Functional genomics of wound healing in *drosophila*

Genòmica funcional del procés de cicatrització en *drosophila*

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Functional genomics of wound healing in *drosophila.*

Genòmica functional del próces de cicatrització en *drosophila*

Memoria presentada por

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To my loving parents and brother

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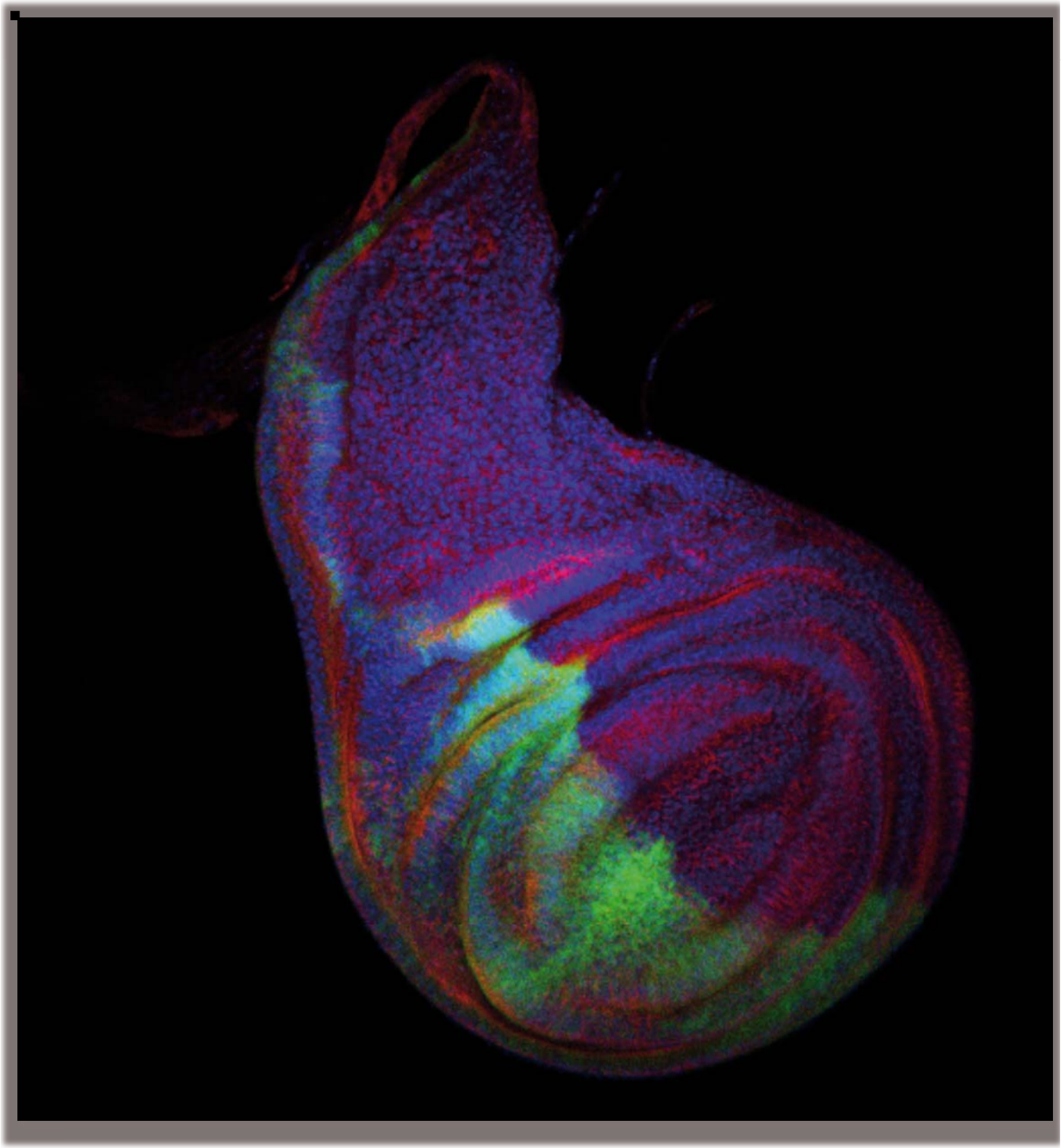


INTRODUCTION

Wound healing is an essential homeostatic mechanism that maintains epithelial barrier integrity after tissue damage [1]. Healing and tissue repair are fundamental properties of multicellular organisms from sponges to humans. For decades *Drosophila* has been considered an excellent model to dissect epithelial repair at cell and molecular levels and, indeed, many of the key molecules and proteins involved in *Drosophila* epithelial healing are also involved in mammalian tissue repair.

Although, in outline, we know the main events participating in the healing of a wound, many of the underlying molecular mechanisms remain unclear. Fruit flies provide a fantastic opportunity for studying these mechanisms, as they are an ideal system to marry genetic techniques with live imaging of the repair process. In fact, researchers have been wounding insects for nearly a hundred years [2] to investigate patterning mechanisms and regenerative capacities.

Drosophila imaginal discs have been widely used for wound healing studies. Two early discoveries were fundamental in establishing imaginal discs as a model for regeneration. First, the discovery by Hadorn and colleagues [3, 4] of the capability of imaginal discs to regenerate after fragmentation; and second, the finding that imaginal disc tissue can recover and regenerate following massive cell death [5]

Life cycle of *Drosophila melanogaster*

Drosophila melanogaster, commonly known as the fruit fly, belongs to the class Diptera. *Drosophila* is a holometabolous insect, that is, with complete metamorphosis, whose life cycle proceeds after egg ecclosion through three larval moults and a prolonged pupal stage up to adult emergence.

Drosophila eggs are approximately one-half mm in length, white, oval, and slightly laterally flattened. Following fertilization, mitosis (nuclear division) gets triggered in the absence of cytokinesis (cytoplasm compartmentalisation) resulting into a multinucleate embryonic (blastoderm) syncytium. The common cytoplasm allows morphogen gradients to play key roles in pattern formation and the major body axes and segment boundaries are determined.

Larval development occurs in 3 phases (instars). After hatching from eggs (24 hours after egg laying), the first instar larva (L1) emerges as a tiny feeding worm. The first

instar larvae grow and moult their cuticle after 24 hrs., later leading to second instar larva (L2). The third instar larvae emerge by moulting 72 hours after egg laying (AEL). During the larval period growth is ensued and further pattern formation of imaginal tissues occurs. The imaginal precursor cells have no functional role in the larva and they are programmed to produce adult structures at metamorphosis. For, some adult structures such as the eyes, antennae, legs, wings, halteres and genitalia, the imaginal precursors are contained as physically distinct primordia - the imaginal discs. The different discs are specifically determined, but do not differentiate until the late larval and subsequent pupal stages when they form the adult structures [6]. In *Drosophila* there are 17 imaginal discs in total, 8 contralateral pairs and 1 genital disc. The imaginal discs are ectodermal monolayered sacs that derive from the non-proliferating ectoderm of the embryo and grow extensively during the larval stages and the first 24h of pupal development.

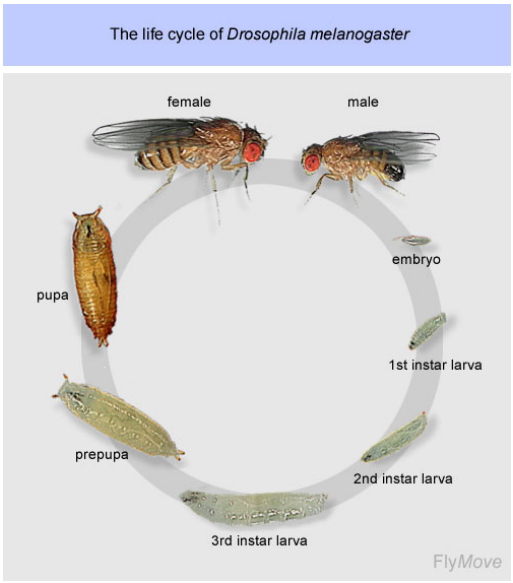


Figure 1. Life Cycle of *Drosophila melanogaster*. Cartoon depicting the four different stages of *Drosophila* development (embryo to adult). Adapted from Flymove [7].

A complex tissue reorganisation (metamorphosis) occurs during pupation. In the prepupa, the thoracic discs evert and fuse with each other replacing the larval

epidermis [8-10]. For the adult thorax, the lateral region (pleura) and dorsal region (notum) are formed by the prothoracic, wing and haltere discs; the latter two also form the dorsal appendages, wings, and halteres. The ventral thorax (sternum) and the legs are formed from three pairs of leg discs. Within 4 days the adult emerges from the pupal case (Figure 1).

Imaginal Discs in *Drosophila*

Drosophila is a holometabolous insect i.e., the adult form (imago) derives from complete metamorphosis during the pupal stage. The transition from larval to adult body shape is mediated by specialized epithelial cell sacs called imaginal discs, which develop from precursors that are specified during embryonic development [10]. The larvae can thus be seen as a two-faced animal, one (the larva) hosting the other (the future adult). By the end of third instar larval stage, sharp variations in the titre of the steroid hormone 20-hydroxyecdysone induces the entry of the larvae into pre-pupal development and discs begin their dramatic morphogenesis [10, 11]. Within 12 hours, the monolayered imaginal discs transform into recognizable adult structures (wings, thorax, legs, head) by a series of morphogenetic changes. These include elongation/shaping and eversion of individual discs (the so-called evagination), followed by spreading and fusion of neighbouring discs. During this metamorphic process, adult tissues will replace larval ones, thus forming a continuum of adult, neo-formed epidermis [10].

Most of the adult structures formed from the imaginal discs of the larva, are composed of clusters of determined but undifferentiated imaginal cells. The imaginal cells represent, on one hand, a homogeneous group with many characteristics in common, and on the other hand, a heterogeneous group spanning a broad spectrum of determined cell types (Figure 2). Some of the common characteristics that distinguish the imaginal cells from other cells of the larva are the following: diploid instead of polytene chromosomes, small size and undifferentiated appearance, and rapid proliferation throughout larval development. Imaginal cells also have the unique capacity of maintaining their determined state during prolonged periods of proliferation in culture [12]

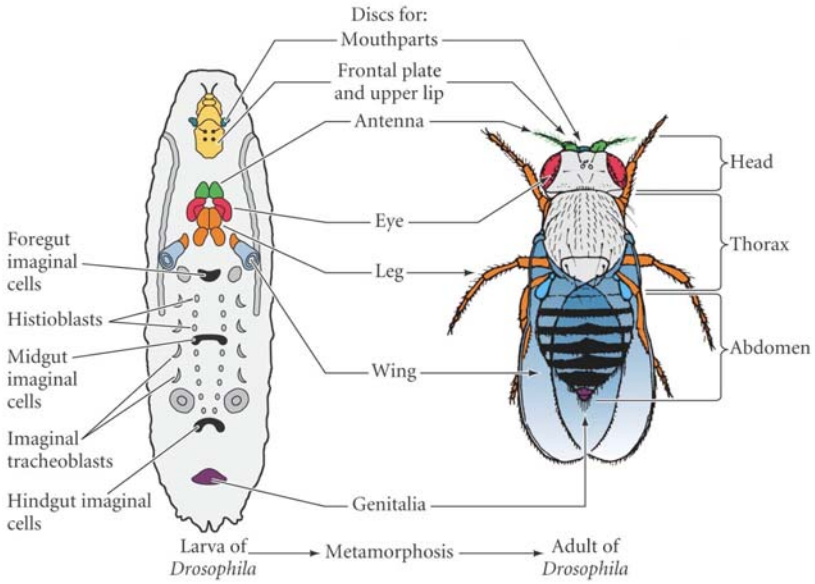


Figure 2. Imaginal discs of *Drosophila.* Imaginal discs of a third instar larva and the corresponding body parts of the adult fly - adapted from [13].

