grateful to Flybase, the Bloomington, Vienna, and Kyoto stock centers, as well as H. Bellen for BAC constructs. We thank B. Ronsin (Toulouse RIO Imaging) for help with microscopy and S. Marques for technical assistance. We also thank all members of the labs for helpful suggestions and critical reading of the manuscript. This work was supported by ANR (Chrononet), Azm & Saade Association, and by the JST PRESTO program and MEXT KAKENHI (20370091) to YK.

References

- Andersen, D.S., Colombani, J., and Leopold, P. (2013). Coordination of organ growth: principles and outstanding questions from the world of insects. *Trends in cell biology* **23**, 336-344.
- Andres, A.J., and Thummel, C.S. (1994). Methods for quantitative analysis of transcription in larvae and prepupae. *Methods Cell Biol* **44**, 565-573.
- Andrew, D.J., and Baker, B.S. (2008). Expression of the *Drosophila* secreted cuticle protein 73 (*dsc73*) requires Shavenbaby. *Dev Dyn* **237**, 1198-1206.
- Andrews, S.J., and Rothnagel, J.A. (2014). Emerging evidence for functional peptides encoded by short open reading frames. *Nature reviews Genetics* **15**, 193-204.
- Antoniewski, C., Laval, M., Dahan, A., and Lepesant, J.A. (1994). The ecdysone response enhancer of the *Fbp1* gene of *Drosophila melanogaster* is a direct target for the EcR/USP nuclear receptor. *Molecular and cellular biology* **14**, 4465-4474.
- Ashburner, M. (1974). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. II. The effects of inhibitors of protein synthesis. *Developmental biology* **39**, 141-157.
- Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., and Hogness, D.S. (1997). *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* **91**, 777-788.
- Chanut-Delalande, H., Fernandes, I., Roch, F., Payre, F., and Plaza, S. (2006). Shavenbaby couples patterning to epidermal cell shape control. *PLoS biology* **4**, e290.
- Chanut-Delalande, H., Ferrer, P., Payre, F., and Plaza, S. (2012). Effectors of tridimensional cell morphogenesis and their evolution. *Seminars in cell & developmental biology* **23**, 341-349.
- Chanut-Delalande, H., Hashimoto, Y., Pelissier-Monier, A., Spokony, R., Dib, A., Kondo, T., Bohere, J., Niimi, K., Latapie, Y., Inagaki, S., *et al.* (2014). Pri peptides are mediators of ecdysone for the temporal control of development. *Nature cell biology* **16**, 1035-1044.
- Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., and Cherbas, P. (2003). EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development (Cambridge, England)* **130**, 271-284.
- Crocker, J., Abe, N., Rinaldi, L., McGregor, A.P., Frankel, N., Wang, S., Alsawadi, A., Valenti, P., Plaza, S., Payre, F., *et al.* (2015). Low affinity binding site clusters confer hox specificity and regulatory robustness. *Cell* **160**, 191-203.
- Delon, I., Chanut-Delalande, H., and Payre, F. (2003). The Ovo/Shavenbaby transcription factor specifies actin remodelling during epidermal differentiation in *Drosophila*. *Mechanisms of development* **120**, 747-758.
- Enya, S., Ameku, T., Igarashi, F., Iga, M., Kataoka, H., Shinoda, T., and Niwa, R. (2014). A Halloween gene *noppera-bo* encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in *Drosophila*. *Scientific reports* **4**, 6586.
- Fernandes, I., Chanut-Delalande, H., Ferrer, P., Latapie, Y., Waltzer, L., Affolter, M., Payre, F., and Plaza, S. (2010). Zona pellucida domain proteins remodel the apical compartment for localized cell shape changes. *Developmental cell* **18**, 64-76.
- Frankel, N., Davis, G.K., Vargas, D., Wang, S., Payre, F., and Stern, D.L. (2010). Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490-493.

- Galindo, M.I., Pueyo, J.I., Fouix, S., Bishop, S.A., and Couso, J.P. (2007). Peptides encoded by short ORFs control development and define a new eukaryotic gene family. *PLoS biology* 5, e106.
- Gauhar, Z., Sun, L.V., Hua, S., Mason, C.E., Fuchs, F., Li, T.R., Boutros, M., and White, K.P. (2009). Genomic mapping of binding regions for the Ecdysone receptor protein complex. *Genome research* 19, 1006-1013.
- Gilbert, L.I. (2004). Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. *Molecular and cellular endocrinology* 215, 1-10.
- Inagaki, S., Numata, K., Kondo, T., Tomita, M., Yasuda, K., Kanai, A., and Kageyama, Y. (2005). Identification and expression analysis of putative mRNA-like non-coding RNA in *Drosophila*. *Genes Cells* 10, 1163-1173.
- Koelle, M.R., Talbot, W.S., SeGRAves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59-77.
- Kondo, T., Hashimoto, Y., Kato, K., Inagaki, S., Hayashi, S., and Kageyama, Y. (2007). Small peptide regulators of actin-based cell morphogenesis encoded by a polycistronic mRNA. *Nature cell biology* 9, 660-665.
- Kondo, T., Plaza, S., Zanet, J., Benrabah, E., Valenti, P., Hashimoto, Y., Kobayashi, S., Payre, F., and Kageyama, Y. (2010). Small peptides switch the transcriptional activity of Shavenbaby during *Drosophila* embryogenesis. *Science* (New York, NY 329, 336-339.
- Kozlova, T., and Thummel, C.S. (2003). Essential roles for ecdysone signaling during *Drosophila* mid-embryonic development. *Science* (New York, NY 301, 1911-1914.
- Kuhn, R., Kuhn, C., Borsch, D., Glatzer, K.H., Schafer, U., and Schafer, M. (1991). A cluster of four genes selectively expressed in the male germ line of *Drosophila melanogaster*. *Mechanisms of development* 35, 143-151.
- Lagha, M., Bothma, J.P., and Levine, M. (2012). Mechanisms of transcriptional precision in animal development. *Trends Genet* 28, 409-416.
- Li, T.R., and White, K.P. (2003). Tissue-specific gene expression and ecdysone-regulated genomic networks in *Drosophila*. *Developmental cell* 5, 59-72.
- Mathelier, A., Zhao, X., Zhang, A.W., Parcy, F., Worsley-Hunt, R., Arenillas, D.J., Buchman, S., Chen, C.Y., Chou, A., Ienasescu, H., *et al.* (2014). JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. *Nucleic acids research* 42, D142-147.
- Menoret, D., Santolini, M., Fernandes, I., Spokony, R., Zanet, J., Gonzalez, I., Latapie, Y., Ferrer, P., Rouault, H., White, K.P., *et al.* (2013). Genome-wide analyses of Shavenbaby target genes reveals distinct features of enhancer organization. *Genome biology* 14, R86.
- Moussian, B. (2010). Recent advances in understanding mechanisms of insect cuticle differentiation. *Insect biochemistry and molecular biology* 40, 363-375.
- Muratoglu, S., Hough, B., Mon, S.T., and Fossett, N. (2007). The GATA factor Serpent cross-regulates lozenge and u-shaped expression during *Drosophila* blood cell development. *Developmental biology* 311, 636-649.
- Namiki, T., Niwa, R., Sakudoh, T., Shirai, K., Takeuchi, H., and Kataoka, H. (2005). Cytochrome P450 CYP307A1/Spook: a regulator for ecdysone synthesis in insects. *Biochemical and biophysical research communications* 337, 367-374.
- Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., and Kataoka, H. (2004). CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of *Bombyx* and *Drosophila*. *The Journal of biological chemistry* 279, 35942-35949.

- Niwa, R., Namiki, T., Ito, K., Shimada-Niwa, Y., Kiuchi, M., Kawaoka, S., Kayukawa, T., Banno, Y., Fujimoto, Y., Shigenobu, S., *et al.* (2010). Non-molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the 'Black Box' of the ecdysteroid biosynthesis pathway. *Development (Cambridge, England)* *137*, 1991-1999.
- Niwa, R., and Niwa, Y.S. (2014). Enzymes for ecdysteroid biosynthesis: their biological functions in insects and beyond. *Bioscience, biotechnology, and biochemistry* *78*, 1283-1292.
- Ono, H., Rewitz, K.F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J.T., Marques, G., Shimell, M.J., *et al.* (2006). Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. *Developmental biology* *298*, 555-570.
- Ou, Q., and King-Jones, K. (2013). What goes up must come down: transcription factors have their say in making ecdysone pulses. *Current topics in developmental biology* *103*, 35-71.
- Ozturk-Colak, A., Moussian, B., Araujo, S.J., and Casanova, J. (2016). A feedback mechanism converts individual cell features into a supracellular ECM structure in *Drosophila* trachea. *eLife* *5*.
- Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E., *et al.* (2004). Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. *Nat Genet* *36*, 288-292.
- Pauli, A., Valen, E., and Schier, A.F. (2015). Identifying (non-)coding RNAs and small peptides: challenges and opportunities. *Bioessays* *37*, 103-112.
- Payre, F. (2004). Genetic control of epidermis differentiation in *Drosophila*. *The International journal of developmental biology* *48*, 207-215.
- Payre, F., Vincent, A., and Carreno, S. (1999). *ovo/svb* integrates Wingless and DER pathways to control epidermis differentiation. *Nature* *400*, 271-275.
- Perry, M.W., Boettiger, A.N., Bothma, J.P., and Levine, M. (2010). Shadow enhancers foster robustness of *Drosophila* gastrulation. *Current biology : CB* *20*, 1562-1567.
- Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., and O'Connor, M.B. (2003). Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proceedings of the National Academy of Sciences of the United States of America* *100*, 13773-13778.
- Pi, H., Huang, Y.C., Chen, I.C., Lin, C.D., Yeh, H.F., and Pai, L.M. (2011). Identification of 11-amino acid peptides that disrupt Notch-mediated processes in *Drosophila*. *Journal of biomedical science* *18*, 42.
- Potier, D., Seyres, D., Guichard, C., Iche-Torres, M., Aerts, S., Herrmann, C., and Perrin, L. (2014). Identification of cis-regulatory modules encoding temporal dynamics during development. *BMC genomics* *15*, 534.
- Pueyo, J.I., and Couso, J.P. (2008). The 11-aminoacid long Tarsal-less peptides trigger a cell signal in *Drosophila* leg development. *Developmental biology* *324*, 192-201.
- Pueyo, J.I., and Couso, J.P. (2011). Tarsal-less peptides control Notch signalling through the Shavenbaby transcription factor. *Developmental biology* *355*, 183-193.
- Pueyo, J.I., Magny, E.G., and Couso, J.P. (2016). New Peptides Under the s(ORF)ace of the Genome. *Trends in biochemical sciences*.
- Rehorn, K.P., Thelen, H., Michelson, A.M., and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila*. *Development (Cambridge, England)* *122*, 4023-4031.

- Riddiford, L.M. (1993). Hormone receptors and the regulation of insect metamorphosis. *Receptor* 3, 203-209.
- Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., and Engels, W.R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* 118, 461-470.
- Ruaud, A.F., Lam, G., and Thummel, C.S. (2010). The *Drosophila* nuclear receptors DHR3 and betaFTZ-F1 control overlapping developmental responses in late embryos. *Development (Cambridge, England)* 137, 123-131.
- Savard, J., Marques-Souza, H., Aranda, M., and Tautz, D. (2006). A segmentation gene in *tribolium* produces a polycistronic mRNA that codes for multiple conserved peptides. *Cell* 126, 559-569.
- Shlyueva, D., Stelzer, C., Gerlach, D., Yanez-Cuna, J.O., Rath, M., Boryn, L.M., Arnold, C.D., and Stark, A. (2014). Hormone-responsive enhancer-activity maps reveal predictive motifs, indirect repression, and targeting of closed chromatin. *Molecular cell* 54, 180-192.
- Skalska, L., Stojnic, R., Li, J., Fischer, B., Cerda-Moya, G., Sakai, H., Tajbakhsh, S., Russell, S., Adryan, B., and Bray, S.J. (2015). Chromatin signatures at Notch-regulated enhancers reveal large-scale changes in H3K56ac upon activation. *The EMBO journal* 34, 1889-1904.
- Spitz, F. (2016). Gene regulation at a distance: from remote enhancers to 3D regulatory ensembles. *Seminars in cell & developmental biology*.
- Stern, D.L., and Frankel, N. (2013). The structure and evolution of cis-regulatory regions: the shavenbaby story. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 368, 20130028.
- Stoiber, M., Celniker, S., Cherbas, L., Brown, B., and Cherbas, P. (2016). Diverse Hormone Response Networks in 41 Independent *Drosophila* Cell Lines. *G3 (Bethesda)* 6, 683-694.
- Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., Singh, C.M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H.L., *et al.* (2004). A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nat Genet* 36, 283-287.
- Thomas, H.E., Stunnenberg, H.G., and Stewart, A.F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* 362, 471-475.
- Thummel, C.S. (1995). From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. *Cell* 83, 871-877.
- Thummel, C.S. (2001). Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. *Developmental cell* 1, 453-465.
- Tupy, J.L., Bailey, A.M., Dailey, G., Evans-Holm, M., Siebel, C.W., Misra, S., Celniker, S.E., and Rubin, G.M. (2005). Identification of putative noncoding polyadenylated transcripts in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 102, 5495-5500.
- Venken, K.J., Carlson, J.W., Schulze, K.L., Pan, H., He, Y., Spokony, R., Wan, K.H., Koriabine, M., de Jong, P.J., White, K.P., *et al.* (2009). Versatile P[acman] BAC libraries for transgenesis studies in *Drosophila melanogaster*. *Nature methods* 6, 431-434.
- Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., Dauphin-Villemant, C., O'Connor, M.B., and Gilbert, L.I. (2002). Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 99, 11043-11048.
- Warren, J.T., Petryk, A., Marques, G., Parvy, J.P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M.B., *et al.* (2004). Phantom encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx*

mori: a P450 enzyme critical in ecdysone biosynthesis. *Insect biochemistry and molecular biology* 34, 991-1010.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 117, 1223-1237.

Yamanaka, N., Rewitz, K.F., and O'Connor, M.B. (2013). Ecdysone control of developmental transitions: lessons from *Drosophila* research. *Annual review of entomology* 58, 497-516.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* 71, 63-72.

Zanet, J., Benrabah, E., Li, T., Pelissier-Monier, A., Chanut-Delalande, H., Ronsin, B., Bellen, H.J., Payre, F., and Plaza, S. (2015). Pri sORF peptides induce selective proteasome-mediated protein processing. *Science (New York, NY)* 349, 1356-1358.

Zanet, J., Chanut-Delalande, H., Plaza, S., and Payre, F. (2016). Small Peptides as Newcomers in the Control of *Drosophila* Development. *Current topics in developmental biology* 117, 199-219.

Figure Legends

Figure 1: the *polished rice* functional unit extends over a large genomic region

A. Schematic representation of the genomic region encompassing the *pri* locus. The different genes are drawn as blue arrows, reflecting the sense of their transcription, and deleted regions in the *pri*¹ and *pri*³ null alleles are indicated as brackets. Purple lines show the genomic regions carried by BAC transgenes and the table on the right summarizes their respective rescuing activity for the restoration of embryonic trichomes of adult viability and fertility. **B.** Cuticle preparations of whole embryos (top row) and close-ups of the ventral region of abdominal segments A3-A4 (bottom). The denticle belts featuring wild type embryos are completely lacking in *pri*¹/*pri*³ mutant embryos. Introduction of the BAC-08H01 in this *pri* null background fully restores denticle formation.

Figure 2: Functional identification of *pri* embryonic enhancers

A. Grey boxes represent the genomic location of the regions tested using LacZ-reporter transgenic lines. **B.** Time course of *pri* mRNA expression and enhancers from mid to late embryogenesis, as revealed by *in situ* hybridization (top). Expression driven by the three main embryonic enhancers (*priA*, *priB* and *priG*) at corresponding stages of development was revealed by anti-β-Gal immunostaining. Embryos are oriented with the anterior at the left, and the ventral region at the bottom of each picture.

Figure 3: Ecdysone controls *pri* expression and the activity of embryonic enhancers

A. Snapshot of the UCSC genome browser focusing on the *pri* genomic region and surrounding genes; *pri* regions tested in transgenic lacZ-reporter lines are drawn as grey boxes. The different tracks represent signal intensity profiles of *in vivo* EcR Chip-Seq 4h and 33h after puparium formation, APF (orange), EcR ChIP-seq in S2 cells with or without ecdysone treatment (blue and green, respectively), STARR-seq profiling of active enhancers with or without ecdysone (brown and dark red respectively) in S2 cells, as well as STARR-seq profiling with or without ecdysone (red) in Ovarian Somatic Cells (OSC). Data from *in vivo* EcR ChIP-seq are from Chanut-Delalande et al (2014), all data for cultured cells are from (Shlyueva et al., 2014). **B.** Expression of the endogenous *pri* gene (left panels) and of the *priA*, *priB* and *priG* enhancers in control conditions (top) and in *phm^{E7}* mutant embryos that are unable to synthesize ecdysone (Warren et al., 2004).

Figure 4: EcR binding sites are required for the activity of *pri* epidermal enhancer

A. Scheme of the *priA* full enhancer and sub-regions of it that have been tested in lacZ-transgenic reporter lines. Red ovals represent the EcR binding sites shown in C. **B.** Expression of *priA*, *priAb2* and *priAse*, as revealed by fluorescence staining (β -gal is in green, actin in red). The right panels show black and white pictures of the β -gal channel to better appreciate quantitative variations. All pictures have been acquired using the same set-ups for confocal microscopy. **C.** The logo represents a position weight matrix of consensus EcR binding sites. Sequence alignment of EcR binding sites in *priA*, showing their evolutionary conservation across *Drosophila* species (*melanogaster*, *sechellia*, *yakuba*, *erecta*, *elegans*, *ananassae* and *pseudoobscura*). Nucleotides mutated in *priA-EcRmut* are in red. **D.** Drawing of the molecular dissection of the *priG* epidermal enhancer. **E.** Expression of *priG-lacZ*, *priG2-lacZ* and *priG5-lacZ*. **F.** Sequence alignment of EcR binding sites of *priG2* and effect of their mutation (*priG2-EcRmut*) on the enhancer activity (ventral closeup).

Figure 5: Expression of the *pri* enhancer in leg primordia at the larval/prepupal transition and regulation by EcR

A. Time course of the expression of *pri-lacZ* in larval/prepupal imaginal leg discs, as revealed by X-gal staining. **B.** Expression of the LacZ gene in the *PlacW-tal^{S011041}* reporter line in late larval leg disc. **C.** Immunostaining showing the activity of *pri-LacZ* (in red), co-stained with anti-Engrailed antibody (in green), in control leg disc and in disc expressing EcR-DN under the control of the *en-Gal4* driver. **D.** Pictures of adult legs showing tarsal defects and a lack of joints when EcR-DN is expressed under the *dll-Gal4* driver. Knocking-down *pri* function in the *dll* domain also impairs leg development.

Figure 6: Expression of the *pri* enhancer in the adult eye-antenna primordium at the larval/ prepupal transition and regulation by EcR

A. Time course of the expression of *pri* mRNA in larval/prepupal imaginal eye-antenna discs, as revealed by *in situ* hybridization. **B.** X-gal staining showing the activity of the *pri-lacZ* transgenic reporter, in imaginal eye-antenna discs of comparable stages of development. **C.** Expression of *pri-lacZ* in late larval eye disc in a wild-type context, or following *GMR-Gal4* driven expression of EcRDN (C'). C'' shows the expression of *UAS-lacZ* in the eye disc under the control of the *GMR-Gal4* driver.

Figure 7: *pri* is required for the differentiation of adult eyes and compensates defects induced by the targeted expression of EcRDN.

A. Mosaic genetic analysis of *pri* function in the adult eye. *pri⁴* mutant cells are lacking the *white* marker, allowing their distinction from wild type sibling cells (red). While small clones of *pri* mutant cells do not affect eye formation, larger clones

induce a necrotic appearance of affected cells in the center of large mutant clone. As control, the right-most picture shows a mosaic eye with wild type cells and cells only lacking the *white* marker. Whole mutant eyes generated by depleting wild-type cells appeared necrotic and were covered solely by melanized materials. **B.** *GMR-gal4* driven expression of EcR-DN strongly affects eye size and differentiation. Concomitant expression of wild-type *pri* mRNA, or a synthetic construct encoding only the first smORF of *pri* is sufficient to rescue EcR-DN-induced defects. In contrast, a mutated version that frame shifts each of the four *pri* smORF is devoid of rescuing activity. **C.** Semi-thin plastic-mounted sections of wild type and *pri*-mutant eyes were stained with Toluidine Blue. Double-headed arrows indicate the retinal region.