- **Structure of imaginal discs**

All imaginal discs in *Drosophila* are made up of a layer of columnar epithelium or the disc proper (CE) and a layer of squamous epithelium, the peripodial membrane (PE). Together they form a bag-like double epithelium. Both CE and PE cells arise from common precursor cells that form the embryonic imaginal primordium (Figure 3). The CE differentiates into adult tissues where PE's main role is to drive discs eversion. During metamorphosis the imaginal discs are completely remodelled in an evagination process comprising two discrete stages: elongation of the columnar epithelium mostly driven by cell shape changes and eversion, when contraction, invasion of the larval tissue and fenestration of the PE drive the appendages through the larval epidermis [14].

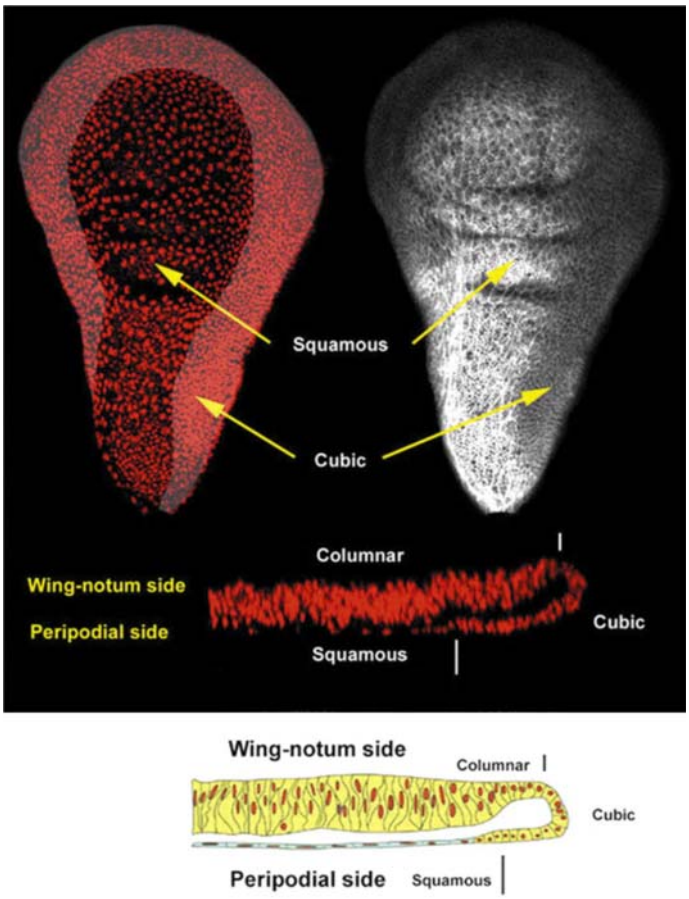


Figure 3. Development of the peripodial and columnar sides of the wing disc. PE structure in a third instar wing disc. Characteristic low-density nuclear distribution and squamous cell morphology are noticed in the PE. Nuclei are stained with the DNA dye TO-PRO-3 (red) and Arm localization in the *zonula adherens* (white) reveals cell shape. A white shadow delimits the surrounding cubic cells. The different cell types are described in a transverse section of a disc [15].

- **Development of the wing**

The wing is derived from the wing imaginal disc, formed from the embryonic ectoderm by an invagination at the intersection of a dorsal/ventral stripe of *wingless* with an anterior-posterior stripe of *decapentaplegic* (*dpp*) at the second thoracic segment [16].

The disc is structured into three axes. The anterior/posterior axis is specified by the segment polarity genes *engrailed/hedgehog* and *cubitus interruptus* on either side of a

stripe expressing *dpp*. The proximal/distal axis is specified by the genes *distalless* and *aristaless*. The dorso-ventral axis is specified by *vestigial*. Both the A/P and the D/V boundaries act as organizing centres. The secreted Wnt protein, Wingless (Wg) acts as an organizer at the D/V boundary in a manner similar to Dpp at the A/P [17] (Figure 4).

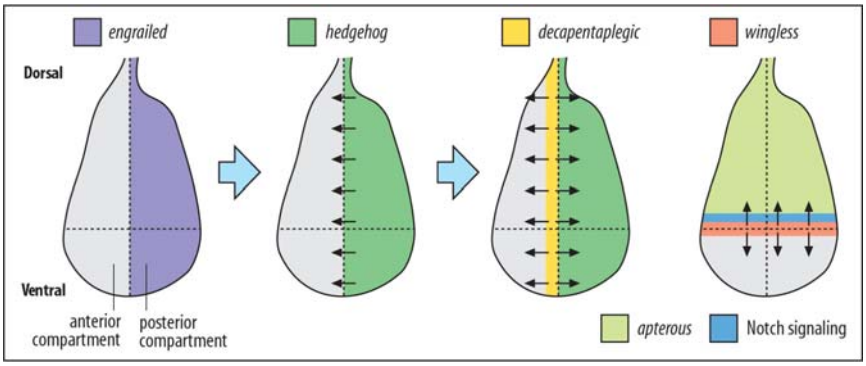


Figure 4. Signals from the A/P and D/V boundaries pattern the wing imaginal discs. Cartoon showing the expression pattern of different segment polarity genes during wing imaginal disc development. The A/P boundary is specified by the coordinated expression of different genes. *en* (in purple) is specifically expressed in posterior. *hh* (green), whose expression depends on *en* is also expressed in posterior and sends a short-range signal to anterior. This leads to the activation of *dpp* (in yellow) along the A/P border, which helps in patterning both anterior and posterior compartments. On the other hand, the formation of D/V boundary depends on short-range interactions between dorsal and ventral cells. *ap* (light green) expressed in the dorsal compartment induces the expression of *serrate* and *fringe*. Serrate then induces the ventral cells to produce Notch that directs the localised expression of *wg* (in pink) along the D/V boundary [18-20]

Epithelial Morphogenesis

Epithelial cell sheets line organ and body surfaces. They constitute cellular layer with apico-basal polarity that provide specialized barrier functions and regulate the exchange of substances with the outside environment and between different body compartments. Epithelia play a role in a wide range of physiological processes such as digestion, excretion, and trafficking.

The morphogenesis of epithelial tissues plays a fundamental role in animal development. Epithelial sheets fold, involute and migrate to give rise in vertebrates to internal structures during the processes of gastrulation, neurulation and

organogenesis, and are responsible for lending structure and shape to invertebrate embryos [21]. In addition, during development, some epithelia form transient primitive structures, including the neural tube and somites, which are essential for the further development of complex organs. The characterization of the molecular mechanisms involved in epithelial morphogenesis is essential and has recently highlighted the roles of the cytoskeleton, cell-cell adhesion and cell-matrix adhesion and their regulation [22].

An important particular process in which epithelial sheets participate both in vertebrate and invertebrate systems during morphogenesis is epithelial fusion. Fusion contributes to organ formation and body structural shape and is evident during amphibian and avian epiboly, gastrulation, neural tube closure, palate formation, and eyelid fusion in vertebrates, ventral enclosure in *Caenorhabditis elegans*, and embryonic dorsal closure in *Drosophila*. Importantly, several observations have led to the conclusion that wound healing and tissue regeneration recapitulate these particular embryonic morphogenetic processes [23].

Models of epithelial fusion in vertebrates

- **Neural tube closure**

Neural tube closure is a remarkable example of coordinated tissue movements. Neurulation is a fundamental embryonic process that leads to the development of the neural tube, which is the precursor of the brain and spinal cord. Building a neural tube is an extremely complex phenomenon where cells need to change in shape, migrate and differentiate to form a hollow tube from a flat sheet of thickened epithelial cells, the neural plate [24] (Figure 5).

The neural tube begins as a flat, wide sheet, the neural plate. Through convergent extension, the neural plate narrows along its medio-lateral axis, and elongates along its anterior-posterior axis. Convergent extension is driven in large part by forces arising from within the neural plate itself [25] The neural tube closes as the paired neural folds are brought together at the dorsal midline. The folds adhere to each other, and the cells from the two folds merge.

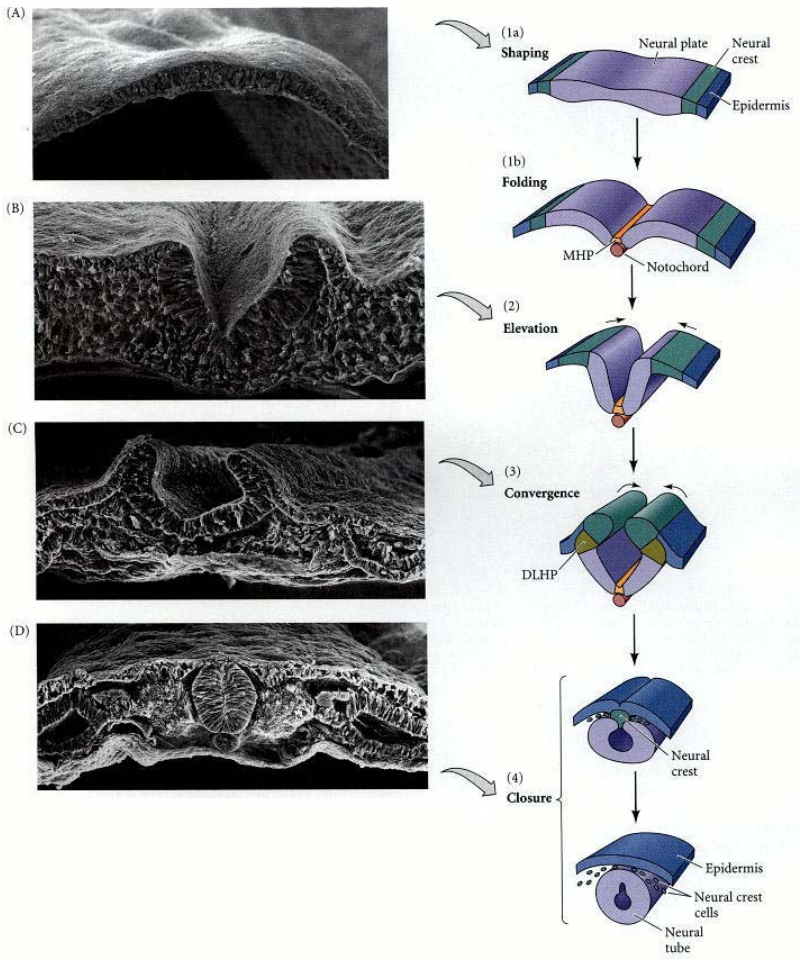


Figure 5. Neural tube closure showing different stages. Primary neurulation: neural tube formation in the chick embryo. A) Cells of the neural plate can be initially distinguished as elongated cells in the dorsal region of the ectoderm. Folding begins as the medial neural hinge point (MHP) cells anchor to notochord and change their shape, while the presumptive epidermal cells move towards the centre. B) The neural folds are elevated as the presumptive epidermis continues to move toward the dorsal midline. C) Convergence of the neural folds occurs as the dorso-lateral hinge point (DLHP) cells become wedge-shaped and epidermal cells push towards the centre. D) The neural folds are brought into contact with one another, and the neural crest cells link the neural tube with the epidermis. The neural crest cells then disperse, leaving the neural tube [26].

- **Secondary palate fusion**

Malformations in secondary palate fusion lead to a cleft palate, a common human birth defect. During development, the palatal shelves of most vertebrates undergo a complex reorientation from a vertical position lateral to the tongue to a horizontal position above the tongue fusing to form a single, continuous structure, the secondary

palate. This process involves four distinct stages including growth of the individual shelves, reorientation of the shelves, epithelial cell adhesion and final autolysis [27-31] (Figure 6). The series of developmental events that are necessary for palatal fusion have been shown to be similar both *in vivo* and in an organ culture models *in vitro* [30, 31].

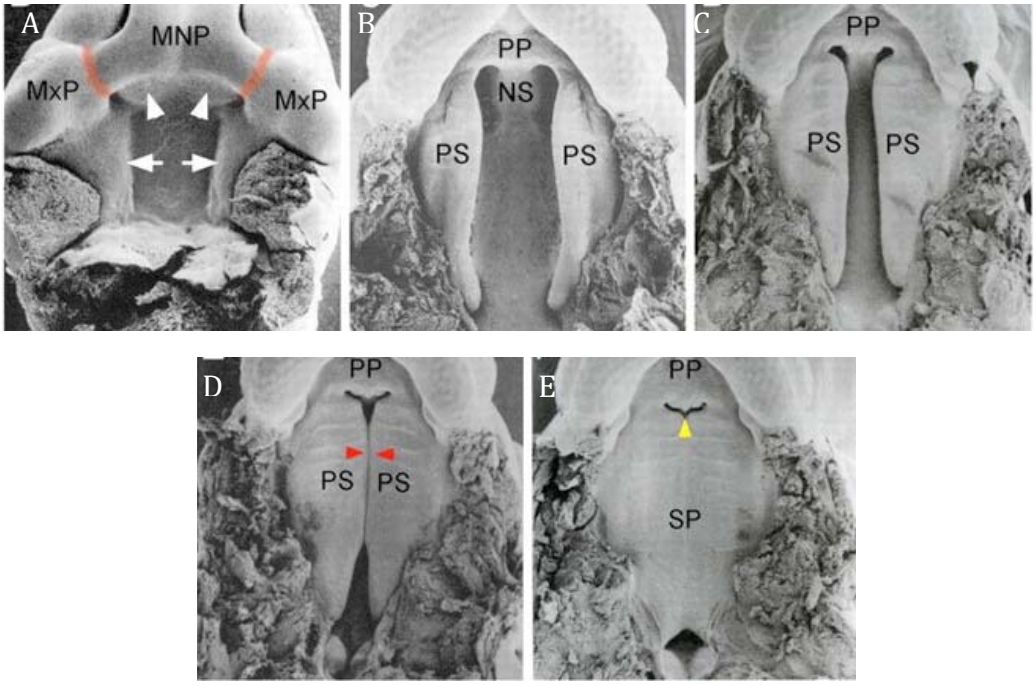


Figure 6. (A-E) Scanning micrographs showing oral views of the secondary palate fusion. The secondary palate arises as paired outgrowths flanking the developing tongue, which initially grow vertically and subsequently reorient to horizontal positions above the dorsum of the tongue in a process known as palatal shelf elevation. Following elevation, the paired palatal shelves grow towards the midline where they meet and fuse with each other (MNP, medial nasal process; MxP, maxillary process; NS, nasal septum; PP, primary palate; PS, palatal shelf; SP, secondary palate) [32].

- **Epiboly in zebrafish**

Gastrulation comprises a series of coordinated cell movements that lead to the organization of the germ layers, ectoderm, mesoderm and endoderm and the establishment of the major body axes of the embryo [33] (Figure 7). One gastrulation movement is epiboly, which involves the thinning and spreading of the multilayered

blastoderm and its closure at the vegetal pole. When epiboly has progressed about half way, the different germ layers that will eventually give rise to every tissue and organ start to become morphologically distinguishable [34]. Epiboly involves a series of cellular rearrangements evolutionary conserved in other phyla.

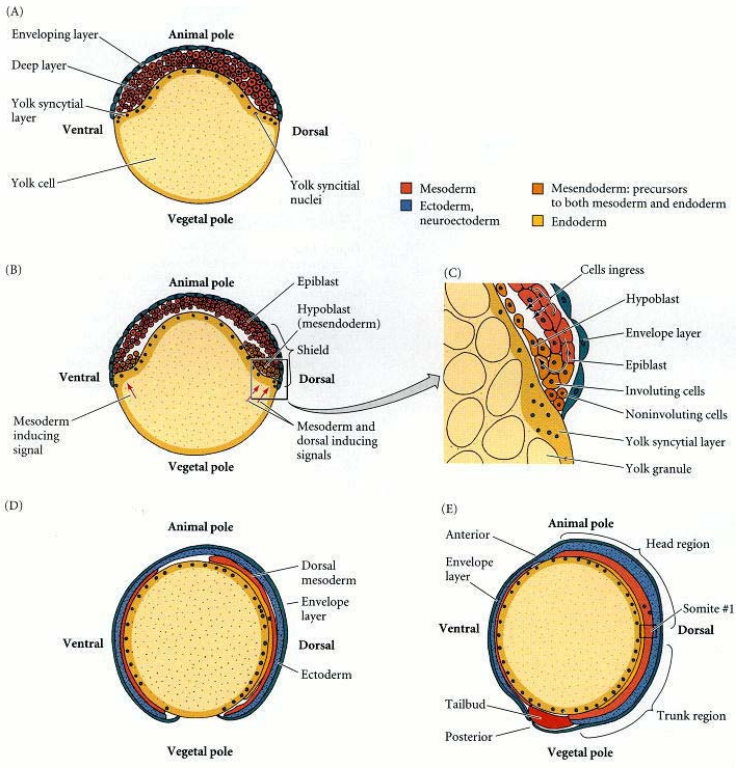


Figure 7. Cell movements during gastrulation of the zebrafish. A) The blastoderm at 30% epiboly (about 4.7 hours). B) Formation of the hypoblast (6 hours). C) Close-up of the marginal region. D) At 90% epiboly (9 hours) the mesoderm surrounds the yolk between the endoderm and the ectoderm. E) Completion of gastrulation (10.3 hours) [35].

Models of epithelial fusion in invertebrates

- **Ventral enclosure in *C. elegans***

The epiboly of the *C. elegans* hypodermis involves the bilateral spreading of a thin epithelial sheet from the dorsal side around the embryo to meet at the ventral midline. Ventral enclosure by the epidermis occurs in two steps [21] (Figure 8).

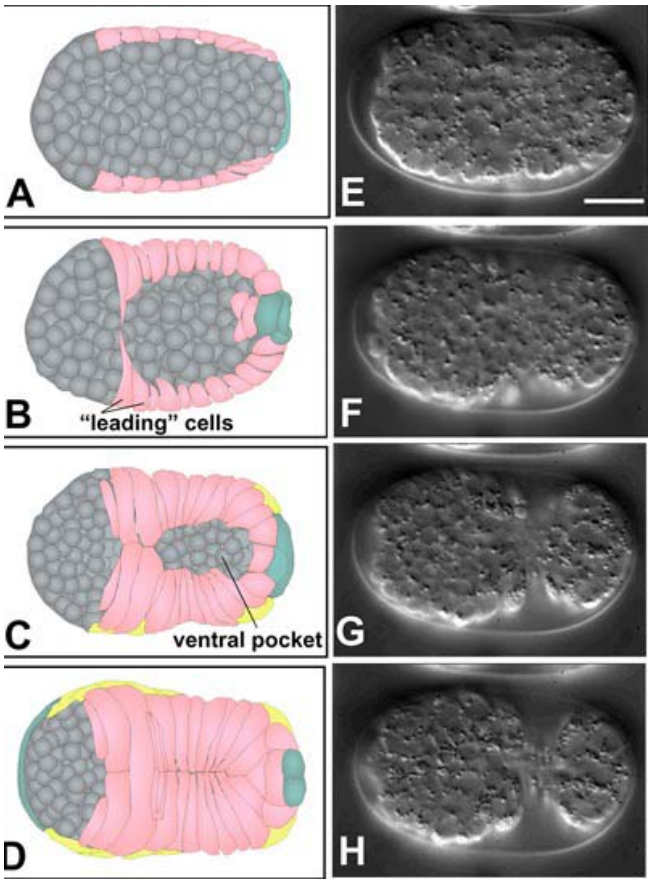


Figure 8. Epidermal enclosure. Schematics (left column, A-D), Nomarski images (right column, E-H, ventral view) of ventral enclosure. Ventral cells are shown in pink in the left-hand column; seam epidermal cells in yellow, and dorsal cells, which wrap around the tail, in teal. Neuroblasts and other internal cells are depicted in grey. The first two pairs of ventral cells (leading cells) extend long protrusions towards the midline. After the leading cells make contact at the ventral midline, cells posterior to these cells (pocket cells) reach the midline. Finally, the anterior epidermal cells enclose the head [36].

First, four anterior epidermal cells lead the migration of the epidermis to the ventral midline by extending actin-rich filopodia over substrate neurons [37]. This transformation occurs in the absence of cell proliferation and is driven by cell migration and shape changes within the surface layer of embryonic epidermal cells (also referred to as the "hypodermis" in nematodes). Second, approximately 300 min post fertilization, the epidermal cells found in three pairs of rows straddling the posterior dorsal midline of the embryonic surface undergo three major morphogenetic movements. Initially, the two rows of epidermal cells immediately adjacent to the dorsal midline intercalate with one another. As dorsal intercalation completes, the

ventral-most rows of epidermal cells undergo epiboly and migrate to the ventral midline to enclose the embryo. On completion, an actin mediated contraction of the lateral row of epidermal cells causes these cells, and the embryo, to contract, resulting in a four fold longitudinal lengthening [38].

- **Dorsal closure in *Drosophila***

Some epithelial movements during *Drosophila* development are analogous to those involved in wound healing and therefore, it has been proposed that embryonic dorsal closure (DC) and thorax fusion might be a model for wound healing [39].