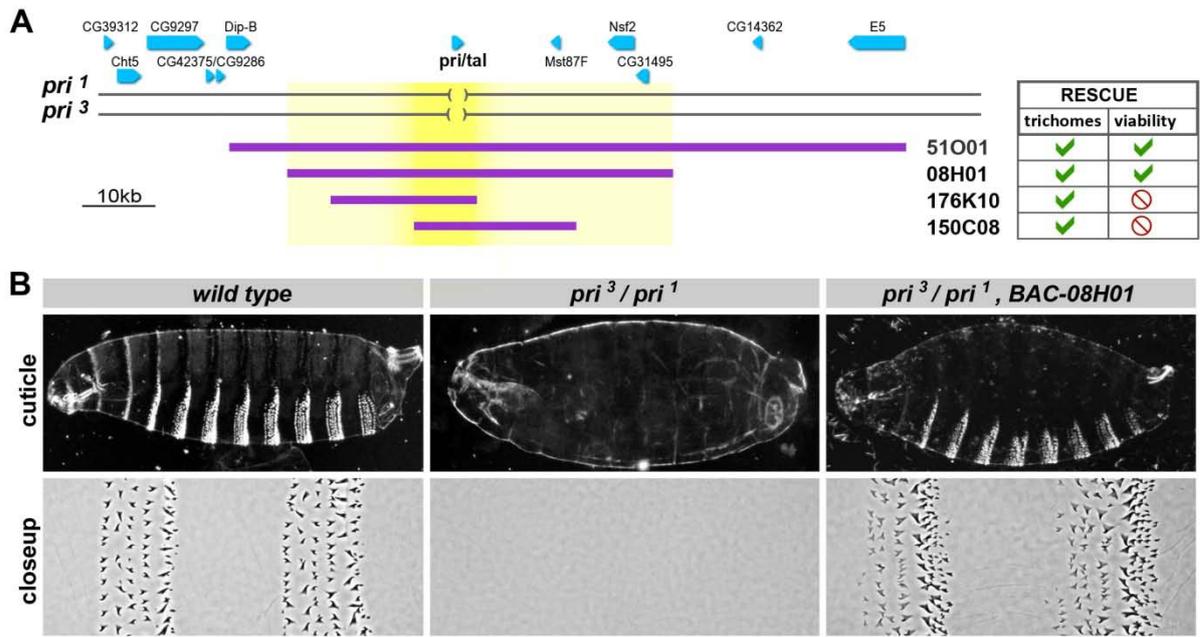


Figure 1

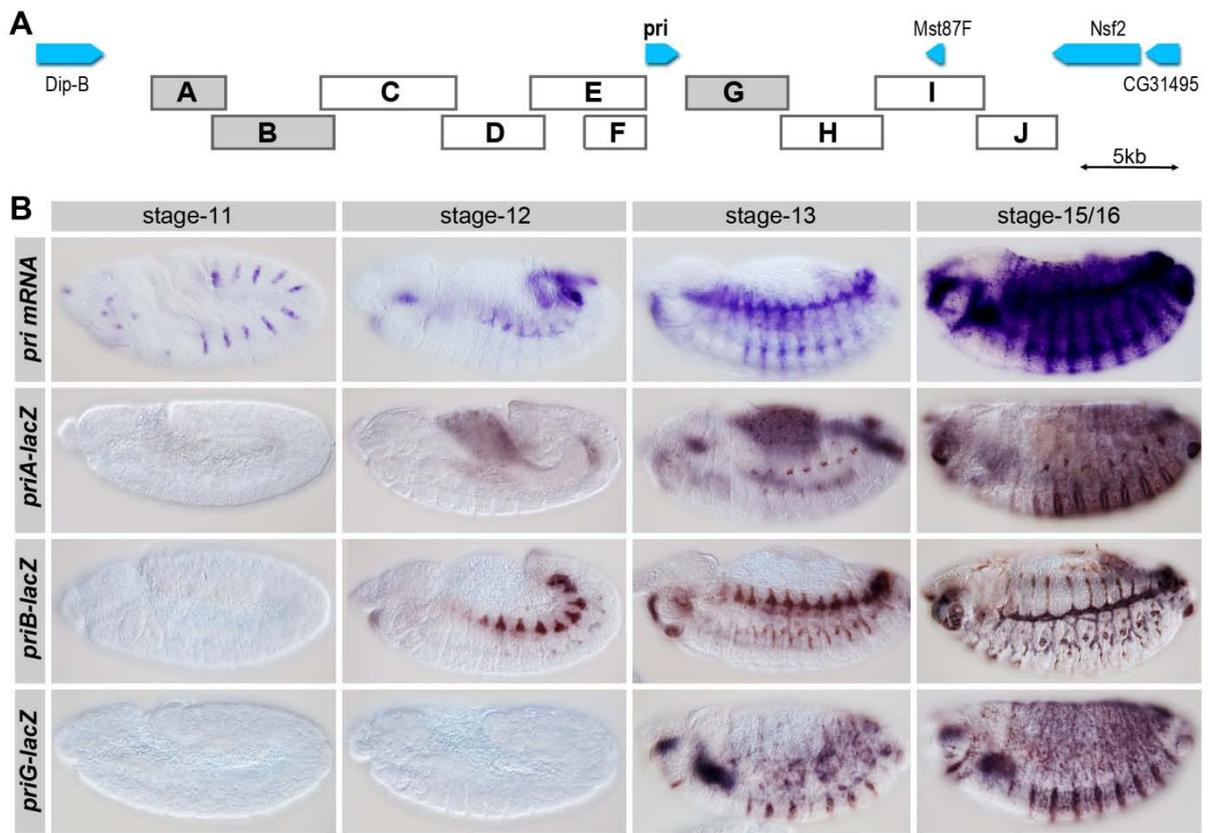


Figure 2

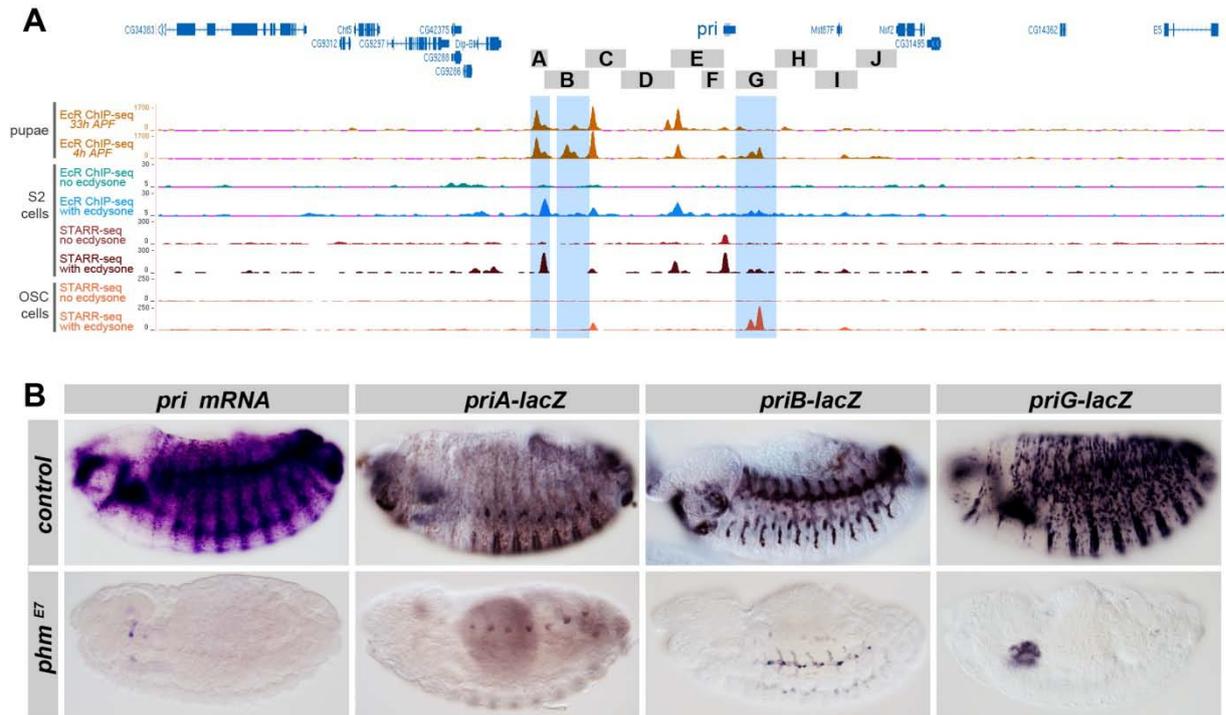


Figure 3

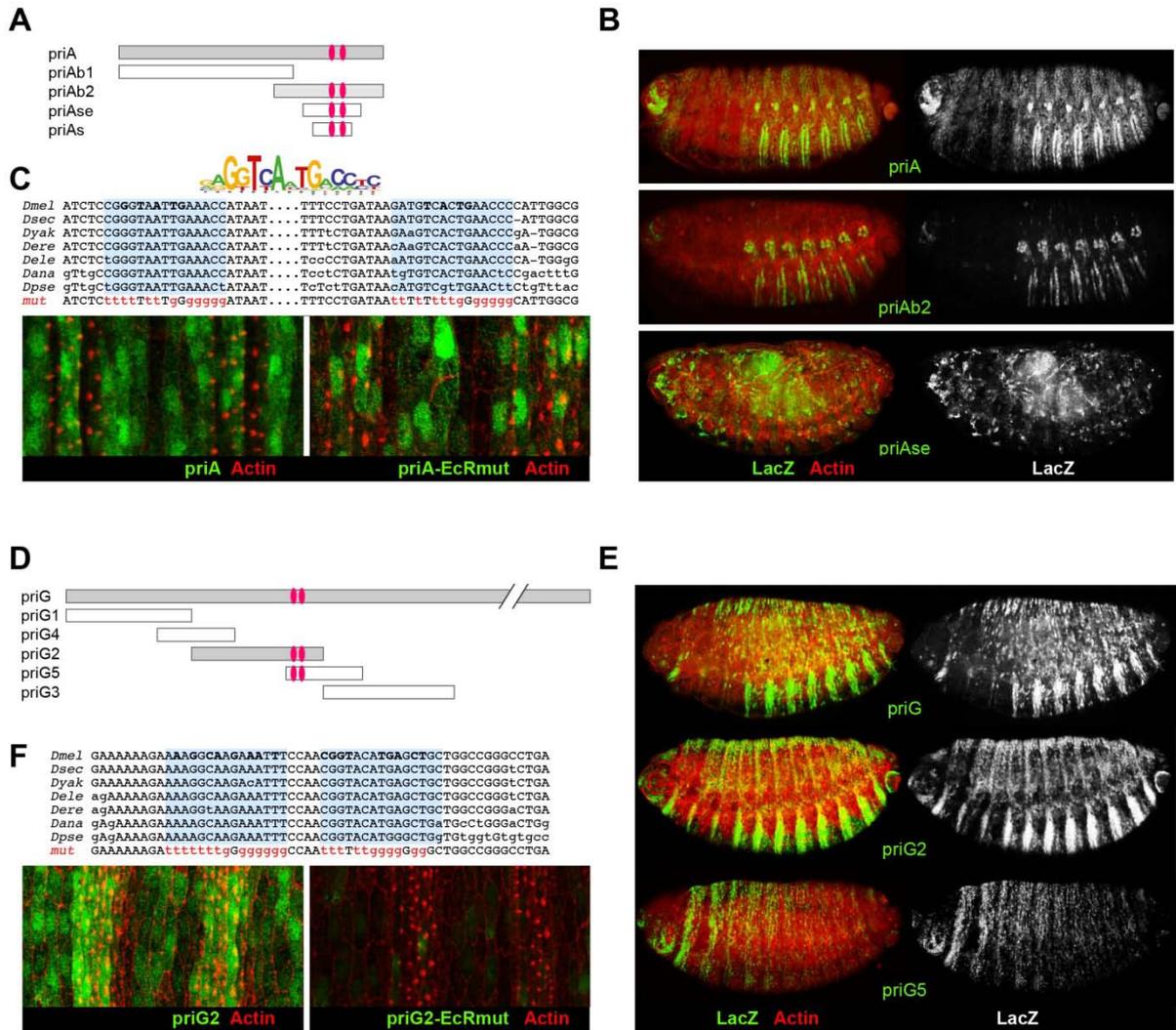


Figure 4

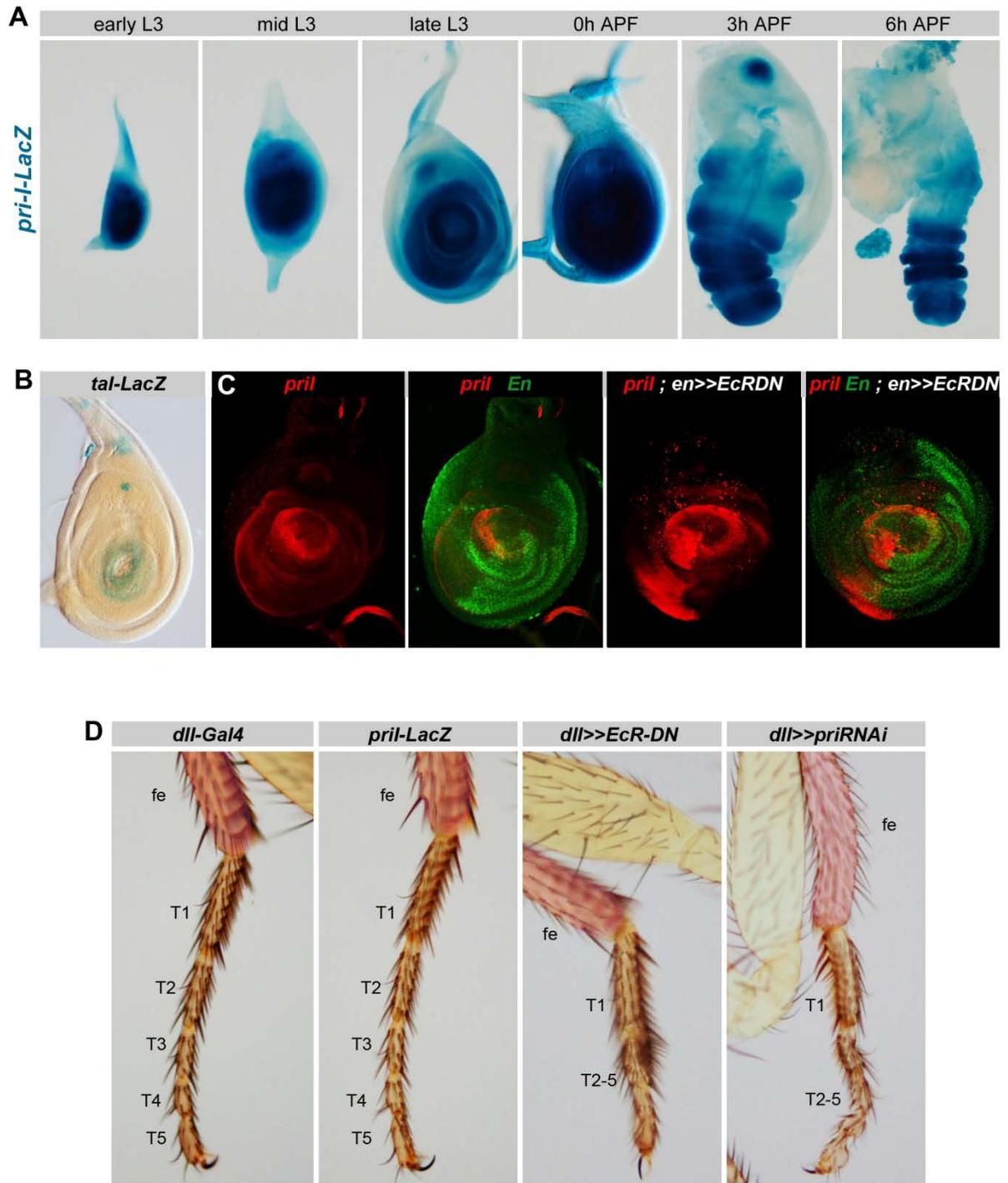


Figure 5

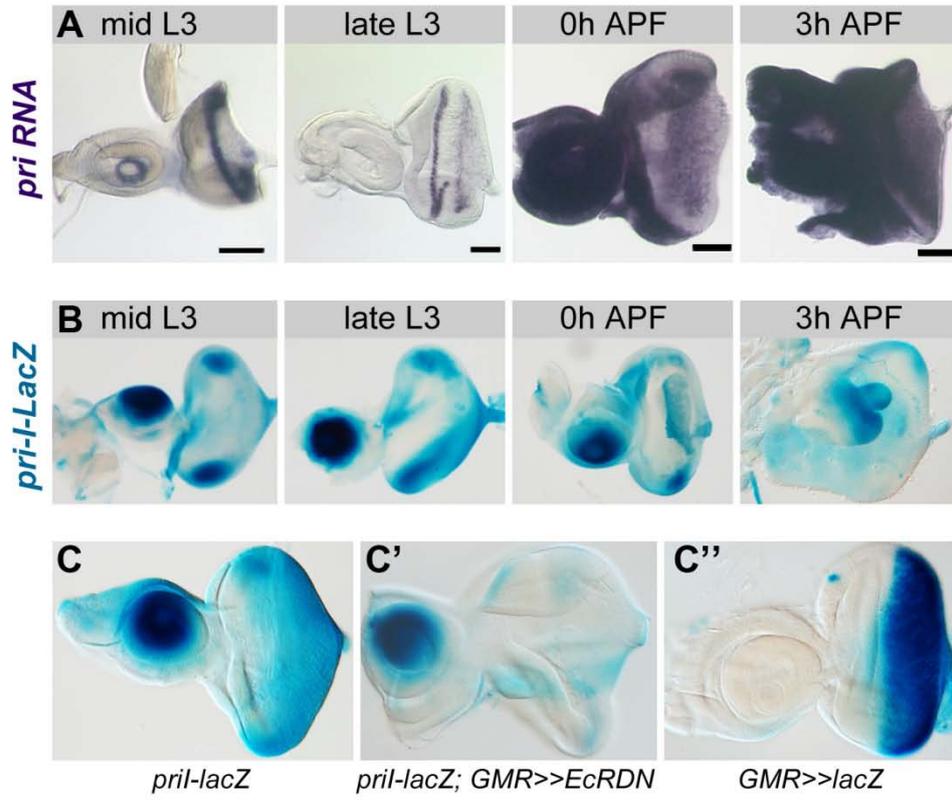


Figure 6

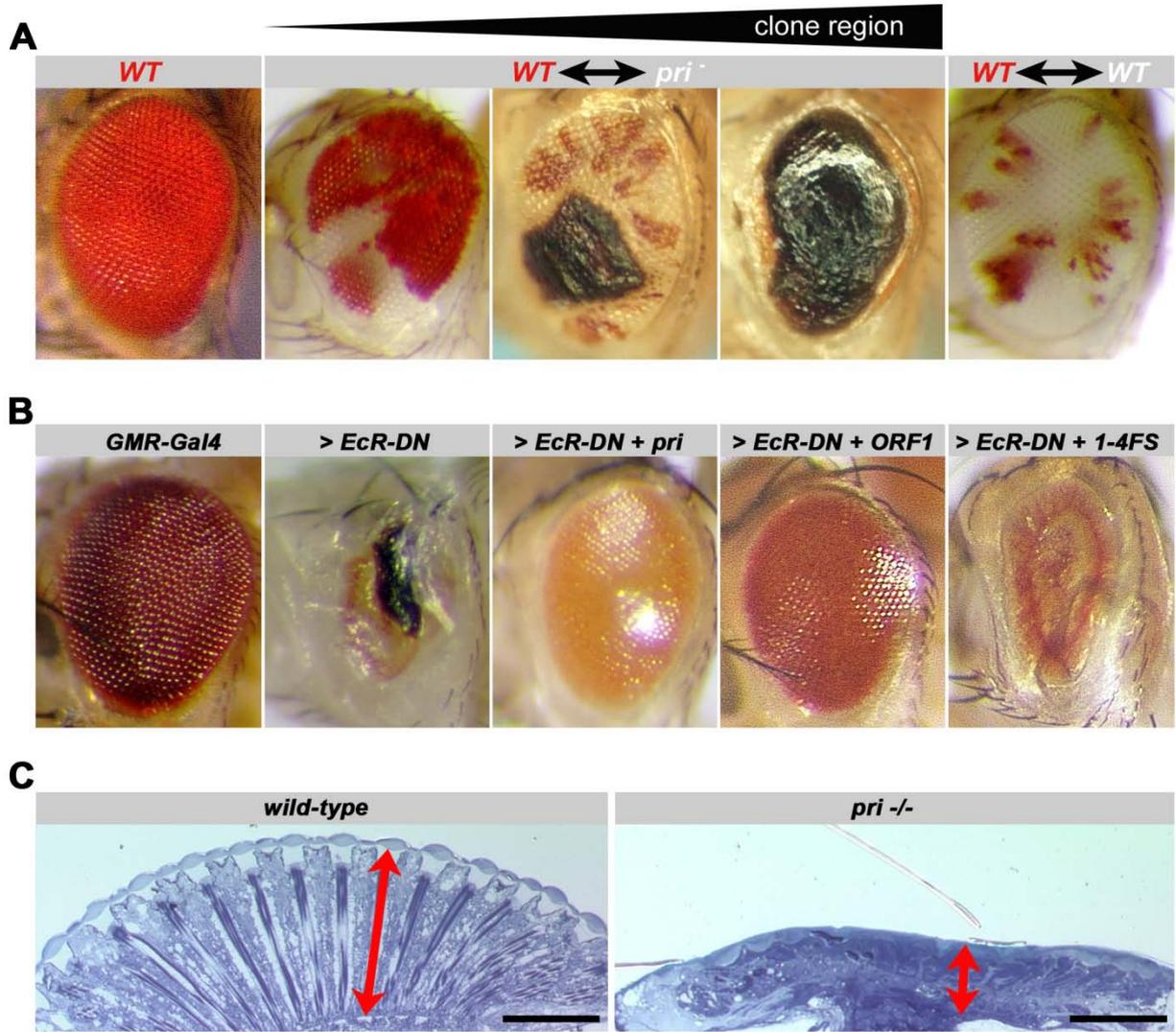


Figure 7

Supplementary material

Figure S1

A. Schematic representation of the *pri* locus. The genomic regions tested using lacZ-reporter transgenic lines are represented as grey boxes. **B.** Activity of *pri* genomic regions C, D, E, H, I and J from stage-11 to 16, showing weak embryonic expression and/or expression in other tissues than the epidermis and trachea.

Figure S2

A. In situ hybridization showing *pri* RNA expression in wild type (left column) and embryos expressing EcR-DN driven by *ptc-Gal4* (right column). The top row shows lateral views, the bottom row ventral views. **B.** LacZ immunostaining showing that *priG* activity is impaired in epidermal cells expressing EcR-DN under the control of the *ptc-Gal4* driver (right panel) compared to control (left panel).

Figure S3

A. Schematic representation of the *pri* mutations generated in this study. *XP d06750* and *XP d01244* are parental insertion strains used to generate the *pri* mutants; *pri⁴* and *pri⁵* are amorphic alleles lacking a promoter and all four functional ORFs, resulting in embryonic lethality and the complete loss of ventral denticles and dorsal trichomes (data not shown). **B.** Mosaic flies showing clones of *pri* mutant cells. Under moderate heat shock conditions (heat shock for one hour during 48-72 hours after egg laying), *hs-Flp; FRT82B RpS3/FRT82B pri⁴* flies showed malformed appendages, with kinked femur segments (fe) and fused tarsal leg

segments (T2-5). Mitotic clones of *pri* mutant cells (*hs-FLP;FRT82B y+ RpS20/FRT82B pri^d*) in the thoracic epidermis (right panel), showing loss of dorsal hairs at the center of large mutant clones.

Table S4

Genomic position of the DNA fragments used for lacZ-reporter lines and transgenic rescuing BACs. The sequence of oligonucleotide primers used for these studies is also indicated.