The DC of the *Drosophila* embryo involves the sealing of a hole in the epidermis through the migration of the epidermal flanks over the tissue occupying the hole, the amnioserosa. In this way, the lateral epithelium at both sides of the embryo is drawn up seaming at the midline. The whole closure process comprises a series of overlapping phases requiring the participation of both the lateral epithelium and the amnioserosa.

1. Initiation — Stage 12, Starts ~8 Hours after Egg Laying (AEL).

The dorsal closure initiates once the germ band has fully retracted to the posterior end of the developing embryo, leaving the dorsal amnioserosa fully exposed. Subsequent closure progresses spreading from the anterior and posterior ends toward the centre of the embryo. At its onset, the epithelial cells of the leading edge form a somewhat disorganized, scalloped edge. During this phase, the epithelial edge slowly advances showing no apparent movement over the amnioserosa, suggesting that closure at this time is largely a consequence of amnioserosa cell contraction. Indeed, amnioserosa cells reduce their apical surface substantially during this phase.

2. Epithelial Sweeping — Stage 13, Starts ~9:30 Hours AEL.

Immediately after the “initiation” phase, leading edge cells begin to polarize as filamentous actin accumulates at their apical edge to form a thick actin cable. The assembly of this cable coincides with, and is probably responsible for, the transformation of the leading edge from a scalloped edge into a taut, neat row of cells. Simultaneously, the leading edge cells elongate in a dorso-ventral direction. It is not clear whether they actively elongate or are tugged forward by the purse-string activity

of the contractile actin cable at their “free edge”. Throughout this stage the epithelial hole continues to reduce in size, presumably through a combination of amnioserosal cell contraction and contraction of the actin cable plus epithelial cell elongation. In addition, it is during this “epithelial sweeping” phase that filopodia and, to a lesser extent, lamellae are first seen protruding from the lead edge cells.

3. Zippering — Stage 14, Starts ~11 Hours AEL.

The zippering phase of dorsal closure begins once opposing leading edges at the most anterior and posterior ends of the hole are close enough for filopodia to reach across and touch the opposite epithelial edge [40]. These actin protrusions act to zip the opposing epithelial surfaces together and may also play a key role in ensuring the correct matching of the embryonic segments as the hole closes. However, in addition to filopodia, it is highly likely that both contractility of the actin cable and amnioserosa cell contraction still play roles in bringing the sides together.

4. Termination — Stage 15, Ends ~13 Hours AEL.

As for the other phases of dorsal closure, the final stage of this process exhibits a temporal progression, spreading like a wave within the seams behind the two advancing terminal zipper fronts. A crucial aspect of termination is that once leading edge cells from opposing sides have made contact, they stop moving and form a tight seam along the midline. A further aspect of termination is that, as well as stopping, the abutting epithelial cells must also convert the transient adhesions formed between filopodia into tight, permanent, adherens junctions [40] (Figure 9).

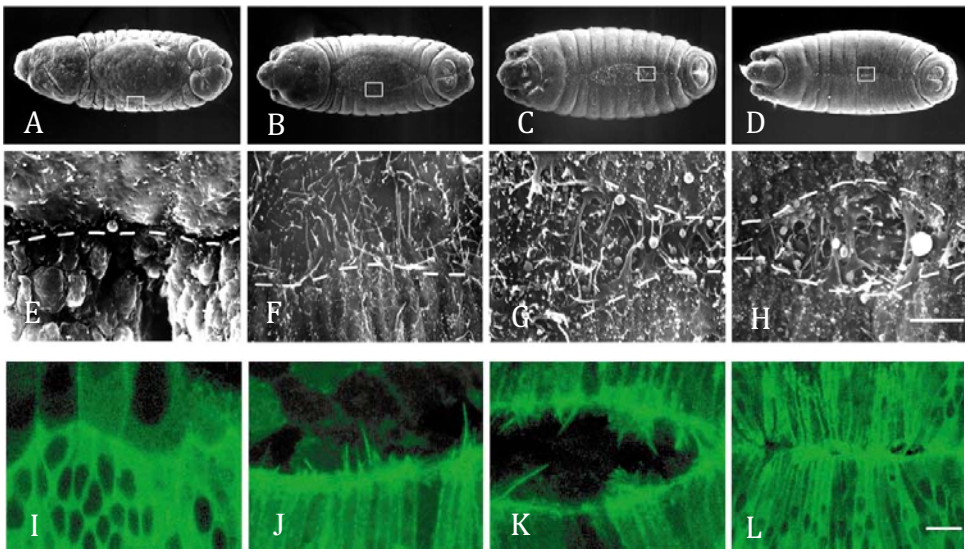


Figure 9. (A–D) Scanning electron micrographs (SEM) of embryos: A) before dorsal closure; B) at early dorsal closure; C) zippering stage D) final stage. (E-H) High-magnification SEM of the boxed regions of (A–D): E) smooth leading edge without filopodia; F) filopodial extensions; G) filopodia accompanied by lamellae at the zipper front where opposing epithelial edges meet; and H) final lamellar ruffling as the dorsal hole is about to close. (I–L) Images of GFP–actin equivalent to the SEM: I) the leading edge still do not show a strong actin cable, neither filopodia; J) leading cells showing a clear actin cable and several filopodial protrusions; K) zippering phase; L) final closure [40].

- **Thorax fusion in *Drosophila***

During metamorphosis, the adult epidermis of *Drosophila* develops from the imaginal discs. During and after puparium formation (pupariation), the imaginal discs evert (the epithelial sac is turned inside out) and then assemble to form the continuous epidermal structure of the future adult. Imaginal discs evert by apposing their peripodial side to the larval epidermis and through the invasion of the larval epidermis by PE cells, which undergo a pseudo-epithelial-mesenchymal transition (PEMT) [14]. At the same time, the larval epidermis is histolysed. The dorsal thorax (notum) develops from the dorsal parts of the two wing imaginal discs. They approach each other from both sides and fuse at the midline between 6 and 8 hours after pupariation [10]. During thorax closure, the leading edge cells of the two imaginal discs are specified and execute coordinated forward movements by dramatically changing their shape (either contraction or stretching) and move over the substratum till the opposing sides of the two discs meet [41] (Figure 10). They then fuse in the antero-dorsal midline forming a seamless joint to form one contiguous T2 thorax. Similar to dorsal closure, thorax closure is also characterized by prominent actin organization at the midline [42]. However, in contrast to the simple epithelial stretching of embryonic dorsal closure, the morphogenetic movements involved in thorax closure appear to be more complex: most cells are of polygonal shape and not obviously elongated along the dorso-ventral axis [42]. The entire process is mediated by changes in cell shape and there is no cell proliferation or cell recruitment [43].

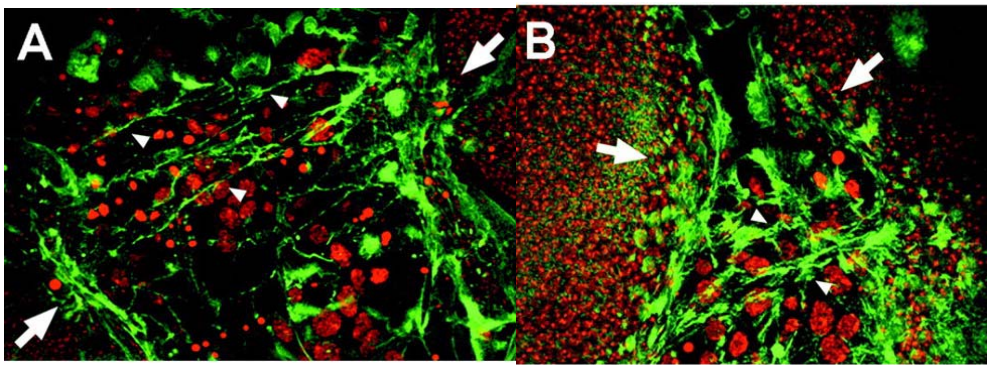


Figure 10. Imaginal cells extend filopodia that connect contralateral discs. A) Confocal image projection of a 5 hours APF pupa stained with phalloidin to label polymerized actin and TO-PRO3 to mark nuclei. Long, thick filopodia extend from the wing imaginal disc edges, expand over the larval tissue, and eventually connect the confronting discs (arrowheads). B) At 6 hours APF, after discs contracted anteriorly, filopodia (arrowheads) extend from more posterior areas of the leading edge [41].

The JNK signalling cascade

The Jun-N-terminal Kinase pathway (JNK), known also as stress activated protein kinase pathway (SAPK), is an eukaryotic evolutionarily conserved signalling pathway. The JNK signal transduction pathway has been implicated in the regulation of gene expression [44, 45]. From a purported evolutionarily ‘‘ancient’’ function as stress mediator, the JNK pathway has evolved in multicellular eukaryotes to permanent roles in development, without leaving its original function. In *Drosophila*, it is required for follicle cell morphogenesis, embryonic dorsal closure, pupal thoracic closure and genital disc rotation, all processes with requisite cell shape changes. Besides, it is activated during wound healing and in response to stress (UV irradiation, oxidative stress), where it may signal cell death or proliferation.

Regulation of the JNK pathway appears majorly negative, with phosphatases, transcription factors and proteins of novel structure “holding back’’ on JNK activation in different tissues. This particular mode of regulation may hark back to the pathway’s origin as stress detector and responder, implying readiness to respond, from which the developmental roles may have evolved as conditions demanding obligate and predicted stress responses (i.e., embryonic dorsal closure viewed as a ‘‘wound of development’’). These pathways use protein phosphorylation to convey signals intracellularly. MAPKs have been found in all eukaryotic cells studied. Classically,

the MAPK superfamily has been subdivided into three principal groups: the extracellular regulated kinase (ERK) group, named after the first MAPK cloned [46, 47], the p38 group, and the Jun-N-terminal Kinase (JNK) group [48, 49]. Eukaryotes ranging from unicellular organisms to mammals variously possess members of these groups. Besides having all different types of MAPKs, vertebrates usually have multiple members of any one pathway: for example, they have two partially redundant ERKs, p42 and p44, and also three partially redundant JNK genes [50-52]. These pathways, centred upon a tiered-stage of three sequential phosphorylations, are conserved both structurally and functionally. The core MAPK module encompasses three kinases: a MAPKKK (or MAPK kinase kinase), a ser/thr kinase that phosphorylates and activates a MAPKK (or MAPK kinase), which is a dual specificity kinase, phosphorylating a TXY motif in the target requisite MAPK, and the MAPK proper, a ser/thr kinase whose nuclear and cytoplasmic targets are normally transcription factors, but include cytoskeleton associated proteins and other kinases as well. Each of these module kinases and the cascades they take part in, have specific spatial and temporal regulators that are usually conserved. Once activated, MAPKs command a pivotal role in signalling, leading to cellular change and modification of gene expression. This kind of regulation allows for specific and immediate changes in the activity of the module. In *Drosophila*, the three main MAPK pathways are well represented, with little or no redundancy. Here, the JNK pathway has been the focus of multiple studies.