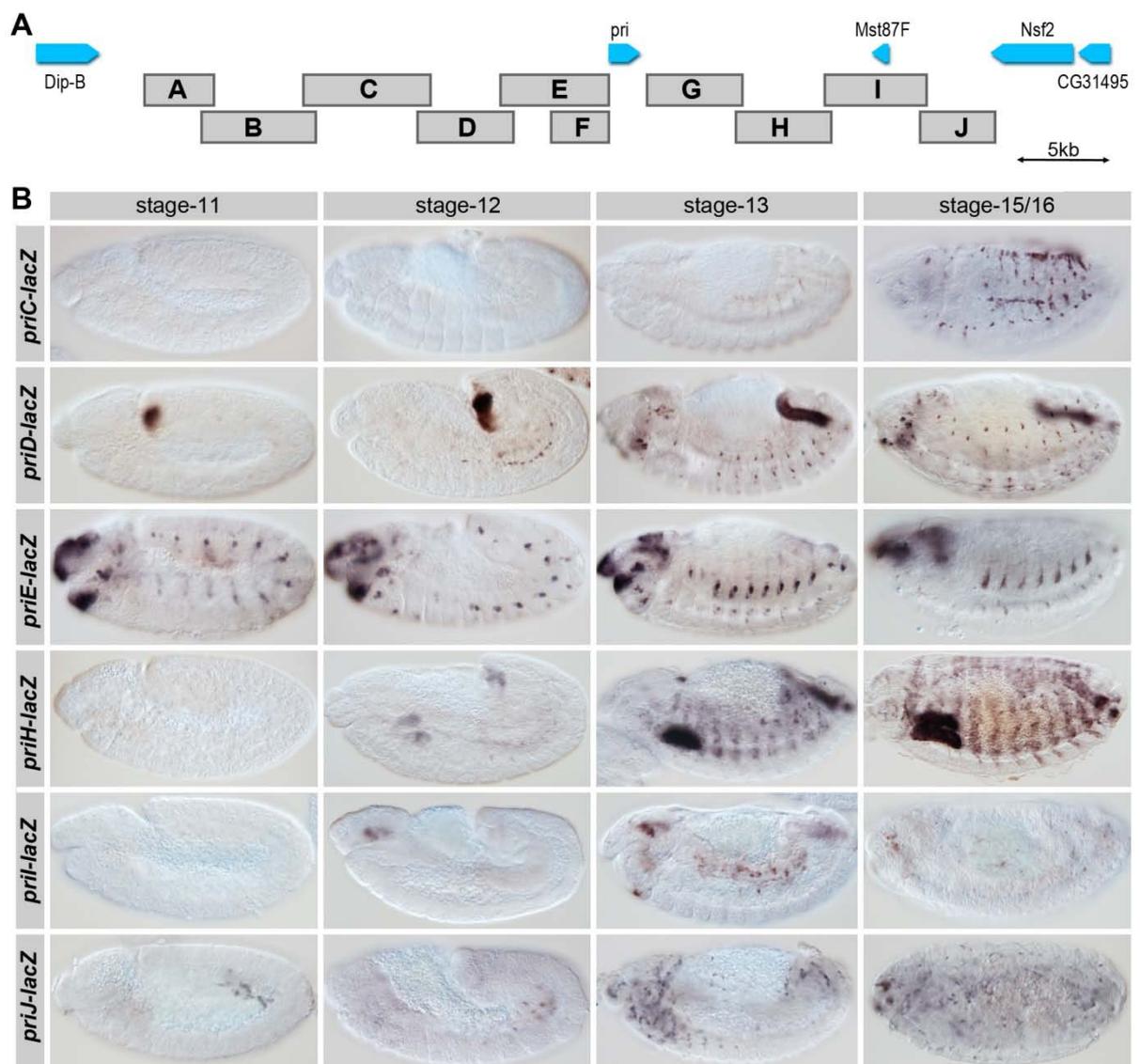


Figure S1

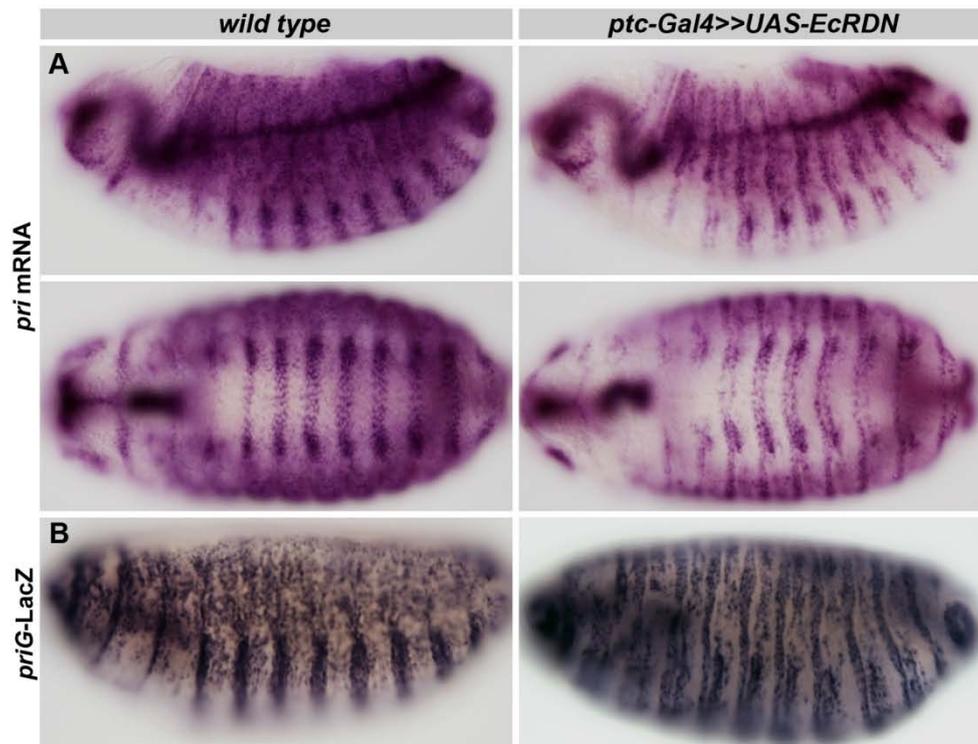


Figure S2

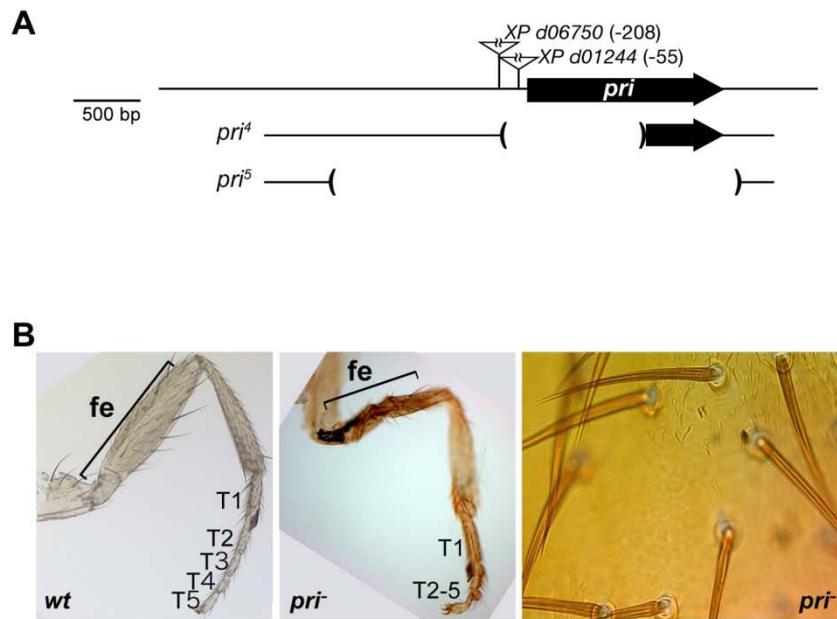


Figure S3

<i>release R5</i>			
constructs	start (3R:)	end (3R:)	size (bp)
<i>priA-LacZ</i>	9 615 076	9 617 126	2 051
<i>priA-EcR1mut-LacZ</i>	9 615 076	9 617 126	2 051
<i>priA-EcR2mut-LacZ</i>	9 615 076	9 617 126	2 051
<i>priA-EcRmut-LacZ</i>	9 615 076	9 617 126	2 051
<i>priAb1-LacZ</i>	9 615 071	9 616 426	1 355
<i>priAb2-LacZ</i>	9 616 323	9 617 126	803
<i>priAse-LacZ</i>	9 616 475	9 616 896	421
<i>priAs-LacZ</i>	9 616 557	9 616 883	326
<i>priB-LacZ</i>	9 616 627	9 622 245	5 619
<i>priC-LacZ</i>	9 621 746	9 626 730	4 985
<i>priD-LacZ</i>	9 626 231	9 632 835	6 605
<i>priEA-LacZ</i>	9 632 336	9 639 247	6 912
<i>priES-LacZ</i>	9 632 336	9 638 545	6 210
<i>priFA-LacZ</i>	9 636 122	9 639 247	3 126
<i>priFS-LacZ</i>	9 636 122	9 638 545	2 424
<i>priG-LacZ</i>	9 640 288	9 645 061	4 773
<i>priG1-LacZ</i>	9 640 288	9 641 279	991
<i>priG2-LacZ</i>	9 641 262	9 642 281	1 019
<i>priG2-EcRmut-LacZ</i>	9 641 262	9 642 281	1 019
<i>priG3-LacZ</i>	9 642 258	9 643 278	1 020
<i>priG4-LacZ</i>	9 641 019	9 641 602	583
<i>priG5-LacZ</i>	9 642 001	9 642 584	583
<i>priH-LacZ</i>	9 645 042	9 650 045	5 003
<i>priI-LacZ</i>	9 650 026	9 655 081	5 055
<i>priJ-LacZ</i>	9 655 070	9 660 195	5 125

Table S4

Discussion

- **Spatio-temporal expression of *pri* during *Drosophila* development**

pri distribution is dynamic in several tissues during morphogenesis.

Understanding when and where developmental genes are expressed often provides insights into their function and underlying mechanisms. Several studies have monitored the developmental expression of *pri* RNA across tissues and stages throughout the development of insects and this has helped disclosing various developmental functions of *pri*.

A characteristic feature of *pri* is its specific expression in a restricted set of developing tissues, as well as highly dynamic changes between successive developmental stages of *Drosophila* embryogenesis (Inagaki et al., 2005; Tupy et al., 2005). In a same vein, the highly specific expression in the posterior growth zone of *mille pattes*, the *pri* ortholog in *Tribolium*, was a first hint of its function in the segmentation of the beetle embryo (Savard et al., 2006). In flies, the dynamics of *pri* mRNA often prefigures tissues the differentiation of which are altered in *pri* mutant embryos. *pri* is well expressed in the developing tracheal system from stages 9 to 15 (Kondo et al., 2007), and the lack of *pri* function leads to various defects in its initial formation (Galindo et al., 2007) and later differentiation (Kondo et al., 2010; Ozturk-Colak et al., 2016). *pri* mutant embryos display breaks in the dorsal trunk branches of the trachea, as seen during mid-embryogenesis (Kondo et al., 2007; Kondo et al., 2010) and, later on, *pri* mutant tracheal cells fail to differentiate actin-rich tenidia, which are apical supra-cellular structures involved in the physical properties of the tracheal system (Kondo et al., 2010; Ozturk-Colak et al., 2016). *pri* also displays a transient but prominent wave of expression in the embryonic epidermis and *pri* mutants display severe defects in epidermal differentiation including the complete lack of trichomes (Chanut-Delalande et al., 2014; Galindo et al., 2007; Kondo et al., 2007; Kondo et al., 2010). However, other specific domains of *pri* expression remain hitherto unlinked to any known function of *pri*. Blastoderm embryos display a clear segmental expression of *pri*, evoking that of *even-skipped* for example, but previous studies failed to detect obvious defects in segmentation, including in embryos depleted for both the maternal and zygotic contribution of *pri* (Galindo et al., 2007; Kondo et al., 2007) (H. Chanut, unpublished results). Whether this expression represents an evolutionary remnant (*tribolium* etc...) of *pri* function in the segmentation of more basal insect species is a possibility. In addition, work in our team has not detected segmentation defects in maternal and zygotic E3 ligase *Ubr3* (*Ubr3^{mat+zyg}*) mutant embryos. It should be noted yet that *Svb* displays early expression in two anterior ventral stripes in blastoderm embryos and work is ongoing to test a possible role of *pri/ubr3/svb* in the proper formation of head segments, which segmentation is more delicate to analyze than in the trunk. There are additional embryonic tissues showing specific *pri* expression, such as the foregut and hindgut primordia (See fig. 23) and testing a possible function of *pri* in these tissues will await further studies.

The highly dynamic expression of *pri* is also obvious upon monitoring whole body levels of *pri* RNA, both from RNA-seq data and northern blot experiments, across embryonic and post-embryonic stages (Chanut-Delalande et al., 2014). These temporal waves of *pri*

RNA expression strikingly match those of the early-late 20E-response gene *DHR3/Hr46* (*Drosophila hormone receptor 3*, aka *Hr46*), while they are clearly distinct from the intermoult transcript β -*FtzF1* (Chanut-Delalande et al., 2014), consistent with the hypothesis that *pri* behaves as a primary ecdysone responsive gene. *In situ* hybridization of *pri* RNA at the larval/pupal transition shows a specific expression in leg discs that begins at mid L3 touches-down at late L3 and then becomes strong again and relatively ubiquitous between 0 and 3h APF (Chanut-Delalande et al., 2014). Interestingly, this expression of *pri* is also required for adult leg development and tarsal joint differentiation (Galindo et al., 2007; Pueyo and Couso, 2008, 2011). Similarly, *pri* expression displays highly dynamic spatiotemporal patterns in the eye/antenna and wing discs during third instar development and larval/prepupal transition (see fig. 25).

Hence the remarkable dynamics of *pri* expression is suggestive of functions for Pri peptides in a wide variety of tissues. In addition, the temporal waves of *pri* expression seen over developmental stages may also indicate its implication in the control of developmental timing.

Identification of pri cis-regulatory regions.

To better understand the role of *pri* during development, it thus was of interest to identify the cis-regulatory regions responsible for its complex expression pattern, both across tissues and over time.

As a first step, we sought to define the functional unit of the *pri* gene during development, using genetic rescuing assays of null *pri* mutant alleles that completely remove the *pri* transcription unit (Kondo et al., 2007) as well as the trichomes. We generated Bacterial Artificial Chromosomes (BAC) genomic constructs and tested their ability to rescue the lack of *pri* activity, when introduced in a trans-heterozygous *pri* mutant combination. Concerning the embryonic trichomes, we observed a rescuing activity in all four BACs used in order to cover the entire region of *pri*, allowing us to restrict the minimal region indispensable in the embryonic epidermis to an 8.7 kb DNA region that represents the overlapping region of all BACs. On the other hand, the smaller BAC capable of restoring the full development and adult viability of *pri* mutants spans over more than 50kb. This region outlines the common region of the two BACs that rescue the lethality.

Thus the function of the *pri* gene, which encodes an intron-less RNA of 1.5 kb, requires an unexpectedly large genomic region. To go further and decode the expression of the regulatory sequences of this area, we next dissected this genomic region into ten DNA fragments, called *priA* to *priJ*; *priA* is about 2 kb in length while the others are almost 5 kb each. We inserted them upstream of a LacZ reporter, generated corresponding transgenic targeted lines and their activity was profiled using immunocoloration. In the embryonic tracheal system, we observed an expression that begins at stage-12, *i.e.* little after the endogenous expression of *pri* that starts at stage-11 of embryogenesis. On the other hand, we noticed an embryonic epidermal expression in both the *priA* and *priG* enhancers, with different expression patterns. *priG* represents sort of a mirror of the endogenous expression of *pri* in the embryonic epidermis, and thus it is strongly expressed in cells which give trichomes, while *priA* is expressed in both naked and trichome epidermal cells. The activity of both epidermal enhancers starts at stage 13, as the timing of the onset of endogenous expression. Interestingly, only 3 kb from the 5' extremity of *priG* are included in the minimal region indispensable for the function of *pri* in the embryonic epidermis, while *priE* and *priF*

are not. This allows us to restrict the region necessary for *pri* expression in the embryonic epidermis from 8.7kb to 3kb. Surprisingly, *priA* that resides almost 20 kb upstream of the *pri* promoter is not included in this minimal region. Thereby, *priA* may likely be considered as a shadow enhancer (Frankel et al., 2010; Lagha et al., 2012) of *pri* in the embryonic epidermis, whereas *priG* is the main enhancer responsible of *pri* expression pattern. Since *pri* is expressed at early stages during *Drosophila* development, it was of interest to know which enhancers are able to recapitulate this early expression. Thus we aimed to have an overview of the enhancer pattern in this context, and we found two enhancers, *priC* and *priE*, which possess some early expression in embryos. However, none of them were sufficient to faithfully capture endogenous expression of *pri* at early blastoderm stages.

As mentioned above, *pri* is expressed in the larval leg primordium and plays a pivotal role in adult leg formation, so we purposed to look for the enhancers responsible for driving the expression of *pri* in imaginal discs at larval and prepupal stages. We therefore systematically assayed for the expression of our series of ten *pri* enhancers, at several times from the early third instar larvae till 8 hours after puparium formation (APF). While we noticed a relatively weak and transient expression of *priA*, *priB* and *priG* in the leg primordium, the *priI* enhancer appears as the main element driving expression in the developing leg. *Pri-I* is highly expressed in the leg disc, specifically in the central region that ultimately gives rise to the tarsal segments. *PriI* is not solely expressed in leg discs, it also drives strong expression both in the eye/antennal and wing discs, at larval and prepupal stages. Remarkably, the *Male-specific transcript at 87F* gene (*Mst87F*) is included in the *priI* enhancer. It was shown in the literature that this gene has little expression in the fat body of larvae and not in other larval tissue. Contrariwise, a significant expression has been reported in the adult male testis (Kuhn et al., 1991). This probably rules out *Mst87F* being expressed in leg and eye-antennae discs, but it does not prevent to test this hypothesis eventually.

- **Pri regulation and the crosstalk with the ecdysone hormone.**

Several regulatory regions driving pri expression are under the control of ecdysone.

Steroid hormones are well known for their role in the temporal coordination of development, and the ecdysone secreted from the prothoracic gland is the major insect hormone that controls the larval/prepupal transition (Ou and King-Jones, 2013; Yamanaka et al., 2013). How the systemic action of ecdysone is ultimately implemented within genetic programs governing tissue-specific differentiation remains not fully understood. Our studies show that the expression of *pri* is under direct control of ecdysone signaling, providing an additional piece of evidence to better understand the hormonal control of *Drosophila* development.

The first hint supporting a possible link between *pri* and ecdysone signaling came from indirect evidence gleaned in the literature. A study on ecdysone function during embryogenesis, has reported that mutants unable of ecdysone synthesis (*sad* and *shd*) display defects both in head involution and tracheal morphogenesis (Chavoshi et al., 2010), both phenotypes having been noticed in *pri* mutant embryos. In addition, the proper development of the tracheal system requires both EcR and Usp functions, and Eip75B (E75; an early 20E-response gene) consistently displays an ecdysone- dependent expression manifest in tracheae (Chavoshi et al., 2010). Finally, defective air-filling of the tracheal lumen in late embryogenesis was observed in *DHR3* and *Ftz-f1* mutants (Ruaud et al., 2010), a phenotype that was likewise observed in *pri* mutants (Galindo et al., 2007; Kondo et al., 2007).

More compelling evidence was provided by a forward genetic screening performed in the lab, systematically examining consequences of short genomic deletions on the differentiation of trichomes. Screening of the whole second chromosome identified a single region whose deletion fully prevents trichome formation. Consistently, the expression of trichome effectors was lost in this background, a similar phenotype of that observed in a *pri* mutant. However, the expression of *shavenbaby* mRNA was unaffected, suggestive of an impaired maturation of the Svb TF, as further demonstrated by the persistence of the large-sized repressor form. Indeed, these epidermal defects resulted from a complete absence of *pri* expression. Rescuing experiments making use of BAC constructs and a series of smaller transgenes identified the gene responsible for this phenotype, encoding a Glutathione S-Transferase enzyme, called GstE14 (Chanut-Delalande et al., 2014) or *noppera-bo* (Enya et al., 2014). When the screening of the third chromosome started, it became clear that several regions displayed the same phenotype and available mutants rapidly identified known components of the ecdysone biosynthetic pathway. Several pieces of evidence further demonstrated that GstE14 was a novel member of the ecdysone synthetic pathway. First, GstE14 was expressed in the prothoracic gland, from late embryogenesis to larval stages. Second, the lack of GstE14 or its specific depletion in the ring gland produces developmental arrest accompanied by a global increase into sterol levels. Importantly, the lethality of *GstE14* embryos was suppressed when mothers were fed with high cholesterol diet. Finally, the epidermal defects observed in the absence of *GstE14* function could be largely rescued by incubation of mutant embryos with ecdysone. Furthermore, we observed an abolishment of the endogenous expression of the *svb* target gene *dyl* in absence of ecdysone, similar result observed for *pri* mRNA in GstE14 mutant. Together, these data therefore showed that *pri* expression in the embryo requires ecdysone. In later stages of development, *pri* RNA levels display temporal cycling at the larval to prepupal transition. These temporal waves of *pri* expression were also obvious within individual tissues, as well illustrated in the developing leg disc. Furthermore, we found that incubation of dissected leg discs with ecdysone was sufficient to induce premature expression of *pri* in this tissue.

Whereas these data established that ecdysone is required, and somehow sufficient, to induce *pri* expression, molecular profiling demonstrated direct *in vivo* binding of EcR on presumptive *pri* cis-regulatory elements, showing that *pri* is a primary response gene (Chanut-Delalande et al., 2014). Chip-seq analyses for EcR in pupal stages well known for their high ecdysone titer outline the fact that EcR binds to regions flanking the *pri* gene, contrasting with the absence of EcR peaks in neighboring genes (Chanut-Delalande et al., 2014). In addition the independent Chip-seq analysis done in cultured cells, shows that EcR binds to the *pri* locus, and specifically upon the addition of ecdysone (Shlyueva et al., 2014). The same study also confirmed that EcR/20E binding promotes the transcription of *pri* in S2 cells.

Interestingly several of the major peaks of EcR binding, either *in vivo* or *ex vivo* overlaps with the location of *pri* enhancers, which were identified by our transgenic assays. As observed for the endogenous expression of *pri* RNA, we found that the activity of the three main embryonic enhancers is compromised in mutants devoid of ecdysone. For example, introducing *priG-LacZ* in a *phm* mutant background abolished its epidermal expression. We performed the same experience to test the *priA* enhancer and, as expected, its expression is likewise under the control of ecdysone. Still in the embryo, the *priB* enhancer also undergoes a significant reduction of its expression in *phm* mutant embryos. Subsequently, in order to delineate the smallest region capable of driving epidermal expression, we dissected *priG* into five shorter segments (*priG1-5*). While *priG1* and *priG4* didn't display detectable expression, *priG3* and *priG5* drove only weak expression in the

embryonic epidermis. In contrast, we observed a strong epidermal expression driven by *priG2* reminiscent to that of *pri* RNA. As for endogenous *pri*, and the *priG* enhancer, the expression of *priG2* was also under the control of ecdysone. We then used the JASPAR database to look for EcR binding sites in that enhancer and it predicted two putative EcR/Usp putative binding sites. The mutation of both conserved EcR binding sites found in *priG2* leads to the abolishment of epidermal activity of *priG2*, thus we defined *priG2* about 1 kb in length as a sufficient DNA fragment to drive epidermal activity directly dependent on EcR/20E binding.

Concerning the *priA* enhancer, it contains a short region recently identified as one of the strongest ecdysone-dependent enhancer in S2 cells (Shlyueva et al., 2014). Furthermore, the mutagenesis of this fragment showed that its activity relies on direct EcR/20E binding, as well as on the integrity of two DNA motifs that resemble GATA Binding sites (Shlyueva et al., 2014). However, when assayed *in vivo* neither this short region (*priAs*), nor an extended region of it (*priAse*), were able to drive any epidermal expression. Instead, we observed a likely ectopic expression in embryonic hemocytes, and it is noteworthy that the GATA factor Serpent (Srp) is a regulator of blood cell differentiation (Rehorn et al., 1996) and is active in S2 cells (Ramet et al., 2002). Taken together, these results suggest that the expression of *priAse* in hemocytes could be the fruit of GATA activity. We found that the mutation of both EcR binding sites in the backbone of *priA* does not significantly affect its epidermal expression; a work is in progress to define whether the mutation of both mutated EcR binding sites is sufficient to affect the activity of *priAb2*, the smallest subregion of *priA* we delineated to drive expression in the embryonic epidermis.

Therefore, these results identified three ecdysone-dependent enhancers driving *pri* expression during embryogenesis. They all correspond to prominent peaks of EcR binding, as confirmed by our and independent ChIP-seq studies. Focusing on epidermal expression, the two remote *pri* enhancers contain functional EcR/Usp binding sites, required for their activity as demonstrated for *priG2* *in vivo*, and as supported for *priA* by *ex vivo* data.

Pri relays ecdysone signaling for the temporal control of epidermal differentiation

Our work demonstrates that the onset of the differentiation of embryonic trichome cells is triggered by ecdysone. Indeed, others and we have observed drastic defects in epidermal differentiation following the inactivation of any ecdysone synthesizing enzyme (Chanut-Delalande et al., 2014; Chavez et al., 2000; Enya et al., 2014; Namiki et al., 2005; Niwa et al., 2004; Niwa et al., 2010; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2004; Yoshiyama et al., 2006). More specifically, we show that ecdysone is required for the early steps of trichome cell remodeling, even before cuticle deposition, as revealed by the absence of any sign of actin reorganization in embryonic epidermal cells lacking ecdysone (Chanut-Delalande et al., 2014). In addition, we provide evidence that *pri* expression, and thereby Svb maturation, are under the direct control of ecdysone signaling. Importantly, we found that restoring *pri* expression in the epidermis is sufficient for a significant rescue of trichome formation in the complete absence of ecdysone. Therefore, the ecdysone pathway drives epidermal trichome remodeling through the regulation of *pri* and more broadly the expression of *pri* in the epidermis is sufficient to outdo the lack of ecdysone. These results thus provide an uninterrupted molecular pathway to explain the action of ecdysone in the terminal differentiation of trichome cells.

Knowing that ecdysone signaling plays a crucial role in the control of developmental timing (Yamanaka et al., 2013), it was our aim to decipher if the main role of *pri* peptides is likewise to provide a temporal control of the execution of the trichome differentiation program. Consistent with this idea, the onset of *pri* expression occurs at stage-13/14, in wild type embryonic epidermal cells, *i.e.* is nicely concomitant with the timing of Svb maturation. To further challenge this notion, we assayed whether anticipating *pri* expression was sufficient to trigger a premature expression of the transcriptional program of trichome differentiation. Indeed, we found that pushing an early *pri* expression in dorsal epidermal cells is sufficient to induce premature expression of trichome effectors in these cells, likely because of a precocious activation of the Svb TF, since we observed a similar phenotype when driving a constitutive activator form of Svb. Hence these results show that *pri* mediates EcR/20E-dependent temporal control of the expression of trichome effectors in the embryonic epidermis.

Epidermal differentiation repeatedly occurs before each of the developmental transitions, which are well known to be temporally controlled by ecdysone pulses (Yamanaka et al., 2013). Therefore we assayed whether the same ecdysone/*pri*/*svb* circuit could be reactivated during post-embryonic development, investigating the terminal differentiation of adult epidermal cells. Indeed, most adult epidermal cells make trichomes and previous work has shown that *svb* is required for their differentiation (Delon et al., 2003). In addition, the cellular effectors identified as direct targets of Svb in the embryo (Andrew and Baker, 2008; Chanut-Delalande et al., 2006; Chanut-Delalande et al., 2012; Chanut-Delalande et al., 2014; Fernandes et al., 2010; Menoret et al., 2013) are also required for the differentiation of adult trichomes (Adler et al., 2013; Bilousov et al., 2012; Lu et al., 2010; Nagaraj and Adler, 2012; Ren et al., 2006; Ren et al., 2005; Roch et al., 2003; Sobala and Adler, 2016; Yan et al., 2008).

To address this issue in more depth, we explored the timing of adult epidermal cell remodeling, focusing on the dorsal thorax (notum) as a model system. We found that the first signs of trichome formation as monitored by actin remodeling occur at 38-40h APF, when epidermal cells have finished their proliferation and have well established apico-basal and PCP polarity (Bosveld et al., 2012; Herszterg et al., 2013). Interestingly, we observed that the Svb repressor accumulates in epidermal cell nuclei from early stages of prepupal/pupal development, and its maturation adequately coincides with the onset of trichome differentiation. Furthermore, the expression of *pri* RNA in the pupal notum is also concomitant, displaying a transient wave of expression at 38-42h. Several pieces of evidence further support the conclusion that the ecdysone-dependent expression of *pri* also times the onset of trichome differentiation in the adult notum. First, the artificial expression of a constitutive activator form of Svb is sufficient to induce, in a cell autonomous manner, the premature formation of trichomes when neighboring cells are still kept undifferentiated through the activity of the endogenous Svb repressor. Reciprocally, the expression of a constitutive repressor form of Svb, which is thus insensitive to *pri*, is capable of blocking the formation of trichomes. Together, these data show the importance of the temporal control of Svb maturation in adult epidermal cells. Second, the expression of *pri* in clones of early epidermal cells is sufficient to promote premature differentiation of trichomes. Finally, the expression of a dominant negative form of the EcR nuclear receptor, which prevents *pri* expression, also causes epidermal cells to be refractory to trichome formation (Chanut-Delalande et al., 2014). We therefore interpret these results to imply that a main role of Pri peptides is to provide, in the response to ecdysone, a strict temporal control of the differentiation of trichome cells throughout embryonic and post-embryonic *Drosophila* development.

Additional functions of pri as a mediator of ecdysone signaling during development.

Having firmly established that *pri* plays a key role in mediating the ecdysone signal for proper developmental timing of epidermal trichomes, I will present here additional evidence that Pri peptides are also involved in relaying ecdysone action throughout other stages and tissues.

pri RNA expression in the leg disc at larval/prepupal transition requires ecdysone, which is further sufficient to induce a premature burst of *pri* expression in larval leg discs upon their *in vitro* incubation with synthetic ecdysone hormone (Chanut-Delalande et al., 2014). Following our profiling of the transcriptional activity of *pri* cis-regulatory regions, we found that *priI* captures, at least in large part, the regulation of *pri* in this tissue. The *priI* enhancer is expressed in leg primordia, where it accumulates a series of concentric rings in the central region, which will ultimately give rise to the distal structures of legs (Estella et al., 2012; Guarner et al., 2014; Lecuit and Cohen, 1997) including the tarsal segments affected in the absence of *pri* (Galindo et al., 2007; Pueyo and Couso, 2008, 2011; Suzanne, 2016). Disrupting ecdysone signaling through targeted expression of a dominant negative form of the ecdysone receptor (EcR-DN) (Cherbas et al., 2003) down regulated *priI* activity in the leg disc. Furthermore, we found that EcR-DN also strongly impaired adult leg formation, which lack well-differentiated tarsal segments, as also seen following the RNAi-mediated knockdown of *pri* function under similar conditions. These results thus support that ecdysone is also an important aspect of the regulation of *pri* expression and function for the development of adult legs.

Morphogenesis of the *Drosophila* eye begins early in the third larval stage (L3) and continues during pupal development (Huang et al., 2015). We found a *priI* activity in the developing eye/antenna imaginal disc, which is also influenced by perturbing ecdysone signaling. This expression yet only partly reproduces endogenous *pri* expression, which displays again a very dynamic spatiotemporal pattern across larval and prepupal stages, as monitored by *in situ* hybridization. Collaborative work done in the Kageyama's lab shows that *pri* function is actually indispensable for the proper differentiation of adult eyes. Indeed, while small clones of *pri* mutant cells do not affect eye differentiation, reinforcing by that the conclusion that the action of Pri peptides is not cell autonomous in different tissues (Chanut-Delalande et al., 2014; Kondo et al., 2007; Pueyo and Couso, 2008, 2011), larger clones and eyes completely lacking *pri* function display strong defects, with cells unable of differentiation and presenting instead features of necrotic melanized cells. Driving EcR-DN impinges on the development of various tissues (Cherbas et al., 2003) and it has been shown that endogenous EcR function is required for the differentiation of photoreceptors during pupal stages (Sprecher and Desplan, 2008). Interfering with EcR function in post-mitotic eye cells produces strong defects, with a pronounced reduction in the eye size and the presence of necrotic melanized cells. Importantly, we show that these defects are completely rescued following co-expression of *pri* together with EcR-DN. Furthermore, an artificial construct that encodes a single smORF Pri peptide also displays full rescuing activity of EcR-DN defects, both for eye development and pupal lethality. In marked contrast, a *pri* RNA construct that contains four point mutations frame-shifting the sequence of Pri peptides is devoid of any rescuing activity. These functional data therefore established that *pri* is required for adult eye differentiation. In addition, *pri* re-expression in cells which are unable to interpret the ecdysone signal (in presence of EcRDN) is sufficient to rescue their proper differentiation, suggesting that *pri* is also an important mediator of ecdysone for adult eye development during pupal metamorphosis.

Conclusion

During embryonic development, cells are oriented towards their final destiny, through cell signaling pathways and TFs, which are expressed specifically in those cells. These regulatory cues converge at CRMs, which in turn promote the expression of differentiation genes, thus defining the developmental fate of each cell.

In this dissertation, we presented our progress to shed light on the temporal function and expression of a novel kind of regulatory gene, *pri*, which encodes four small peptides throughout *Drosophila* development. These results provide a conceptual framework to better understand the mechanisms coordinating cell behavior in response to endocrine steroid hormone signals. Our work establishes a continuous molecular pathway allowing the connection from early stages of ecdysone production (GstE14), to the direct control of *pri* enhancers by EcR/20, and their consequences for the temporal control of epidermal trichome differentiation (maturation of the Svb TF and activation of trichome effectors). We hope that these findings may contribute to a better understanding of how a systemic hormonal signal can be integrated in a spatiotemporal pattern of gene activity and directly implement a genetic program of terminal differentiation. Our results further suggest that Pri peptides represent a fruitful entry point to decipher the whole range of ncRNAs and peptides action in mediating steroid function across Shavenbaby-dependent and independent developmental processes. Current work in the laboratory exploits some of these ideas and should not miss to provide additional insights into both the action of smORF peptides and the molecular mechanisms implementing temporal control of development.

Bibliography

Adler, P.N., Sobala, L.F., Thom, D., and Nagaraj, R. (2013). *dusky-like* is required to maintain the integrity and planar cell polarity of hairs during the development of the *Drosophila* wing. *Developmental biology* 379, 76-91.

Aerts, S. (2012). Computational strategies for the genome-wide identification of cis-regulatory elements and transcriptional targets. *Current topics in developmental biology* 98, 121-145.

Albuquerque, J.P., Tobias-Santos, V., Rodrigues, A.C., Mury, F.B., and da Fonseca, R.N. (2015). small ORFs: A new class of essential genes for development. *Genetics and molecular biology* 38, 278-283.

Andersen, D.S., Colombani, J., and Leopold, P. (2013). Coordination of organ growth: principles and outstanding questions from the world of insects. *Trends in cell biology* 23, 336-344.

Andres, A.J., and Thummel, C.S. (1994). Methods for quantitative analysis of transcription in larvae and prepupae. *Methods Cell Biol* 44, 565-573.

Andres, A.J., and Thummel, C.S. (1995). The *Drosophila* 63F early puff contains E63-1, an ecdysone-inducible gene that encodes a novel Ca(2+)-binding protein. *Development (Cambridge, England)* 121, 2667-2679.

Andrew, D.J., and Baker, B.S. (2008). Expression of the *Drosophila* secreted cuticle protein 73 (*dsc73*) requires *Shavenbaby*. *Dev Dyn* 237, 1198-1206.

Andrews, J., Garcia-Estefania, D., Delon, I., Lu, J., Mevel-Ninio, M., Spierer, A., Payre, F., Pauli, D., and Oliver, B. (2000). OVO transcription factors function antagonistically in the *Drosophila* female germline. *Development (Cambridge, England)* 127, 881-892.

Andrews, S.J., and Rothnagel, J.A. (2014). Emerging evidence for functional peptides encoded by short open reading frames. *Nature reviews Genetics* 15, 193-204.

Antoniewski, C., Laval, M., Dahan, A., and Lepesant, J.A. (1994). The ecdysone response enhancer of the *Fbp1* gene of *Drosophila melanogaster* is a direct target for the EcR/USP nuclear receptor. *Molecular and cellular biology* 14, 4465-4474.

Antoniewski, C., Mugat, B., Delbac, F., and Lepesant, J.A. (1996). Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster*. *Molecular and cellular biology* 16, 2977-2986.

Arif, S., Kittelmann, S., and McGregor, A.P. (2015). From *shavenbaby* to the naked valley: trichome formation as a model for evolutionary developmental biology. *Evolution & development* 17, 120-126.

Ashburner, M. (1974). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. II. The effects of inhibitors of protein synthesis. *Developmental biology* 39, 141-157.

Ashburner, M. (1980). Chromosomal action of ecdysone. *Nature* 285, 435-436.

Baek, K.H., and Lee, K.Y. (1999). Signal transduction pathway for anterior-posterior development in *Drosophila*. *Journal of biomedical science* 6, 314-319.

Bainbridge, S.P., and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster*. *Journal of embryology and experimental morphology* 66, 57-80.

- Baker, K.D., Shewchuk, L.M., Kozlova, T., Makishima, M., Hassell, A., Wisely, B., Caravella, J.A., Lambert, M.H., Reinking, J.L., Krause, H., *et al.* (2003). The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell* *113*, 731-742.
- Bayer, C.A., Holley, B., and Fristrom, J.W. (1996). A switch in broad-complex zinc-finger isoform expression is regulated posttranscriptionally during the metamorphosis of *Drosophila* imaginal discs. *Developmental biology* *177*, 1-14.
- Beaulaton, J.A. (1968). [Ultrastructural modifications of the secretory cells of the prothoracic gland of the silkworm during the last two larval stages. I. The chondriome and its relations to the agranular reticulum]. *The Journal of cell biology* *39*, 501-525.
- Becker, H.J. (1959). [The puffs of salivary gland chromosomes of *Drosophila melanogaster*. Part 1. Observations on the behavior of a typical puff in the normal strain and in two mutants, giant and lethal giant larvae]. *Chromosoma* *10*, 654-678.
- Beckstead, R.B., Lam, G., and Thummel, C.S. (2005). The genomic response to 20-hydroxyecdysone at the onset of *Drosophila* metamorphosis. *Genome biology* *6*, R99.
- Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., and Hogness, D.S. (1997). *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* *91*, 777-788.
- Bialecki, M., Shilton, A., Fichtenberg, C., Seagraves, W.A., and Thummel, C.S. (2002). Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Developmental cell* *3*, 209-220.
- Billas, I.M., Moulinier, L., Rochel, N., and Moras, D. (2001). Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. *The Journal of biological chemistry* *276*, 7465-7474.
- Bilousov, O.O., Kozeretska, I.A., and Katanaev, V.L. (2012). Role of the gene *Miniature* in *Drosophila* wing maturation. *Genesis* *50*, 525-533.
- Bonn, S., Zinzen, R.P., Girardot, C., Gustafson, E.H., Perez-Gonzalez, A., Delhomme, N., Ghavi-Helm, Y., Wilczynski, B., Riddell, A., and Furlong, E.E. (2012). Tissue-specific analysis of chromatin state identifies temporal signatures of enhancer activity during embryonic development. *Nat Genet* *44*, 148-156.
- Bosveld, F., Bonnet, I., Guirao, B., Tlili, S., Wang, Z., Petitalot, A., Marchand, R., Bardet, P.L., Marcq, P., Graner, F., *et al.* (2012). Mechanical control of morphogenesis by Fat/Dachsous/Four-jointed planar cell polarity pathway. *Science (New York, NY)* *336*, 724-727.
- Buchler, N.E., Gerland, U., and Hwa, T. (2003). On schemes of combinatorial transcription logic. *Proceedings of the National Academy of Sciences of the United States of America* *100*, 5136-5141.
- Bulger, M., and Groudine, M. (2011). Functional and mechanistic diversity of distal transcription enhancers. *Cell* *144*, 327-339.
- Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., and Hogness, D.S. (1990). The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. *Cell* *61*, 85-99.
- Butler, J.E., and Kadonaga, J.T. (2001). Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs. *Genes & development* *15*, 2515-2519.
- Caceres, L., Necakov, A.S., Schwartz, C., Kimber, S., Roberts, I.J., and Krause, H.M. (2011). Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. *Genes & development* *25*, 1476-1485.