- **JNK signalling in dorsal closure**

Dorsal Closure (DC) is driven by dynamic signal-dependent changes in cell shape, mobility, and adhesion. JNK/Jun signalling is required for DC and loss-of-function mutations of each pathway component tested (e.g. *slipper*, *hemipterous*, *basket*, *jun*, and *kay*, encoding the JNKKK, JNKK, JNK, Jun, and Fos, respectively) fail in DC (reviewed in [53-55]). Mutations in these genes lead to embryos with severe dorsal and anterior open cuticles indicative of a failure of DC and head involution. Loss of JNK pathway activity has several consequences that contribute to a failure of DC. Mutant embryos exhibit a loss of *dpp* expression in LE cells without perturbing other

elements of the *dpp* expression pattern [56-58]. *puckered (puc)* expression (a gene coding for a JNK-specific phosphatase) is similarly affected [59] (Figure 11). JNK pathway mutants also fail to accumulate the proper cytoskeletal network in LE cells and thus LE cells do not fully elongate dorsally [57, 60]. Lateral cells never elongate when the JNK signalling cascade is blocked by mutations suggesting that Dpp, a JNK pathway transcriptional target and secreted factor, may mediate the ventrally directed wave of cellular elongation observed in wild type embryos. JNK signalling also regulates changes in the cortical actin cytoskeleton in leading edge cells [50, 54, 60-62] controlling the polymerization of actin into a cable, as well as the joining (zipping) of the contra lateral epithelial cell sheets [63].

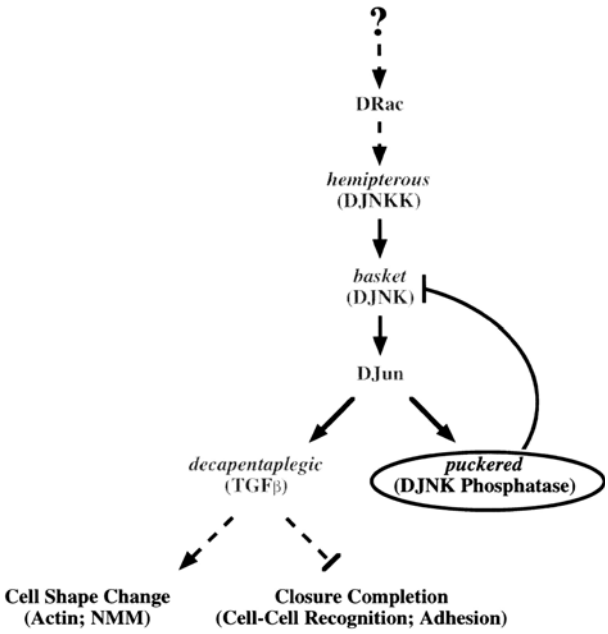


Figure 11. JNK Signalling Cascade. During dorsal closure the JNK signalling is activated in the leading edge where it controls the expression of *puc (*a dual specificity MAPK phosphatase). *puc* regulates its own expression in a negative feedback manner and controls the expression of *dpp* [59].

- **JNK signalling in thorax fusion**

During normal thorax morphogenesis, the JNK pathway appears to determine the leading cells in the moving epithelia and to set up the borders between columnar and squamous epithelia [41] (Figure 12). Under JNK loss-of function conditions, e.g. homozygosity of the *hep*1 allele, or over expression of Puc*,* the thoracic epithelia fail

to reach the midline and/or to fuse contralaterally. These prepupal defects give rise to adults with a thoracic cleft phenotype. In the same manner as in dorsal closure, JNK activity is auto regulated during thorax closure by induction of the negative feedback regulator Puc. Hep activity is both required and sufficient for *puc* expression, suggesting that this gene is entirely under the control of the JNK pathway and, conversely, that *puc* expression reflects JNK activity. Another component of the pathway is D-Fos. The *D-fos* hypomorphic allele *kay2* gives rise to a cleft thorax that can be efficiently rescued by expressing wild-type D-Fos or by eliminating one copy of the *puc* gene. Furthermore, *puc* expression shows a remarkable correlation with high D-Fos levels during thorax closure, and a dominant negative form of D-Fos can decrease *puc* expression in the medial margin cells. It is therefore likely that also during thorax closure, D-Fos acts downstream of the JNK pathway and contributes to the expression of *puc*. Since the expression of D-Jun is identical to D-Fos expression during thorax closure, it is conceivable that D-Fos acts together with D-Jun in a classical AP-1 transcriptional complex, as in embryonic dorsal closure [42].

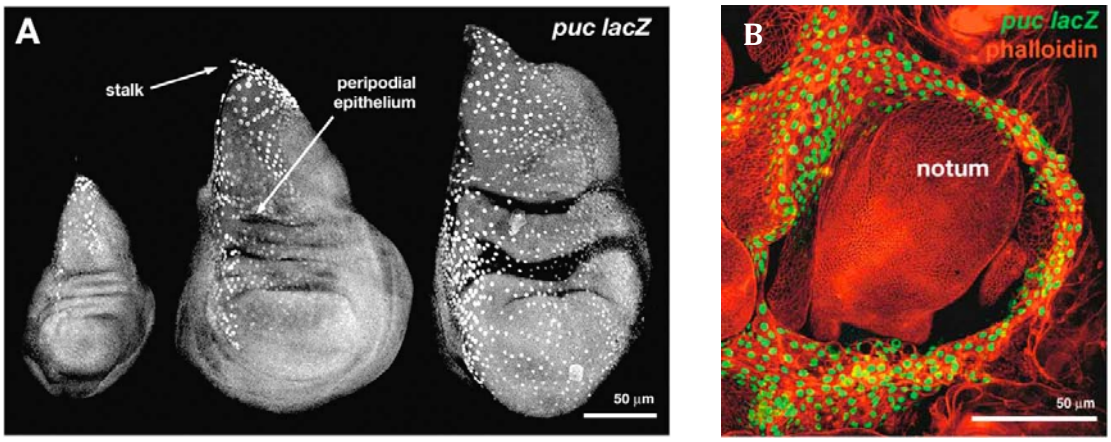


Figure 12. The JNK signalling is active in Peripodial and Stalk Cells during Eversion A) Expression of puc^{E69} LacZ enhancer trap in third instar larval imaginal discs. B) *puc* expression is restricted to cells at the edge of a half-everted wing imaginal disc in a wild-type pupa 4.5 hr. APF. puc^{E69} β-gal expression (green). Actin (phalloidin) is in red [14].

- **JNK signalling in tissue polarity**

Epithelial planar polarity (also called tissue polarity) in *Drosophila* is present in all adult structures derived from imaginal discs [64-67]. Different tissues show distinct

aspects of planar polarization. In the wing, for example, each cell orients itself proximal-to-distally, generating a hair at the distal vertex (reviewed in [68]). In the eye, planar polarity is reflected in the arrangement of the ommatidia in the dorso-ventral axis [69].

Although the role of JNK pathway in *Drosophila* eye development is not clear since the loss of *bsk* or *jun* do not show any gross morphogenetic defects in mutants clones in eye, it is suspected that signalling through Hep and Bsk in the developing eye is functionally redundant and compensated through other JNK and/or p38. Interestingly, however, genetic interaction studies suggested that the JNK signalling is involved in Frizzled (Fz)-mediated planar polarity generation. *puc* causes planar polarity eye phenotypes, establishing that JNK acts in planar polarity signalling [69] (Figure 13). The JNK signalling might not be required for photoreceptor differentiation but might be important for developmental events like rotation of eye facets. It is still intriguing to speculate that perhaps DC also relies on proper planar polarity of the embryonic dorsal epidermis in order to coordinate the directed cellular movements of the epithelium toward the midline [70].

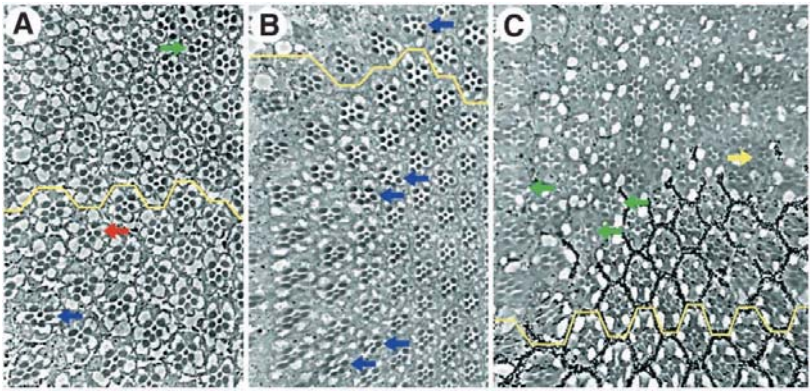


Figure 13. *jun*- clones show polarity defects. Examples of *jun*- mutant eye tissue; the clones are marked by the absence of pigment, a wild-type pigmented area is present in bottom of C). A, B) jun^{IA109} allele; C) $jun^{76\text{-}19}$. Mutant ommatidia can be symmetrical of the R4/R4 type (blue arrows) or display inverted chirality (green arrows) and rarely rotation defects (red arrow). Ommatidia with missing photoreceptors are also found (yellow arrow). The equator is indicated with a yellow line [69].

Wound Healing

Organisms maintain their structural and functional integrity in response to external injuries and physiological dysfunction through a variety of mechanisms, including wound healing/tissue repair and tissue regeneration and cell turnover. The first two processes are both triggered by exogenous damage and can affect multiple cell types, requiring cell replacement over a long time scale time; however, there is a clear distinction between them. The purpose of wound healing is to restore tissue continuity without precise replacement of lost/damaged tissue and is achieved by formation of a scar. On the contrary, tissue regeneration refers to the replacement of lost/damaged tissue so that both morphology and functionality are completely restored. This can occur in combination with wound healing [71]. Improving wound healing is particularly important for victims of burns or blasts and people with slow healing skin lesions, such as patients suffering from diabetes (see [72]). There are also cases of excessive healing (as occurs in hypertrophic and keloidal scars), which can lead to abnormal scars that grow beyond the boundary of the original site of a skin injury.

Furthermore, wound closure is the most important step after surgery and it contributes to the success or failure of surgical interventions. Another motivation to study wound healing is the fact that DNA microarray analyses have shown that the gene expression pattern of healing skin resembles that of malignant tumours [73, 74]; non healing wounds could be a risk factor for malignant transformation (see [75]).

- **Wound healing in mammals**

Cellular studies of mammalian wound healing have shown that it is a complex process that takes weeks to complete and involves not just the damaged epithelial cells and their neighbours, but also fibroblasts and blood vessels in the underlying stroma, and inflammatory cells that are recruited to the wound site [76, 77] (Figure 14).