- Capovilla, M., and Botas, J. (1998). Functional dominance among Hox genes: repression dominates activation in the regulation of Dpp. *Development (Cambridge, England)* *125*, 4949-4957.
- Carbonell, A., Mazo, A., Serras, F., and Corominas, M. (2013). Ash2 acts as an ecdysone receptor coactivator by stabilizing the histone methyltransferase Trr. *Molecular biology of the cell* *24*, 361-372.
- Carney, G.E., Wade, A.A., Sapra, R., Goldstein, E.S., and Bender, M. (1997). DHR3, an ecdysone-inducible early-late gene encoding a Drosophila nuclear receptor, is required for embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America* *94*, 12024-12029.
- Chanut-Delalande, H., Fernandes, I., Roch, F., Payre, F., and Plaza, S. (2006). Shavenbaby couples patterning to epidermal cell shape control. *PLoS biology* *4*, e290.
- Chanut-Delalande, H., Ferrer, P., Payre, F., and Plaza, S. (2012). Effectors of tridimensional cell morphogenesis and their evolution. *Seminars in cell & developmental biology* *23*, 341-349.
- Chanut-Delalande, H., Hashimoto, Y., Pelissier-Monier, A., Spokony, R., Dib, A., Kondo, T., Bohere, J., Niimi, K., Latapie, Y., Inagaki, S., *et al.* (2014). Pri peptides are mediators of ecdysone for the temporal control of development. *Nature cell biology* *16*, 1035-1044.
- Charles, J.P. (2010). The regulation of expression of insect cuticle protein genes. *Insect biochemistry and molecular biology* *40*, 205-213.
- Chavez, V.M., Marques, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Natzle, J.E., and O'Connor, M.B. (2000). The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development (Cambridge, England)* *127*, 4115-4126.
- Chavoshi, T.M., Moussian, B., and Uv, A. (2010). Tissue-autonomous EcR functions are required for concurrent organ morphogenesis in the Drosophila embryo. *Mechanisms of development* *127*, 308-319.
- Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., and Cherbas, P. (2003). EcR isoforms in Drosophila: testing tissue-specific requirements by targeted blockade and rescue. *Development (Cambridge, England)* *130*, 271-284.
- Cherbas, L., Lee, K., and Cherbas, P. (1991). Identification of ecdysone response elements by analysis of the Drosophila Eip28/29 gene. *Genes & development* *5*, 120-131.
- Chipman, A.D., and Akam, M. (2008). The segmentation cascade in the centipede *Strigamia maritima*: involvement of the Notch pathway and pair-rule gene homologues. *Developmental biology* *319*, 160-169.
- Choe, C.P., Miller, S.C., and Brown, S.J. (2006). A pair-rule gene circuit defines segments sequentially in the short-germ insect *Tribolium castaneum*. *Proceedings of the National Academy of Sciences of the United States of America* *103*, 6560-6564.
- Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., and Schwabe, J.W. (2001). The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proceedings of the National Academy of Sciences of the United States of America* *98*, 1549-1554.
- Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S., and Leopold, P. (2005). Antagonistic actions of ecdysone and insulins determine final size in Drosophila. *Science (New York, NY)* *310*, 667-670.
- Crocker, J., Abe, N., Rinaldi, L., McGregor, A.P., Frankel, N., Wang, S., Alsawadi, A., Valenti, P., Plaza, S., Payre, F., *et al.* (2015). Low affinity binding site clusters confer hox specificity and regulatory robustness. *Cell* *160*, 191-203.

- Danielsen, E.T., Moeller, M.E., Yamanaka, N., Ou, Q., Laursen, J.M., Soenderholm, C., Zhuo, R., Phelps, B., Tang, K., Zeng, J., *et al.* (2016). A *Drosophila* Genome-Wide Screen Identifies Regulators of Steroid Hormone Production and Developmental Timing. *Developmental cell* **37**, 558-570.
- Davis, M.B., and Li, T. (2013). Genomic analysis of the ecdysone steroid signal at metamorphosis onset using and mutants. *Genes & genomics* **35**, 21-46.
- Delon, I., Chanut-Delalande, H., and Payre, F. (2003). The Ovo/Shavenbaby transcription factor specifies actin remodelling during epidermal differentiation in *Drosophila*. *Mechanisms of development* **120**, 747-758.
- Delon, I., and Payre, F. (2004). Evolution of larval morphology in flies: get in shape with shavenbaby. *Trends Genet* **20**, 305-313.
- Dequeant, M.L., and Pourquie, O. (2008). Segmental patterning of the vertebrate embryonic axis. *Nature reviews Genetics* **9**, 370-382.
- DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., and Guild, G.M. (1991). The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics* **129**, 385-397.
- Dogini, D.B., Pascoal, V.D., Avansini, S.H., Vieira, A.S., Pereira, T.C., and Lopes-Cendes, I. (2014). The new world of RNAs. *Genetics and molecular biology* **37**, 285-293.
- Dorn, A., and Romer, F. (1976). Structure and function of prothoracic glands and oenocytes in embryos and last larval instars of *Oncopeltus fasciatus* Dallas (Insecta, Heteroptera). *Cell and tissue research* **171**, 331-350.
- Enya, S., Ameku, T., Igarashi, F., Iga, M., Kataoka, H., Shinoda, T., and Niwa, R. (2014). A Halloween gene *noppera-bo* encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in *Drosophila*. *Scientific reports* **4**, 6586.
- Erceg, J., Saunders, T.E., Girardot, C., Devos, D.P., Hufnagel, L., and Furlong, E.E. (2014). Subtle changes in motif positioning cause tissue-specific effects on robustness of an enhancer's activity. *PLoS genetics* **10**, e1004060.
- Estella, C., Voutev, R., and Mann, R.S. (2012). A dynamic network of morphogens and transcription factors patterns the fly leg. *Current topics in developmental biology* **98**, 173-198.
- Faunes, F., and Larrain, J. (2016). Conservation in the involvement of heterochronic genes and hormones during developmental transitions. *Developmental biology*.
- Feigl, G., Gram, M., and Pongs, O. (1989). A member of the steroid hormone receptor gene family is expressed in the 20-OH-ecdysone inducible puff 75B in *Drosophila melanogaster*. *Nucleic acids research* **17**, 7167-7178.
- Fernandes, I., Chanut-Delalande, H., Ferrer, P., Latapie, Y., Waltzer, L., Affolter, M., Payre, F., and Plaza, S. (2010). Zona pellucida domain proteins remodel the apical compartment for localized cell shape changes. *Developmental cell* **18**, 64-76.
- Florence, B., Guichet, A., Ephrussi, A., and Laughon, A. (1997). Ftz-F1 is a cofactor in Ftz activation of the *Drosophila* engrailed gene. *Development (Cambridge, England)* **124**, 839-847.
- Frankel, N., Davis, G.K., Vargas, D., Wang, S., Payre, F., and Stern, D.L. (2010). Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490-493.
- Frankel, N., Erezylmaz, D.F., McGregor, A.P., Wang, S., Payre, F., and Stern, D.L. (2011). Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. *Nature* **474**, 598-603.
- Galindo, M.I., Pueyo, J.I., Fouix, S., Bishop, S.A., and Couso, J.P. (2007). Peptides encoded by short ORFs control development and define a new eukaryotic gene family. *PLoS biology* **5**, e106.

- Gauhar, Z., Sun, L.V., Hua, S., Mason, C.E., Fuchs, F., Li, T.R., Boutros, M., and White, K.P. (2009). Genomic mapping of binding regions for the Ecdysone receptor protein complex. *Genome research* 19, 1006-1013.
- Gibbens, Y.Y., Warren, J.T., Gilbert, L.I., and O'Connor, M.B. (2011). Neuroendocrine regulation of *Drosophila* metamorphosis requires TGFbeta/Activin signaling. *Development (Cambridge, England)* 138, 2693-2703.
- Gilbert, L.I. (2004). Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. *Molecular and cellular endocrinology* 215, 1-10.
- Gilbert, L.I., Rybczynski, R., and Warren, J.T. (2002). Control and biochemical nature of the ecdysteroidogenic pathway. *Annual review of entomology* 47, 883-916.
- Giudicelli, F., Ozbudak, E.M., Wright, G.J., and Lewis, J. (2007). Setting the tempo in development: an investigation of the zebrafish somite clock mechanism. *PLoS biology* 5, e150.
- Gomez, C., Ozbudak, E.M., Wunderlich, J., Baumann, D., Lewis, J., and Pourquie, O. (2008). Control of segment number in vertebrate embryos. *Nature* 454, 335-339.
- Gonsalves, S.E., Neal, S.J., Kehoe, A.S., and Westwood, J.T. (2011). Genome-wide examination of the transcriptional response to ecdysteroids 20-hydroxyecdysone and ponasterone A in *Drosophila melanogaster*. *BMC genomics* 12, 475.
- Guarner, A., Manjon, C., Edwards, K., Steller, H., Suzanne, M., and Sanchez-Herrero, E. (2014). The zinc finger homeodomain-2 gene of *Drosophila* controls Notch targets and regulates apoptosis in the tarsal segments. *Developmental biology* 385, 350-365.
- Guttman, M., and Rinn, J.L. (2012). Modular regulatory principles of large non-coding RNAs. *Nature* 482, 339-346.
- Halder, G., Callaerts, P., and Gehring, W.J. (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science (New York, NY)* 267, 1788-1792.
- Halfon, M.S., Carmena, A., Gisselbrecht, S., Sackerson, C.M., Jimenez, F., Baylies, M.K., and Michelson, A.M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell* 103, 63-74.
- Hancock, S.P., Ghane, T., Cascio, D., Rohs, R., Di Felice, R., and Johnson, R.C. (2013). Control of DNA minor groove width and Fis protein binding by the purine 2-amino group. *Nucleic acids research* 41, 6750-6760.
- Hare, E.E., Peterson, B.K., Iyer, V.N., Meier, R., and Eisen, M.B. (2008). Sepsid even-skipped enhancers are functionally conserved in *Drosophila* despite lack of sequence conservation. *PLoS genetics* 4, e1000106.
- Hashimoto, Y., Kondo, T., and Kageyama, Y. (2008). Lilliputians get into the limelight: novel class of small peptide genes in morphogenesis. *Development, growth & differentiation* 50 Suppl 1, S269-276.
- Heck, B.W., Zhang, B., Tong, X., Pan, Z., Deng, W.M., and Tsai, C.C. (2012). The transcriptional corepressor SMRTER influences both Notch and ecdysone signaling during *Drosophila* development. *Biology open* 1, 182-196.
- Henrich, V.C., Rybczynski, R., and Gilbert, L.I. (1999). Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. *Vitamins and hormones* 55, 73-125.
- Henrich, V.C., Sliter, T.J., Lubahn, D.B., MacIntyre, A., and Gilbert, L.I. (1990). A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. *Nucleic acids research* 18, 4143-4148.

- Henrich, V.C., Szekely, A.A., Kim, S.J., Brown, N.E., Antoniewski, C., Hayden, M.A., Lepesant, J.A., and Gilbert, L.I. (1994). Expression and function of the ultraspiracle (*usp*) gene during development of *Drosophila melanogaster*. *Developmental biology* *165*, 38-52.
- Herszterg, S., Leibfried, A., Bosveld, F., Martin, C., and Bellaiche, Y. (2013). Interplay between the dividing cell and its neighbors regulates adherens junction formation during cytokinesis in epithelial tissue. *Developmental cell* *24*, 256-270.
- Hirayama, J., and Sassone-Corsi, P. (2005). Structural and functional features of transcription factors controlling the circadian clock. *Current opinion in genetics & development* *15*, 548-556.
- Holley, S.A., Julich, D., Rauch, G.J., Geisler, R., and Nusslein-Volhard, C. (2002). *her1* and the notch pathway function within the oscillator mechanism that regulates zebrafish somitogenesis. *Development (Cambridge, England)* *129*, 1175-1183.
- Howard, K.R., and Struhl, G. (1990). Decoding positional information: regulation of the pair-rule gene *hairy*. *Development (Cambridge, England)* *110*, 1223-1231.
- Hu, X., Cherbas, L., and Cherbas, P. (2003). Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions. *Molecular endocrinology (Baltimore, Md)* *17*, 716-731.
- Huang, Y., Xie, J., and Wang, T. (2015). A Fluorescence-Based Genetic Screen to Study Retinal Degeneration in *Drosophila*. *PloS one* *10*, e0144925.
- Inagaki, S., Numata, K., Kondo, T., Tomita, M., Yasuda, K., Kanai, A., and Kageyama, Y. (2005). Identification and expression analysis of putative mRNA-like non-coding RNA in *Drosophila*. *Genes Cells* *10*, 1163-1173.
- Irvine, K.D., and Wieschaus, E. (1994). Cell intercalation during *Drosophila* germband extension and its regulation by pair-rule segmentation genes. *Development (Cambridge, England)* *120*, 827-841.
- Jackle, H., and Sauer, F. (1993). Transcriptional cascades in *Drosophila*. *Current opinion in cell biology* *5*, 505-512.
- Junion, G., Spivakov, M., Girardot, C., Braun, M., Gustafson, E.H., Birney, E., and Furlong, E.E. (2012). A transcription factor collective defines cardiac cell fate and reflects lineage history. *Cell* *148*, 473-486.
- Kadonaga, J.T. (2012). Perspectives on the RNA polymerase II core promoter. *Wiley interdisciplinary reviews* *1*, 40-51.
- Kastenmayer, J.P., Ni, L., Chu, A., Kitchen, L.E., Au, W.C., Yang, H., Carter, C.D., Wheeler, D., Davis, R.W., Boeke, J.D., *et al.* (2006). Functional genomics of genes with small open reading frames (sORFs) in *S. cerevisiae*. *Genome research* *16*, 365-373.
- Keyte, A.L., and Smith, K.K. (2014). Heterochrony and developmental timing mechanisms: changing ontogenies in evolution. *Seminars in cell & developmental biology* *34*, 99-107.
- Khila, A., El Haidani, A., Vincent, A., Payre, F., and Souda, S.I. (2003). The dual function of *ovo/shavenbaby* in germline and epidermis differentiation is conserved between *Drosophila melanogaster* and the olive fruit fly *Bactrocera oleae*. *Insect biochemistry and molecular biology* *33*, 691-699.
- Kitayner, M., Rozenberg, H., Rohs, R., Suad, O., Rabinovich, D., Honig, B., and Shakked, Z. (2010). Diversity in DNA recognition by p53 revealed by crystal structures with Hoogsteen base pairs. *Nature structural & molecular biology* *17*, 423-429.
- Koelle, M.R., Segraves, W.A., and Hogness, D.S. (1992). DHR3: a *Drosophila* steroid receptor homolog. *Proceedings of the National Academy of Sciences of the United States of America* *89*, 6167-6171.

- Koelle, M.R., Talbot, W.S., Seagraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59-77.
- Kolodkin, A.L., Matthes, D.J., and Goodman, C.S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* 75, 1389-1399.
- Kondo, T., Hashimoto, Y., Kato, K., Inagaki, S., Hayashi, S., and Kageyama, Y. (2007). Small peptide regulators of actin-based cell morphogenesis encoded by a polycistronic mRNA. *Nature cell biology* 9, 660-665.
- Kondo, T., Plaza, S., Zanet, J., Benrabah, E., Valenti, P., Hashimoto, Y., Kobayashi, S., Payre, F., and Kageyama, Y. (2010). Small peptides switch the transcriptional activity of Shavenbaby during *Drosophila* embryogenesis. *Science (New York, NY)* 329, 336-339.
- Koyama, T., Rodrigues, M.A., Athanasiadis, A., Shingleton, A.W., and Mirth, C.K. (2014). Nutritional control of body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis. *eLife* 3.
- Kozlova, T., and Thummel, C.S. (2003a). Essential roles for ecdysone signaling during *Drosophila* mid-embryonic development. *Science (New York, NY)* 301, 1911-1914.
- Kozlova, T., and Thummel, C.S. (2003b). Methods to characterize *Drosophila* nuclear receptor activation and function in vivo. *Methods in enzymology* 364, 475-490.
- Kugler, S.J., Gehring, E.M., Wallkamm, V., Kruger, V., and Nagel, A.C. (2011). The Putzig-NURF nucleosome remodeling complex is required for ecdysone receptor signaling and innate immunity in *Drosophila melanogaster*. *Genetics* 188, 127-139.
- Kuhn, R., Kuhn, C., Borsch, D., Glatzer, K.H., Schafer, U., and Schafer, M. (1991). A cluster of four genes selectively expressed in the male germ line of *Drosophila melanogaster*. *Mechanisms of development* 35, 143-151.
- Kulkarni, M.M., and Arnosti, D.N. (2003). Information display by transcriptional enhancers. *Development (Cambridge, England)* 130, 6569-6575.
- Kumar, S., and Cakouros, D. (2004). Transcriptional control of the core cell-death machinery. *Trends in biochemical sciences* 29, 193-199.
- Kurzchalia, T.V., and Ward, S. (2003). Why do worms need cholesterol? *Nature cell biology* 5, 684-688.
- Lachaise D., C.M.L., David J.R., Lemeunier F., Tsacas L, Ashburner M. (1988). Historical biogeography of the *Drosophila melanogaster* species subgroup. In *Evolutionary Biology*, pp. 159-225.
- Lagha, M., Bothma, J.P., and Levine, M. (2012). Mechanisms of transcriptional precision in animal development. *Trends Genet* 28, 409-416.
- Landry, J.R., Mager, D.L., and Wilhelm, B.T. (2003). Complex controls: the role of alternative promoters in mammalian genomes. *Trends Genet* 19, 640-648.
- Lavorgna, G., Karim, F.D., Thummel, C.S., and Wu, C. (1993). Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proceedings of the National Academy of Sciences of the United States of America* 90, 3004-3008.
- Lavorgna, G., Ueda, H., Clos, J., and Wu, C. (1991). FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu. *Science (New York, NY)* 252, 848-851.
- Lecuit, T., and Cohen, S.M. (1997). Proximal-distal axis formation in the *Drosophila* leg. *Nature* 388, 139-145.

- Lewis, J. (2003). Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator. *Current biology : CB* 13, 1398-1408.
- Li, T.R., and White, K.P. (2003). Tissue-specific gene expression and ecdysone-regulated genomic networks in *Drosophila*. *Developmental cell* 5, 59-72.
- Locke, M., and Krishnan, N. (1971). The distribution of phenoloxidases and polyphenols during cuticle formation. *Tissue & cell* 3, 103-126.
- Lozano, J., Kayukawa, T., Shinoda, T., and Belles, X. (2014). A role for Taiman in insect metamorphosis. *PLoS genetics* 10, e1004769.
- Lu, Q., Yan, J., and Adler, P.N. (2010). The *Drosophila* planar polarity proteins inturnd and multiple wing hairs interact physically and function together. *Genetics* 185, 549-558.
- Mace, K.A., Pearson, J.C., and McGinnis, W. (2005). An epidermal barrier wound repair pathway in *Drosophila* is mediated by grainy head. *Science (New York, NY)* 308, 381-385.
- Markstein, M., Markstein, P., Markstein, V., and Levine, M.S. (2002). Genome-wide analysis of clustered Dorsal binding sites identifies putative target genes in the *Drosophila* embryo. *Proceedings of the National Academy of Sciences of the United States of America* 99, 763-768.
- Mathelier, A., Zhao, X., Zhang, A.W., Parcy, F., Worsley-Hunt, R., Arenillas, D.J., Buchman, S., Chen, C.Y., Chou, A., Ienasescu, H., *et al.* (2014). JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. *Nucleic acids research* 42, D142-147.
- McBrayer, Z., Ono, H., Shimell, M., Parvy, J.P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I., and O'Connor, M.B. (2007). Prothoracicotrophic hormone regulates developmental timing and body size in *Drosophila*. *Developmental cell* 13, 857-871.
- McGregor, A.P., Orgogozo, V., Delon, I., Zanet, J., Srinivasan, D.G., Payre, F., and Stern, D.L. (2007). Morphological evolution through multiple cis-regulatory mutations at a single gene. *Nature* 448, 587-590.
- Menoret, D., Santolini, M., Fernandes, I., Spokony, R., Zanet, J., Gonzalez, I., Latapie, Y., Ferrer, P., Rouault, H., White, K.P., *et al.* (2013). Genome-wide analyses of Shavenbaby target genes reveals distinct features of enhancer organization. *Genome biology* 14, R86.
- Mével-Ninio, M., Terracol, R., and Kafatos, F.C. (1991). The ovo gene of *Drosophila* encodes a zinc finger protein required for female germ line development. *The EMBO journal* 10, 2259-2266.
- Mével-Ninio, M., Terracol, R., Salles, C., Vincent, A., and Payre, F. (1995). ovo, a *Drosophila* gene required for ovarian development, is specifically expressed in the germline and shares most of its coding sequences with shavenbaby, a gene involved in embryo patterning. *Mechanisms of development* 49, 83-95.
- Moeller, M.E., Danielsen, E.T., Herder, R., O'Connor, M.B., and Rewitz, K.F. (2013). Dynamic feedback circuits function as a switch for shaping a maturation-inducing steroid pulse in *Drosophila*. *Development (Cambridge, England)* 140, 4730-4739.
- Monier, B., Astier, M., Semeriva, M., and Perrin, L. (2005). Steroid-dependent modification of Hox function drives myocyte reprogramming in the *Drosophila* heart. *Development (Cambridge, England)* 132, 5283-5293.
- Montagne, J., Lecerf, C., Parvy, J.P., Bennion, J.M., Radimerski, T., Ruhf, M.L., Zilbermann, F., Vouilloz, N., Stocker, H., Hafen, E., *et al.* (2010). The nuclear receptor DHR3 modulates dS6 kinase-dependent growth in *Drosophila*. *PLoS genetics* 6, e1000937.
- Morata, G. (2001). How *Drosophila* appendages develop. *Nature reviews* 2, 89-97.

- Moussian, B. (2010). Recent advances in understanding mechanisms of insect cuticle differentiation. *Insect biochemistry and molecular biology* 40, 363-375.
- Moussian, B., Schwarz, H., Bartoszewski, S., and Nusslein-Volhard, C. (2005). Involvement of chitin in exoskeleton morphogenesis in *Drosophila melanogaster*. *J Morphol* 264, 117-130.
- Moussian, B., Veerkamp, J., Muller, U., and Schwarz, H. (2007). Assembly of the *Drosophila* larval exoskeleton requires controlled secretion and shaping of the apical plasma membrane. *Matrix Biol* 26, 337-347.
- Muratoglu, S., Hough, B., Mon, S.T., and Fossett, N. (2007). The GATA factor *Serpent* cross-regulates *lozenge* and *u-shaped* expression during *Drosophila* blood cell development. *Developmental biology* 311, 636-649.
- Nagaraj, R., and Adler, P.N. (2012). *Dusky-like* functions as a *Rab11* effector for the deposition of cuticle during *Drosophila* bristle development. *Development (Cambridge, England)* 139, 906-916.
- Namiki, T., Niwa, R., Sakudoh, T., Shirai, K., Takeuchi, H., and Kataoka, H. (2005). Cytochrome P450 CYP307A1/*Spook*: a regulator for ecdysone synthesis in insects. *Biochemical and biophysical research communications* 337, 367-374.
- Nijhout, H.F., Riddiford, L.M., Mirth, C., Shingleton, A.W., Suzuki, Y., and Callier, V. (2014). The developmental control of size in insects. *Wiley interdisciplinary reviews* 3, 113-134.
- Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., and Kataoka, H. (2004). CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of *Bombyx* and *Drosophila*. *The Journal of biological chemistry* 279, 35942-35949.
- Niwa, R., Namiki, T., Ito, K., Shimada-Niwa, Y., Kiuchi, M., Kawaoka, S., Kayukawa, T., Banno, Y., Fujimoto, Y., Shigenobu, S., *et al.* (2010). *Non-molting glossy/shroud* encodes a short-chain dehydrogenase/reductase that functions in the 'Black Box' of the ecdysteroid biosynthesis pathway. *Development (Cambridge, England)* 137, 1991-1999.
- Niwa, R., and Niwa, Y.S. (2014). Enzymes for ecdysteroid biosynthesis: their biological functions in insects and beyond. *Bioscience, biotechnology, and biochemistry* 78, 1283-1292.
- Niwa, Y.S., and Niwa, R. (2016). Transcriptional regulation of insect steroid hormone biosynthesis and its role in controlling timing of molting and metamorphosis. *Development, growth & differentiation* 58, 94-105.
- Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795-801.
- Ohno, C.K., and Petkovich, M. (1993). *FTZ-F1 beta*, a novel member of the *Drosophila* nuclear receptor family. *Mechanisms of development* 40, 13-24.
- Ono, H., Rewitz, K.F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J.T., Marques, G., Shimell, M.J., *et al.* (2006). *Spook* and *Spookier* code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. *Developmental biology* 298, 555-570.
- Oro, A.E., McKeown, M., and Evans, R.M. (1990). Relationship between the product of the *Drosophila* ultraspiracle locus and the vertebrate retinoid X receptor. *Nature* 347, 298-301.
- Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K., *et al.* (2004). Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat Genet* 36, 40-45.
- Ou, Q., and King-Jones, K. (2013). What goes up must come down: transcription factors have their say in making ecdysone pulses. *Current topics in developmental biology* 103, 35-71.

- Ou, Q., Magico, A., and King-Jones, K. (2011). Nuclear receptor DHR4 controls the timing of steroid hormone pulses during *Drosophila* development. *PLoS biology* 9, e1001160.
- Ou, Q., Zeng, J., Yamanaka, N., Brakken-Thal, C., O'Connor, M.B., and King-Jones, K. (2016). The Insect Prothoracic Gland as a Model for Steroid Hormone Biosynthesis and Regulation. *Cell reports* 16, 247-262.
- Ozturk-Colak, A., Moussian, B., Araujo, S.J., and Casanova, J. (2016). A feedback mechanism converts individual cell features into a supracellular ECM structure in *Drosophila* trachea. *eLife* 5.
- Palmeirim, I., Henrique, D., Ish-Horowicz, D., and Pourquie, O. (1997). Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* 91, 639-648.
- Pankratz, M.J., Seifert, E., Gerwin, N., Billi, B., Nauber, U., and Jackle, H. (1990). Gradients of Kruppel and knirps gene products direct pair-rule gene stripe patterning in the posterior region of the *Drosophila* embryo. *Cell* 61, 309-317.
- Panne, D. (2008). The enhanceosome. *Current opinion in structural biology* 18, 236-242.
- Parker, S.C., Hansen, L., Abaan, H.O., Tullius, T.D., and Margulies, E.H. (2009). Local DNA topography correlates with functional noncoding regions of the human genome. *Science (New York, NY)* 324, 389-392.
- Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E., *et al.* (2004). Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. *Nat Genet* 36, 288-292.
- Parvy, J.P., Blais, C., Bernard, F., Warren, J.T., Petryk, A., Gilbert, L.I., O'Connor, M.B., and Dauphin-Villemant, C. (2005). A role for betaFTZ-F1 in regulating ecdysteroid titers during post-embryonic development in *Drosophila melanogaster*. *Developmental biology* 282, 84-94.
- Parvy, J.P., Wang, P., Garrido, D., Maria, A., Blais, C., Poidevin, M., and Montagne, J. (2014). Forward and feedback regulation of cyclic steroid production in *Drosophila melanogaster*. *Development (Cambridge, England)* 141, 3955-3965.
- Patwardhan, R.P., Hiatt, J.B., Witten, D.M., Kim, M.J., Smith, R.P., May, D., Lee, C., Andrie, J.M., Lee, S.I., Cooper, G.M., *et al.* (2012). Massively parallel functional dissection of mammalian enhancers in vivo. *Nature biotechnology* 30, 265-270.
- Pauli, A., Valen, E., and Schier, A.F. (2015). Identifying (non-)coding RNAs and small peptides: challenges and opportunities. *Bioessays* 37, 103-112.
- Payre, F. (2004). Genetic control of epidermis differentiation in *Drosophila*. *The International journal of developmental biology* 48, 207-215.
- Payre, F., Vincent, A., and Carreno, S. (1999). *ovo/svb* integrates Wingless and DER pathways to control epidermis differentiation. *Nature* 400, 271-275.
- Perrin, L., Monier, B., Ponzielli, R., Astier, M., and Semeriva, M. (2004). *Drosophila* cardiac tube organogenesis requires multiple phases of Hox activity. *Developmental biology* 272, 419-431.
- Perry, M.W., Boettiger, A.N., Bothma, J.P., and Levine, M. (2010). Shadow enhancers foster robustness of *Drosophila* gastrulation. *Current biology : CB* 20, 1562-1567.
- Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., and O'Connor, M.B. (2003). Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proceedings of the National Academy of Sciences of the United States of America* 100, 13773-13778.

- Pi, H., Huang, Y.C., Chen, I.C., Lin, C.D., Yeh, H.F., and Pai, L.M. (2011). Identification of 11-amino acid peptides that disrupt Notch-mediated processes in *Drosophila*. *Journal of biomedical science* *18*, 42.
- Plaza, S., Chanut-Delalande, H., Fernandes, I., Wassarman, P.M., and Payre, F. (2010). From A to Z: apical structures and zona pellucida-domain proteins. *Trends in cell biology* *20*, 524-532.
- Poortinga, G., Watanabe, M., and Parkhurst, S.M. (1998). *Drosophila* CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *The EMBO journal* *17*, 2067-2078.
- Potier, D., Seyres, D., Guichard, C., Iche-Torres, M., Aerts, S., Herrmann, C., and Perrin, L. (2014). Identification of cis-regulatory modules encoding temporal dynamics during development. *BMC genomics* *15*, 534.
- Pourquie, O. (2011). Vertebrate segmentation: from cyclic gene networks to scoliosis. *Cell* *145*, 650-663.
- Pueyo, J.I., and Couso, J.P. (2008). The 11-aminoacid long Tarsal-less peptides trigger a cell signal in *Drosophila* leg development. *Developmental biology* *324*, 192-201.
- Pueyo, J.I., and Couso, J.P. (2011). Tarsal-less peptides control Notch signalling through the Shavenbaby transcription factor. *Developmental biology* *355*, 183-193.
- Pueyo, J.I., Magny, E.G., and Couso, J.P. (2016). New Peptides Under the s(ORF)ace of the Genome. *Trends in biochemical sciences*.
- Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R.A. (2002). Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. *Nature* *416*, 644-648.
- Rehorn, K.P., Thelen, H., Michelson, A.M., and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila*. *Development (Cambridge, England)* *122*, 4023-4031.
- Reinking, J., Lam, M.M., Pardee, K., Sampson, H.M., Liu, S., Yang, P., Williams, S., White, W., Lajoie, G., Edwards, A., *et al.* (2005). The *Drosophila* nuclear receptor e75 contains heme and is gas responsive. *Cell* *122*, 195-207.
- Ren, N., He, B., Stone, D., Kirakodu, S., and Adler, P.N. (2006). The shavenoid gene of *Drosophila* encodes a novel actin cytoskeleton interacting protein that promotes wing hair morphogenesis. *Genetics* *172*, 1643-1653.
- Ren, N., Zhu, C., Lee, H., and Adler, P.N. (2005). Gene expression during *Drosophila* wing morphogenesis and differentiation. *Genetics* *171*, 625-638.
- Riddiford, L.M. (1993). Hormone receptors and the regulation of insect metamorphosis. *Receptor* *3*, 203-209.
- Riddihough, G., and Ish-Horowicz, D. (1991). Individual stripe regulatory elements in the *Drosophila* hairy promoter respond to maternal, gap, and pair-rule genes. *Genes & development* *5*, 840-854.
- Riddihough, G., and Pelham, H.R. (1987). An ecdysone response element in the *Drosophila* hsp27 promoter. *The EMBO journal* *6*, 3729-3734.
- Ring, J.M., and Martinez Arias, A. (1993). puckered, a gene involved in position-specific cell differentiation in the dorsal epidermis of the *Drosophila* larva. *Development (Cambridge, England)* *119 Suppl*, 251-259.
- Robertson, C.W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. *Journal of morphology* *52*, 351-399.
- Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., and Engels, W.R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* *118*, 461-470.

- Roch, F., Alonso, C.R., and Akam, M. (2003). *Drosophila* miniature and dusky encode ZP proteins required for cytoskeletal reorganisation during wing morphogenesis. *Journal of cell science* *116*, 1199-1207.
- Rogol, A.D., Clark, P.A., and Roemmich, J.N. (2000). Growth and pubertal development in children and adolescents: effects of diet and physical activity. *The American journal of clinical nutrition* *72*, 521S-528S.
- Rohs, R., Jin, X., West, S.M., Joshi, R., Honig, B., and Mann, R.S. (2010). Origins of specificity in protein-DNA recognition. *Annual review of biochemistry* *79*, 233-269.
- Rougvie, A.E. (2001). Control of developmental timing in animals. *Nature reviews Genetics* *2*, 690-701.
- Ruaud, A.F., Lam, G., and Thummel, C.S. (2010). The *Drosophila* nuclear receptors DHR3 and betaFTZ-F1 control overlapping developmental responses in late embryos. *Development (Cambridge, England)* *137*, 123-131.
- Sanson, B. (2001). Generating patterns from fields of cells. Examples from *Drosophila* segmentation. *EMBO reports* *2*, 1083-1088.
- Santolini, M., Mora, T., and Hakim, V. (2014). A general pairwise interaction model provides an accurate description of in vivo transcription factor binding sites. *PLoS one* *9*, e99015.
- Sarrazin, A.F., Peel, A.D., and Averof, M. (2012). A segmentation clock with two-segment periodicity in insects. *Science (New York, NY)* *336*, 338-341.
- Savard, J., Marques-Souza, H., Aranda, M., and Tautz, D. (2006). A segmentation gene in *tribolium* produces a polycistronic mRNA that codes for multiple conserved peptides. *Cell* *126*, 559-569.
- Schirm, S., Jiricny, J., and Schaffner, W. (1987). The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity. *Genes & development* *1*, 65-74.
- Schock, F., and Perrimon, N. (2003). Retraction of the *Drosophila* germ band requires cell-matrix interaction. *Genes & development* *17*, 597-602.
- Schoppmeier, M., and Damen, W.G. (2005). Suppressor of Hairless and Presenilin phenotypes imply involvement of canonical Notch-signalling in segmentation of the spider *Cupiennius salei*. *Developmental biology* *280*, 211-224.
- Shlyueva, D., Stelzer, C., Gerlach, D., Yanez-Cuna, J.O., Rath, M., Boryn, L.M., Arnold, C.D., and Stark, A. (2014). Hormone-responsive enhancer-activity maps reveal predictive motifs, indirect repression, and targeting of closed chromatin. *Molecular cell* *54*, 180-192.
- Skalska, L., Stojnic, R., Li, J., Fischer, B., Cerda-Moya, G., Sakai, H., Tajbakhsh, S., Russell, S., Adryan, B., and Bray, S.J. (2015). Chromatin signatures at Notch-regulated enhancers reveal large-scale changes in H3K56ac upon activation. *The EMBO journal* *34*, 1889-1904.
- Slattery, M., Zhou, T., Yang, L., Dantas Machado, A.C., Gordan, R., and Rohs, R. (2014). Absence of a simple code: how transcription factors read the genome. *Trends in biochemical sciences* *39*, 381-399.
- Small, S., Blair, A., and Levine, M. (1992). Regulation of even-skipped stripe 2 in the *Drosophila* embryo. *The EMBO journal* *11*, 4047-4057.
- Smith, R.P., Taher, L., Patwardhan, R.P., Kim, M.J., Inoue, F., Shendure, J., Ovcharenko, I., and Ahituv, N. (2013). Massively parallel decoding of mammalian regulatory sequences supports a flexible organizational model. *Nat Genet* *45*, 1021-1028.
- Sobala, L.F., and Adler, P.N. (2016). The Gene Expression Program for the Formation of Wing Cuticle in *Drosophila*. *PLoS genetics* *12*, e1006100.