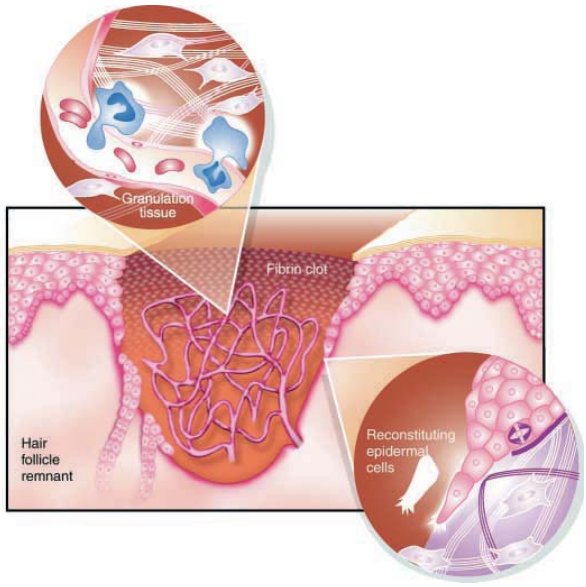


Figure 14. The cellular players in the healing of a skin wound. Wound are first 'plugged' with a fibrin clot, which is infiltrated by inflammatory cells, fibroblasts and a dense plexus of capillary vessels. Secondly, the epidermis migrates forwards from the edges of the wound. Neutrophils and macrophages (blue) emigrate from the wound capillaries into the wound granulation tissue where they kill microbes, engulf cell and matrix debris and release signals that act on the host wounded tissues [76].

Only the first step in mammalian wound healing, the proteolytic cascade that culminates in fibrin deposition and clot formation, is well understood at the molecular level [78]. As the clot forms, platelets bound to it and to the damaged tissue release additional procoagulant proteins as well as growth factors and chemokines that can attract neutrophils and monocytes that mediate an early inflammatory response. Keratinocytes at the wound margin become activated, break down their cell junctions, and assume a lamellipodial crawling morphology as they spread across the wound site to restore epithelial integrity [79, 80]. The early inflammatory cells release additional signals that can attract and activate fibroblasts, macrophages, and blood vessel endothelial cells. These cells infiltrate the wound site and form a specialized stroma called granulation tissue, which facilitates reepithelialisation, helps contract the wound, and is later remodelled to form the scar [81].

- **Wound healing in invertebrates. *Drosophila* as a model for studying epithelial wound repair**

Understanding the molecular basis of wound healing and regeneration in vertebrates is one of the main challenges in biology and medicine. *Drosophila* has been used to model genetic aspects of various human pathologies, and has emerged as a valuable model for studying these processes because the genetic networks and cytoskeletal machinery involved in epithelial movements occurring during embryonic dorsal closure, larval imaginal disc fusion/regeneration, and epithelial repair are similar to those acting during wound healing and regeneration in vertebrates [23]. Fruit flies are an ideal system to combine genetic techniques with live imaging. However, only in the last 10 or so years flies have become recognised as a worthy model to investigate the genetics of tissue repair and the associated inflammatory response [1] (Figure 15).

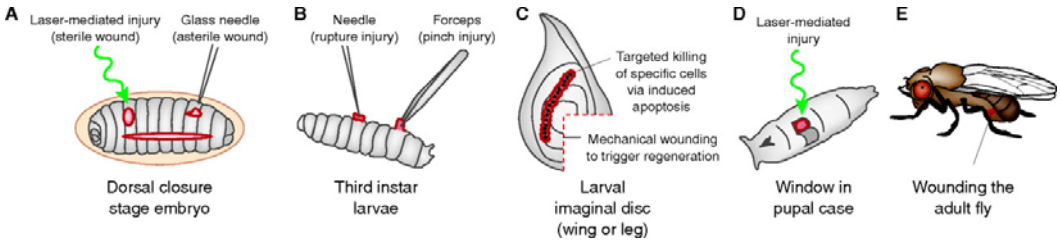


Figure 15. Different stages of *Drosophila* development (from embryo to adult) can be used as wound-healing models. A) Embryos can be wounded by glass needles or by laser ablation. B) Larval wounding is mediated by needle or by pinching with forceps damaging the epidermis. C) Imaginal discs mechanical wounds repair requires grafting back the tissue into adults. Recently targeted killing using the Gal4/UAS system has also be used. D) Pupal stages are wounded by laser ablation (this requires the opening of the pupal case). E) Adult flies can be wounded with the help of tungsten needles [1].

- ***Wound healing in Drosophila embryos***

Simple embryonic epithelial tissues have an extraordinary capacity to reseal small discontinuities very rapidly and efficiently through an epithelial resealing mechanism. This was initially described in the chick embryo [82], but seems to be conserved across species [83-85]. In all systems, small epithelial wounds close via the cooperation of three distinct mechanisms: the assembly of an actomyosin purse string

in the epithelial cells at the wound margin, the protrusive activity of epithelial cells at the margin, and the contraction and ingression of deep cells when those are exposed [86-88]. In *Drosophila* embryos, the cells at the wound margin constrict their apical edges through the action of an actomyosin cable that assembles just minutes after wounding and is linked intercellularly through adherens junctions. Concomitant with the formation of the purse string, cells at the wound margin begin to extend actin-rich protrusions. When opposing wound margins come into close proximity, filopodia and lamellipodia from opposing flanks make contact that mainly function to knit the confronting epithelial edges together to finally seal the wound closed [85] (Figure 16).

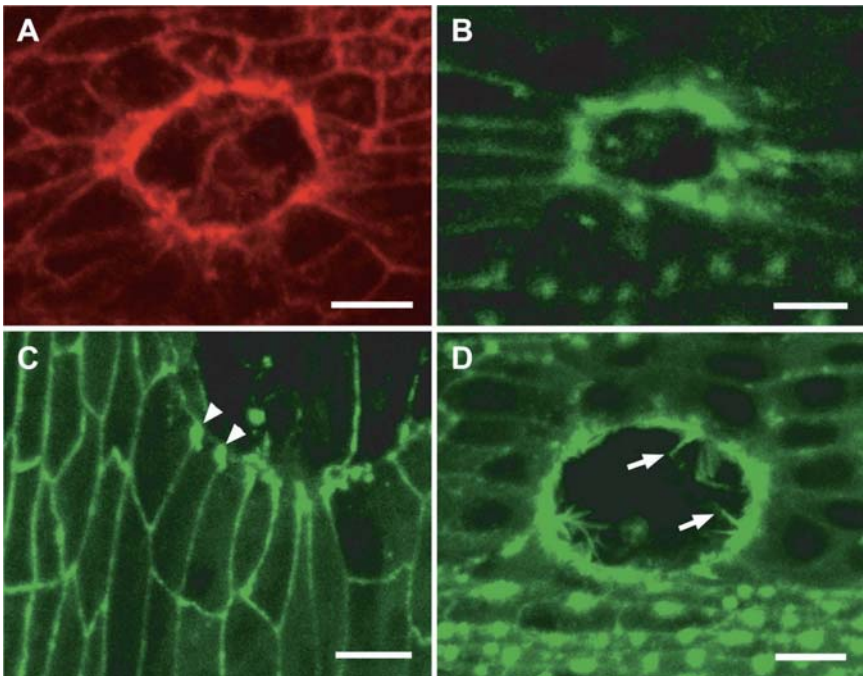


Figure 16. The cytoskeletal machinery of wound closure. A) Phalloidin-staining (red) reveals a dense cable of actin that encompasses the full circumference of the wound. B) Similarly, non-muscle myosin (green) localizes to the leading edge of epithelial cells at the wound margin. C) Wounded α-catenin–GFP embryos have bright spots of positive staining where neighbouring cells abut one another around the wound circumference (arrowheads). These sites probably represent adherens junctions, where intercellular segments of the actin cable are inserted. D) An image from a live GFP–actin wound, revealing both the actin cable and filopodial protrusions (arrows) extending from leading-edge cells (modified from [85]).

- ***Wound healing in Drosophila larvae***

Simple punctures in larval epidermis results in a variable loss of blood (hemolymph) from the wound site. Within 10–15 min, the wound site begins to darken and a plug forms in the gap. This plug is composed of debris, presumably the remnants of necrotic cells damaged by wounding that are disorganized and highly vesiculated and not bound by a cell membrane or basal lamina. The plug may also contain blood coagulation products. By 2 or 3 days after wounding, debris is cleared, the scab resolves, and the exterior of the animal resumes a nearly normal appearance. Epidermal cells that grow back across the wound gap appear to participate in debris clearance, because they extend processes that engulf the debris and contain within their cytoplasm vesiculated material resembling debris. Other components of the plug and scab may be degraded extracellularly or passively shed from the wound site. Over the next 24 hours, the outer part of the plug is converted into a scab. This part of the plug becomes electron dense as the scab enlarges and darkens presumably due to a melanisation reaction. Melanisation affects all of the external structures at the wound site including the debris, the edges of the damaged cuticle, and even entrapped tissues such as tracheae. The JNK pathway is activated in a gradient emanating out from the wound, and the epidermal cells spread along or through the wound plug to re-establish a continuous epithelium and its basal lamina and apical cuticle lining. Inactivation of the JNK pathway inhibits epidermal spreading and reepithelialisation but does not affect scab formation or earlier healing responses. Several studies have demonstrated that the cellular responses of wound healing are coordinated by multiple signals emanating from the wound, including a negative feedback signal between scab formation and the JNK pathway [81] (Figure 17).

A reporter-based *in vivo* RNAi screen in the *Drosophila* larval epidermis has been done to identify genes required for normal wound closure. In addition to JNK pathway components, small Rho-GTPases and MMP1 were found to be essential for epidermal healing in the *Drosophila* larva [89, 90].

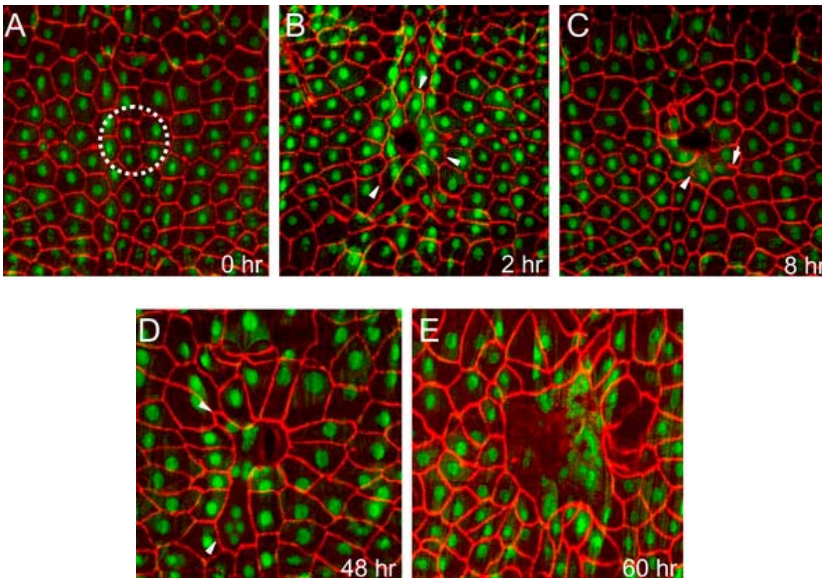


Figure 17. Larval epidermis at different time points after wounding. A) Larval epidermis pre wounding. B) After 2 hours of wounding, cells at the wound elongate and orient towards the wound. C) Cells at the wound margin start to fuse to form a syncytium with 4 nuclei after 8 hours. D) After 48 hours of wounding, the central syncytium contains ten or more nuclei. E) 60 hours after wounding a large syncytium is formed with more than 30 nuclei. Fasciclin III in red and GFP (Nuclear) in green [81].

- ***Wound healing in imaginal discs cultured in vivo***

Wounded imaginal discs have capacity to heal and regenerate after implantation in adult abdomens or after culture in growth permissive conditions. After wounding of wing imaginal discs, the wound edges of both epithelial layers of the disc (CE and PE) curl towards the lumen and the wound surface contracts. Short-lived cell contacts are first established between CE and PE (heterotypic contacts) that switch at 24h to homotypic contacts bearing desmosomes between closely apposed CE cells along the wound [91]. Curling likely results from contraction of an actin-rich cable at the apical end of the cells at the edge of the wound. This cable, present in both epithelia, is more apparent along the wound edge of the PE and at the vertex areas. In both epithelia, contraction of the actin cable induce subsequent changes in the overall morphology of disc fragments, which is already apparent 5 hours after cutting and specially at 12 hours. A second driving force is exerted through filopodial extensions already present along the wound area soon after cutting. At the vertex, such extensions allow contacts

between cells, which start the zippering process by pulling, and knitting wound edges together. This process seems to proceed from proximal (central) to distal (peripheral) areas of the disc until the wound is closed. At the cellular level, zipping appear to start from filopodia at the basal–lateral border of cells at the wound edge, the apical part lagging behind in time. This proximal distal, basal–apical, zippering process is completed at 24 hours [92] (Figure 18).

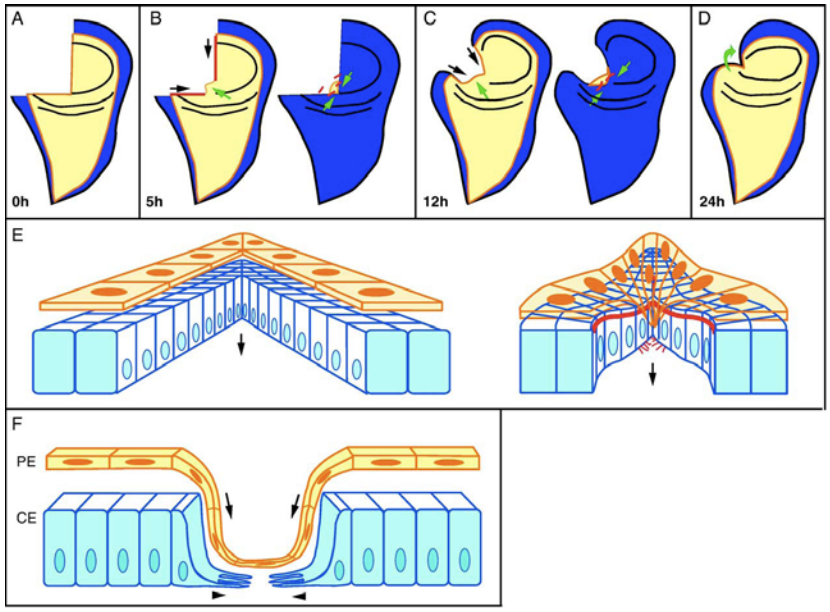


Figure 18. A) Schematic PE view of a 3/4 wing disc fragment soon after cutting. CE is shown in dark blue and PE in orange. B) 5 hours after cutting. Left, PE view. An actin cable forms at the edge of the PE (red lines, left) pulling in the wound edges (black arrows) shrinking the wound surface. PE cells elongate towards the wound vertex (green arrow). Right, CE view. CE cells extend filopodial processes (red lines) to zip wound cells at the vertex. C) 12 hours after cutting. Left, PE view. Increasing numbers of PE cells elongate towards the wound vertex (green arrow) pulling in the wound edges (black arrows). Right, CE view. PE cells still cover the wound vertex, whereas filopodial processes (red lines) progressively zipper the wound (green arrows). D) PE view of a 3/4 wing disc fragment 24 hours after cutting. Wound healing is completed with PE cells covering the healed region (arrow). E) Schematic frontal views of the wound vertex soon after cutting (left) and during the zippering process (right). The wound closes (black arrows) from inside (distal) to outside (proximal) due to the contraction of the apical actin super cable (thick red lines in the CE) and filopodial extensions (basal thin red lines). PE cells (orange) help wound healing elongating towards the wound and covering the vertex. F) Schematic representation of a Z section at the wound vertex. PE (orange) elongates down covering the wound vertex (arrows), whereas CE cells (blue) extend filopodial processes from their basal–lateral surfaces (arrowheads) knitting the CE cells at the wound edge [92]

Altogether, these observations indicate that healing of wing imaginal discs is completed by a "purse-string" contraction of the actin cable together with cellular zippering by filopodial extension and adhesion.

The JNK signalling activity (*puc* expression) is limited in unwound discs to the stalk region and to few rows of the PE. On top of this WT pattern, which is maintained throughout healing and regeneration, *puc* is also expressed at the wound edge of regenerating discs. At 5 hours post wounding, a few scattered positive cells for *puc* are seen in both epithelia spanning an average of 10 to 15 cell rows from the wound edge. By 12 hours, the number of *puc*-GFP-positive cells reaches a maximum, though its range is maintained to 10–15 rows of cells from the wound edge. Such increase occurs in both epithelia Meanwhile, cells elongate towards the wound edge and the distal tips of the wound contracts, in parallel to maximal *puc* expression. At 24 hours, the number of GFP cells has decreased, though its range still spans 10–15 rows of cells, and 3/4 fragments are almost completely healed. From then on, GFP expression persists around the healed wound up to 6–7 days of culture when regeneration is completed [92] (Figure 19).

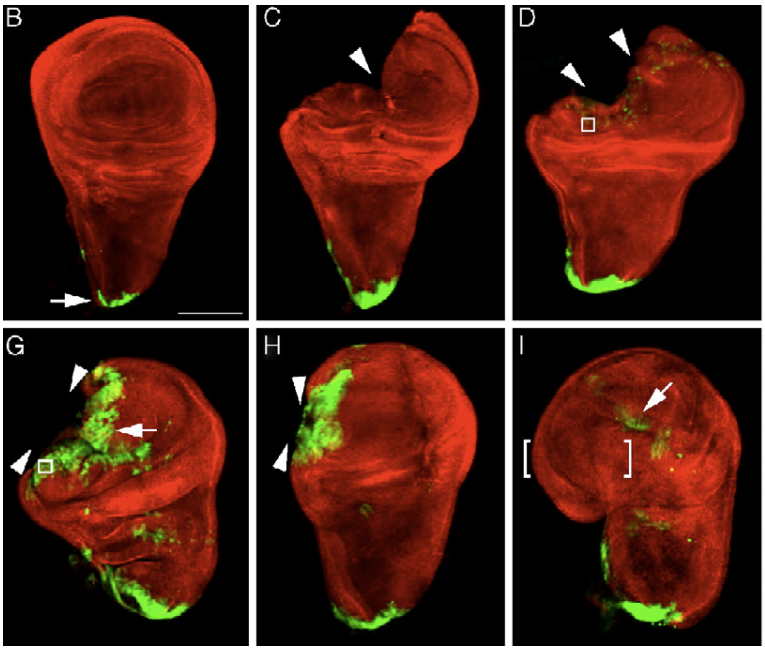


Figure 19. *puc* expression patterns during imaginal disc wound healing. Double staining for *puc* (green; puc^{E69}-A–Gal4; UAS-GFP) and Fasciclin III (red). B) Intact wing disc. *puc* expression at the stalk (arrow) holds along wound healing and regeneration. C) 3/4 fragment immediately after cutting.

puc is expressed at the stalk but not at the wound edge (arrowhead). D) 3/4 fragment after 5 hours of *in vivo* culture. *puc* is weakly detected within several rows of cells from the wound edge (arrowheads). E) Enlargement of white square in D) at the PE confocal plane. *puc* is expressed in a few PE cells. G) 3/4 wing disc fragment after 12 hours *in vivo* culture. *puc* is strongly expressed at the wound edge (arrow) in parallel with wound contraction (arrowheads). H) 3/4 fragment after 24 hours of *in vivo* culture. *puc* is expressed at the wound (arrowheads), which is almost closed. I) 3/4 fragment after 7 days of *in vivo* culture. A few cells express *puc* at the former wound region (arrow). The regenerated CE (bracketed) does not express *puc* [92].

Importantly, wound healing during regeneration fails to initiate or is aberrant in different mutants of the JNK pathway. In particular, regenerating discs from hemizygous *hep* mutants, remain unhealed after 7 days. In these discs, *puc* expression is strongly reduced at the wound confirming the role of *hep* on the activation of *puc.*

- ***Cell death induced regeneration in imaginal discs***

The analysis of the regeneration of *Drosophila* imaginal discs has been until recently very complicated involving skilful microsurgery [93, 94]. Recently, however, genetic means have made possible to investigate regeneration *in situ* in imaginal discs without the need for mechanical manipulation. Cell death is locally induced in certain domains of the disc using the Gal4/UAS binary system in combination with $Gal80^{ts}$ [95] to transiently activate pro-apoptotic genes, thereby mimicking microsurgical ablation. This allows cells to be killed in specific domains for a limited time period, after which the tissue recovers and regenerates [96].

Cell-induced regeneration includes two phases: the first, which occurs near the wound edges, involves JNK activity and is important for healing and rapid local proliferation. The second involves proliferation to compensate for the lost tissue and is extended throughout the damaged compartment. As in normal development, the regenerative growth that occurs in this second phase requires the reconstitution of morphogenetic signals that drive proliferation [97] (Figure 20).