- Spitz, F. (2016). Gene regulation at a distance: from remote enhancers to 3D regulatory ensembles. *Seminars in cell & developmental biology*.
- Spitz, F., and Furlong, E.E. (2012). Transcription factors: from enhancer binding to developmental control. *Nature reviews Genetics* 13, 613-626.
- Sprecher, S.G., and Desplan, C. (2008). Switch of rhodopsin expression in terminally differentiated *Drosophila* sensory neurons. *Nature* 454, 533-537.
- Stella, S., Cascio, D., and Johnson, R.C. (2010). The shape of the DNA minor groove directs binding by the DNA-bending protein Fis. *Genes & development* 24, 814-826.
- Stern, D.L. (2013). The genetic causes of convergent evolution. *Nature reviews Genetics* 14, 751-764.
- Stern, D.L., and Frankel, N. (2013). The structure and evolution of cis-regulatory regions: the shavenbaby story. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 368, 20130028.
- Stoiber, M., Celniker, S., Cherbas, L., Brown, B., and Cherbas, P. (2016). Diverse Hormone Response Networks in 41 Independent *Drosophila* Cell Lines. *G3 (Bethesda)* 6, 683-694.
- Stone, B.L., and Thummel, C.S. (1993). The *Drosophila* 78C early late puff contains E78, an ecdysone-inducible gene that encodes a novel member of the nuclear hormone receptor superfamily. *Cell* 75, 307-320.
- Stowers, R.S., Garza, D., Rasclé, A., and Hogness, D.S. (2000). The L63 gene is necessary for the ecdysone-induced 63E late puff and encodes CDK proteins required for *Drosophila* development. *Developmental biology* 221, 23-40.
- Stowers, R.S., Russell, S., and Garza, D. (1999). The 82F late puff contains the L82 gene, an essential member of a novel gene family. *Developmental biology* 213, 116-130.
- Sucena, E., Delon, I., Jones, I., Payre, F., and Stern, D.L. (2003). Regulatory evolution of shavenbaby/ovo underlies multiple cases of morphological parallelism. *Nature* 424, 935-938.
- Sullivan, A.A., and Thummel, C.S. (2003). Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. *Molecular endocrinology (Baltimore, Md)* 17, 2125-2137.
- Suzanne, M. (2016). Molecular and cellular mechanisms involved in leg joint morphogenesis. *Seminars in cell & developmental biology* 55, 131-138.
- Talamillo, A., Herboso, L., Pirone, L., Perez, C., Gonzalez, M., Sanchez, J., Mayor, U., Lopitz-Otsoa, F., Rodriguez, M.S., Sutherland, J.D., *et al.* (2013). Scavenger receptors mediate the role of SUMO and Ftz-f1 in *Drosophila* steroidogenesis. *PLoS genetics* 9, e1003473.
- Talbot, W.S., Swyryd, E.A., and Hogness, D.S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* 73, 1323-1337.
- Tata, J.R. (2002). Signalling through nuclear receptors. *Nature reviews* 3, 702-710.
- Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., Singh, C.M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H.L., *et al.* (2004). A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nat Genet* 36, 283-287.
- Thomas, H.E., Stunnenberg, H.G., and Stewart, A.F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* 362, 471-475.

- Thompson, J.N., Jr., Ashburner, M., and Woodruff, R.C. (1977). Presumptive control mutation for alcohol dehydrogenase in *Drosophila melanogaster*. *Nature* 270, 363.
- Thummel, C.S. (1995). From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. *Cell* 83, 871-877.
- Thummel, C.S. (2001a). Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. *Developmental cell* 1, 453-465.
- Thummel, C.S. (2001b). Steroid-triggered death by autophagy. *Bioessays* 23, 677-682.
- Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M., and Evans, R.M. (1999). SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Molecular cell* 4, 175-186.
- Tupy, J.L., Bailey, A.M., Dailey, G., Evans-Holm, M., Siebel, C.W., Misra, S., Celniker, S.E., and Rubin, G.M. (2005). Identification of putative noncoding polyadenylated transcripts in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 102, 5495-5500.
- Ueda, H., and Hirose, S. (1990). Identification and purification of a *Bombyx mori* homologue of FTZ-F1. *Nucleic acids research* 18, 7229-7234.
- Vaskova, M., Bentley, A.M., Marshall, S., Reid, P., Thummel, C.S., and Andres, A.J. (2000). Genetic analysis of the *Drosophila* 63F early puff. Characterization of mutations in E63-1 and maggie, a putative Tom22. *Genetics* 156, 229-244.
- Venken, K.J., Carlson, J.W., Schulze, K.L., Pan, H., He, Y., Spokony, R., Wan, K.H., Koriabine, M., de Jong, P.J., White, K.P., *et al.* (2009). Versatile P[acman] BAC libraries for transgenesis studies in *Drosophila melanogaster*. *Nature methods* 6, 431-434.
- Walter, M.F., Black, B.C., Afshar, G., Kermabon, A.Y., Wright, T.R., and Biessmann, H. (1991). Temporal and spatial expression of the yellow gene in correlation with cuticle formation and dopa decarboxylase activity in *Drosophila* development. *Developmental biology* 147, 32-45.
- Wang, S., Tsarouhas, V., Xylourgidis, N., Sabri, N., Tiklova, K., Nautiyal, N., Gallio, M., and Samakovlis, C. (2009). The tyrosine kinase Stitcher activates Grainy head and epidermal wound healing in *Drosophila*. *Nature cell biology* 11, 890-895.
- Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., Dauphin-Villemant, C., O'Connor, M.B., and Gilbert, L.I. (2002). Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 99, 11043-11048.
- Warren, J.T., Petryk, A., Marques, G., Parvy, J.P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M.B., *et al.* (2004). Phantom encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. *Insect biochemistry and molecular biology* 34, 991-1010.
- Webb, A.B., and Oates, A.C. (2016). Timing by rhythms: Daily clocks and developmental rulers. *Development, growth & differentiation* 58, 43-58.
- White, K.P., Rifkin, S.A., Hurban, P., and Hogness, D.S. (1999). Microarray analysis of *Drosophila* development during metamorphosis. *Science (New York, NY)* 286, 2179-2184.
- Wilson, M.D., and Odom, D.T. (2009). Evolution of transcriptional control in mammals. *Current opinion in genetics & development* 19, 579-585.

Woodard, C.T., Baehrecke, E.H., and Thummel, C.S. (1994). A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell* **79**, 607-615.

Xiang, Y., Liu, Z., and Huang, X. (2010). *br* regulates the expression of the ecdysone biosynthesis gene *npc1*. *Developmental biology* **344**, 800-808.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., and Ueda, H. (2000). Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster*. *Development (Cambridge, England)* **127**, 5083-5092.

Yamanaka, N., Rewitz, K.F., and O'Connor, M.B. (2013). Ecdysone control of developmental transitions: lessons from *Drosophila* research. *Annual review of entomology* **58**, 497-516.

Yan, J., Huen, D., Morely, T., Johnson, G., Gubb, D., Roote, J., and Adler, P.N. (2008). The multiple-wing-hairs gene encodes a novel GBD-FH3 domain-containing protein that functions both prior to and after wing hair initiation. *Genetics* **180**, 219-228.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* **71**, 63-72.

Yasugi, T., and Nishimura, T. (2016). Temporal regulation of the generation of neuronal diversity in *Drosophila*. *Development, growth & differentiation* **58**, 73-87.

Yoshida, H., Liu, J., Samuel, S., Cheng, W., Rosen, D., and Naora, H. (2005). Steroid receptor coactivator-3, a homolog of Taiman that controls cell migration in the *Drosophila* ovary, regulates migration of human ovarian cancer cells. *Molecular and cellular endocrinology* **245**, 77-85.

Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H., and Niwa, R. (2006). Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. *Development (Cambridge, England)* **133**, 2565-2574.

Zanet, J., Benrabah, E., Li, T., Pelissier-Monier, A., Chanut-Delalande, H., Ronsin, B., Bellen, H.J., Payre, F., and Plaza, S. (2015). Pri sORF peptides induce selective proteasome-mediated protein processing. *Science (New York, NY)* **349**, 1356-1358.

Zanet, J., Chanut-Delalande, H., Plaza, S., and Payre, F. (2016). Small Peptides as Newcomers in the Control of *Drosophila* Development. *Current topics in developmental biology* **117**, 199-219.

Zavolan, M., van Nimwegen, E., and Gaasterland, T. (2002). Splice variation in mouse full-length cDNAs identified by mapping to the mouse genome. *Genome research* **12**, 1377-1385.

Zdarek, J., and Slama, K. (1972). Supernumerary larval instars in cyclorrhaphous diptera. *The Biological bulletin* **142**, 350-357.

Zhang, C., Robinson, B.S., Xu, W., Yang, L., Yao, B., Zhao, H., Byun, P.K., Jin, P., Veraksa, A., and Moberg, K.H. (2015). The ecdysone receptor coactivator Taiman links Yorkie to transcriptional control of germline stem cell factors in somatic tissue. *Developmental cell* **34**, 168-180.

Zhou, T., Yang, L., Lu, Y., Dror, I., Dantas Machado, A.C., Ghane, T., Di Felice, R., and Rohs, R. (2013). DNASHape: a method for the high-throughput prediction of DNA structural features on a genomic scale. *Nucleic acids research* **41**, W56-62.

Zinzen, R.P., Girardot, C., Gagneur, J., Braun, M., and Furlong, E.E. (2009). Combinatorial binding predicts spatio-temporal cis-regulatory activity. *Nature* **462**, 65-70.

Summary

Recent advances in genomics have revealed that most species produce a broad variety of long non-coding RNAs, whose functions remain generally not well understood. A growing body of evidence yet indicates that apparently non-coding RNAs can often encode peptides from small Open-Reading Frames (smORFs). While additional data clearly support their translation in cells, an important issue is to elucidate the putative mode of action of smORF peptides and whether these peptides could contribute to the regulation of differentiation or development.

Our team is studying the development of epidermal derivatives in flies. Previous work has identified a key transcription factor, OvoL/Shavenbaby (Svb), that governs the differentiation of epidermal trichomes, which are cuticle extensions contributing to different aspects of the insect life. Svb is both required and sufficient to determine trichome formation, and thus Svb expression defines which subsets of cells form trichomes. Recent studies showed that Svb directly activates the expression of a large number of genes encoding cellular effectors, collectively responsible for trichome differentiation. Unexpectedly, trichome formation also requires an atypical RNA, called *polished rice/tarsal less/mille pattes* (*pri*), which was initially considered as non-coding but that acts through the production of four smORF peptides (11-32aa). The absence of *pri* leads to embryos lacking any trichomes, as seen following the inactivation of Svb, thus suggesting a functional interaction between Pri & Svb. Indeed, a collaborative work has demonstrated that Pri peptides induce a post-translational maturation of the Svb protein, switching its activity from a transcriptional repressor to an activator. Therefore, whereas Svb expression defines the spatial pattern of epidermal cells forming trichomes, Pri peptides are required to turn ON the genetic program of trichome differentiation. While recent work in the team now unravels the molecular mechanisms by which Pri peptides achieve Svb maturation, the developmental rationale of such a complex process remained to be explored.

To address this question, the aim of my PhD has been to investigate the transcriptional control of *pri* expression. This issue appeared important since this is ultimately the onset of *pri* expression that defines when the transcriptional program of trichome is executed, in Svb positive cells. In a first step, I used a series of bacterial artificial chromosomes to functionally delineate the extent of the *pri* genetic locus. Although *pri* is an intron-less RNA of approx. 1.5kb, rescuing assays showed that *pri* function relies on distant genomic regions, spanning more than 50 kb. Using a battery of *in vivo* reporter constructs, I then characterized *pri* genomic regions and found that they include a large array of cis-regulatory regions driving *pri* expression in different tissues, and at several stages of embryonic and post-embryonic development. In collaboration with other members of the team, our studies further demonstrate that *pri* expression is regulated by the ecdysone steroid hormone, a signaling pathway well known for providing a temporal control of developmental transitions. We collected a set of complementary pieces of evidence showing that the Ecdysone Receptor activates the expression of *pri*, directly binding to different enhancers that drive various spatiotemporal patterns of *pri* expression. All together, these data establish that a main role of *pri* is to mediate the systemic signal of steroid hormone to precisely time the execution of epidermal differentiation, at the successive stages of *Drosophila* development. This allows us to explain the developmental importance of Pri peptides in the temporal control of epidermis differentiation, and additional results suggest a broader implication of Pri in implementing ecdysone signaling for the timing of different programs of development.

Résumé

Les avancées de la génomique montrent que les êtres vivants produisent de nombreux long ARNs noncodant, dont les fonctions restent globalement mal connues. Des données récentes indiquent que ces long ARNs apparemment noncodants peuvent cependant traduire des peptides à partir de petits cadres ouverts de lecture (smORFs). Si différentes approches établissent l'existence de ces smORF peptides, un enjeu important est d'élucider leur mode d'action et de déterminer s'ils peuvent participer à la régulation du développement.

Notre équipe étudie le développement de l'épiderme chez la drosophile. Des travaux antérieurs ont bien établi le rôle clé d'un facteur de transcription, OvoL/Shavenbaby (Svb), qui gouverne la différenciation des cellules à trichomes de l'épiderme. Des études récentes de l'équipe ont permis d'identifier le répertoire des gènes cibles de Svb, qui codent différents effecteurs cellulaires collectivement responsables de la formation des trichomes. De manière inattendue, la différenciation des trichomes nécessite aussi la fonction d'un ARN atypique: *polished rice / tarsal less / mille pattes (pri)*. Initialement découvert comme un long ARN noncodant, *pri* agit en réalité par la production de quatre smORF peptides (11-32aa). Une collaboration internationale a permis de démontrer que les peptides Pri induisent une maturation post-traductionnelle de la protéine Svb, la transformant d'un répresseur à un activateur de transcription. Ainsi, alors que l'expression de Svb définit le registre spatial des cellules à trichome, les peptides Pri sont requis pour mettre en route le programme transcriptionnel de leur différenciation. Si les travaux de l'équipe viennent d'identifier les mécanismes moléculaires de l'activation de Svb par les peptides Pri, la logique développementale de cette régulation complexe restait à explorer.

Pour aborder cette question, mes travaux de thèse se sont concentrés sur la recherche des mécanismes transcriptionnels contrôlant l'expression du gène *pri*. En effet, cette problématique apparaissait particulièrement importante car c'est finalement l'expression de *pri* qui va déclencher la formation des trichomes dans les cellules Svb positives. Dans une première étape, j'ai utilisé une série de chromosomes bactériens artificiels introduits chez la drosophile pour délimiter l'étendue du locus génétique indispensable à la fonction de *pri*. Bien que *pri* code un ARN sans intron d'environ 1,5 kilobases (kb), mes tests génétiques ont montré que l'unité fonctionnelle du gène *pri* s'étend sur plus de 50kb ! J'ai construit une batterie de lignées transgéniques rapportrices, qui ont permis d'identifier un ensemble de régions cis-régulatrices distinctes, dirigeant l'expression de *pri* dans différents tissus et stades de développement. En collaboration avec les autres membres de l'équipe, nos travaux ont montré que l'expression de *pri* est sous le contrôle de l'ecdysone, une hormone stéroïde bien connue pour son rôle clé dans la régulation temporelle des transitions développementales, incluant les mues larvaires et la métamorphose. Des données complémentaires soutiennent un rôle direct du récepteur nucléaire à l'ecdysone (EcR) pour l'expression de *pri*, et j'ai complété ces résultats en montrant l'importance du site de liaison à EcR pour l'activité de la région cis-régulatrice dirigeant la transcription de *pri* dans l'épiderme. L'ensemble de ces données établit donc qu'un rôle majeur de *pri* est de relayer l'action systémique de l'hormone stéroïde pour définir précisément la temporalité d'exécution de la différenciation épidermique, aux stades successifs du développement embryonnaire et post-embryonnaire. Ces résultats permettent ainsi d'expliquer l'importance développementale des peptides Pri dans le contrôle temporel de la différenciation de l'épiderme, et des données additionnelles suggèrent une implication plus large de Pri dans l'implémentation du signal ecdysone pour la régulation temporelle de différents programmes transcriptionnels du développement.