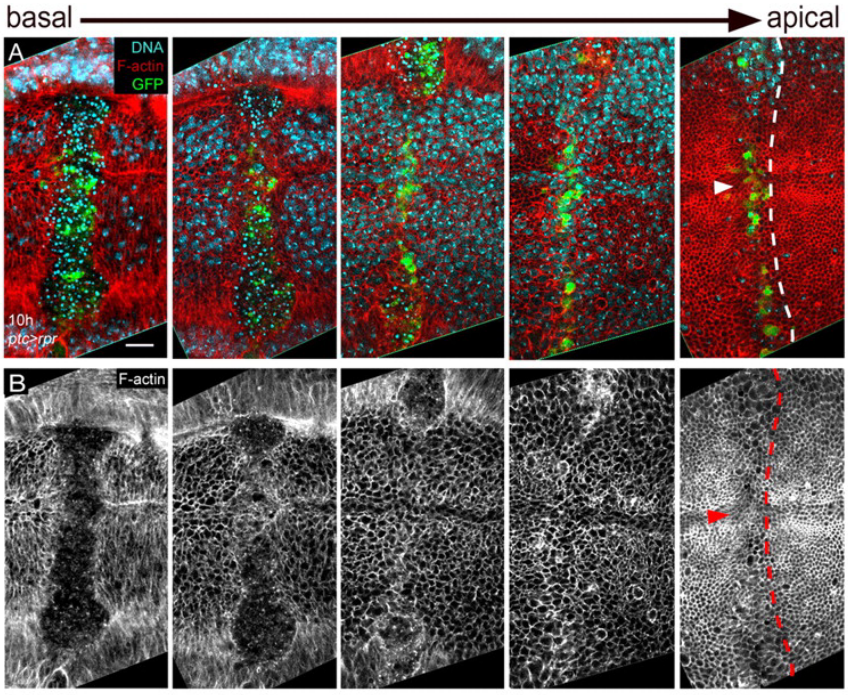


Figure 20. Confocal sections of phalloidin-labelled wing disc at 10 hours of Rpr induction. A) Merges of F-actin (red), Ptc-Gal4, UAS-GFP (green) and nuclei (TO-PRO3, blue). B) Single-channel labelling of F-actin. The healing process can be observed from basal (left) to apical (right). It starts rapidly from the dorso-ventral (DV) boundary (arrowhead) and extends progressively to proximal regions until it is almost complete in more apical sections. The antero-posterior (AP) boundary is indicated with a dashed line. Anterior is to the left and posterior to the right [97].

- ***Adult Drosophila as model for studying wound healing***

Adult *Drosophila* has also been used as a model to study wound healing. After mechanical wounding of adult flies with either razor blades or iridectomy scissors the epithelium is healed by epithelial cells protruding lamellipodia or filopodia that undergo cell shape changes. The JNK signalling pathway also participates in wound healing in adult flies as indicated by the expression of puc^{E69}, a *puc* enhancer trap line. Furthermore, this induction was controlled by *kay*, which encodes D-Fos, one of the transcriptional effectors of the JNK pathway. In absence of D-Fos, the wounded epidermis could not initiate epithelial repair [98] (Figure 21).

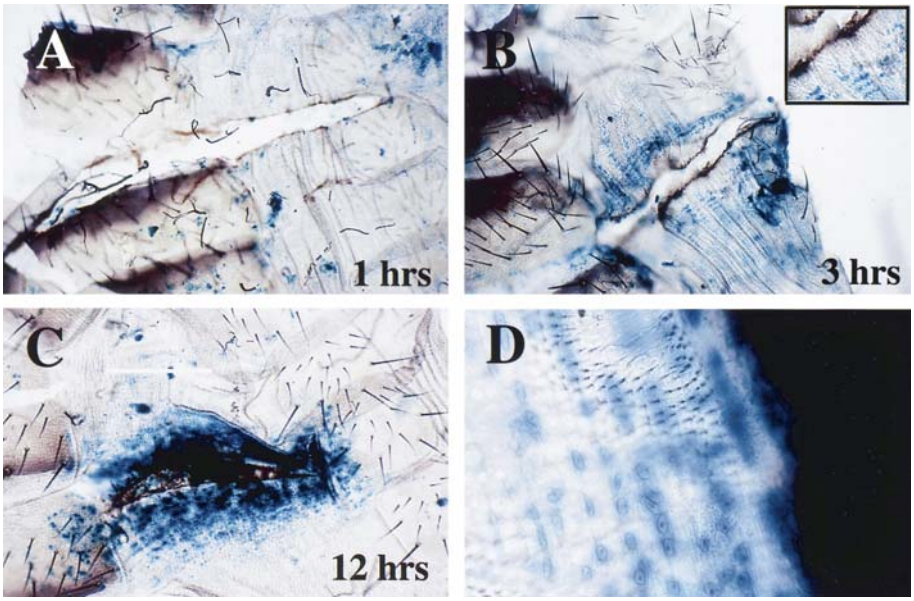


Figure 21. Time course of *puc-LacZ* gene expression. *puc* gene expression was assayed in a puc^{E69} strain at different time post wounding: A) 1 hour, B) 3 hours, C) 12 hours. Small top-right square in B is a higher magnification of the leading edge observed at 3 hours post wounding. The leading edge seems to be retracted from the edge of the wound. D) 100X magnification showing that *puc* is expressed from the wound edge over several cell rows in a decreasing gradient (at 12 hours post wounding) [98].

- **JNK activity regulation in wound healing**

In the context of wound healing, the specific signal(s) required to trigger JNK activation is (are) still unknown. Growth factors, mechanical stretching of the woundedge cells, loss of cell polarity or necrotic signals are all candidates for upstream activators [98-100]. Indeed, several small GTPases of the Rho family are upstream activators of JNK signalling during wound healing both in mammals and *Drosophila* [53, 101-105]. Alternatively, release of intracellular ATP or Ca^{2+} by damage or signals secreted by inflammatory cells recruited to the wound may participate in JNK activation [106].

In mammals, inhibition of JNK signalling results in defects in fibroblasts migration during wound healing [107, 108]. Furthermore, mammalian AP-1 transcription factors regulate the expression of several genes involved in healing, such as those encoding matrix metalloproteinases (MMPs), integrins, cytokines, and growth factors [109-112] and act during the inflammatory/proliferative phases and in keratinocyte migration

[107, 113].

In *Drosophila*, JNK activity can be reported by the expression of *puc*. In both larval [81] and adult [98] epithelial wound edges, *puc* expression is seen from as early as 4 hours after wounding. Wounding of larval imaginal discs also induces JNK activity, extending several cell diameters from the wound edge [92]. The presence of JNK activity corresponds to the cells that will participate in the wound healing response [92]. During all stages of *Drosophila* development thus far examined, impaired healing has been reported to result from expression of a dominant-negative form of Basket (the *Drosophila* homologue of JNK) [81], expression of various interfering RNAs targeted against proteins in the JNK pathway [89] or loss-of-function mutations in Kayak (the *Drosophila* homologue of Fos) [98].

Cytoskeletal machineries that drive wound repair/re-epithelialisation

Cells show dynamic reorganization of cytoskeletons to migrate and change their shapes. Multiple studies have revealed striking similarities in signalling mechanisms, cytoskeletal modulation and cell shape remodelling between epithelial morphogenesis and wound healing [114]. Specific molecular elements like actin, myosin, microtubules, and the plasma membrane respond dynamically during wound repair and its been shown that perturbations of each of these components yield abnormal healing.

- **Actin**

Wound re-epithelialisation in *Drosophila* embryos requires an active and dynamic actin machinery. A contractile actomyosin cable closes epithelial holes as a purse string, while dynamic filopodia (finger-like actin-rich membrane protrusions) knit the confronting epithelial edges together to seal the wound [85]. The function of the actomyosin cable is dependent on a Rho-GTPase, Rho1. *rho1* mutant *Drosophila* embryos cannot assemble an actomyosin cable although compensate its absence with extensive filopodial protrusions. These zip neighbouring cells together dragging the closing of the wound [85]. On the contrary, the expression of a dominant-negative mutant version of the small Rho GTPase Cdc42 in the embryonic epidermis leads to inhibition of filopodia formation and a failure of the wound edges to seal [85].

- **Microtubules**

Microtubules (MT) are an important component of the cell cytoskeleton, and the interaction between microtubules and actin is fundamental for many cell processes [115]. MTs reorganization in non-dividing cells has been observed both during developmental differentiation processes and after tissue wounding. The origins of such MT reorganizations are not known. During dorsal closure, MTs form stable arrays that align parallel to the elongation axis and are restricted to the apical cell cortex. Indeed, at the zippering stage, dynamic MTs intrude into lamellipodia and filopodia. Radial MTs engagements associated with F-actin have also been found in *Xenopus* oocytes.

Wounding of *Xenopus* leads to a dramatic reorganization of the microtubule cytoskeleton and inhibition of microtubule polymerization results in a failure of actomyosin purse-string formation and blocks repair. This indicates that MTs are required for delivery of the components of the actomyosin purse-string to the wound site [116]. Further, MTs are also reorganized after scratching in cultured epithelial cells [117], which is believed to support cell migration towards the wound [118] (Figure 22). On the contrary, not significant accumulation or rearrangements of MTs have been found in *Drosophila* embryo wounds. Still, the perturbation of the MT network in this model has a negative effect on repair and embryos treated with colchicine show defects in actin ring assembly [119].

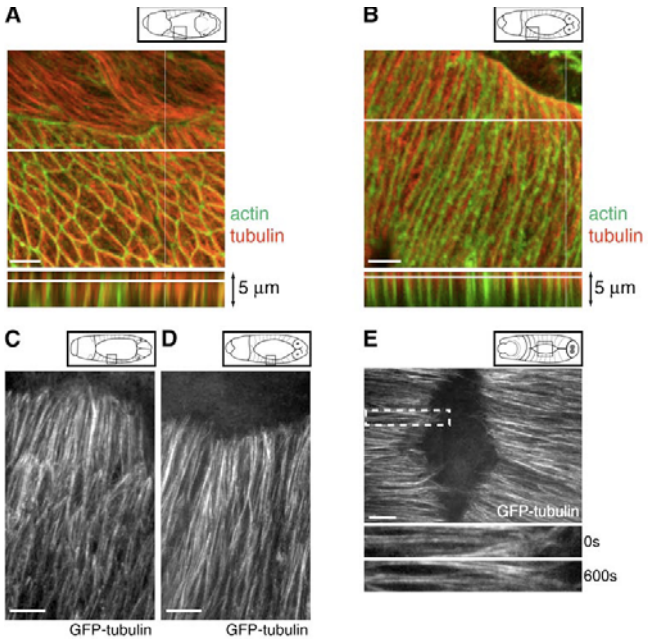


Figure 22. MT Distribution during DC. Fixed wild-type embryos stained with anti-tubulin antibody for MTs (red) and phalloidin for actin and the cells outline (green). The bottom panels show cross-sections parallel to the A/P cell axis. A) Embryo at the onset of DC. B) Zippering-stage embryo. Note the more apical tubulin in the cross-section as compared to A). C and D) Images of a living embryo showing MT array formation in approximately three rows of GFP-tubulin expressing epithelial cells. C) Living embryo at the onset of DC. Leading edge cells have rearranged their MTs. D) One hour later, MTs have also rearranged in the remaining epithelium. E) Frames taken from a movie sequence showing GFP-tubulin-expressing cells in an embryo toward the end of DC. The first frame shows a dorsal overview. The boxed area is enlarged in the bottom frames showing two time points. MT arrays in the cell body (left half) do not change much, but MTs at the leading edge (on the right) are dynamic [118].

- **Myosin**

Myosin is required for epithelial morphogenesis in *Drosophila* and also plays an important role during epithelial wound healing. In both scenarios, cell migration is coordinated through the contraction of a thick cable of actin and myosin in the leading edge of marginal cells [114]. Laser wounded embryos, co-expressing GFP-Spaghetti squash (myosin regulatory light chain, sqh-GFP) and actin (sChMCA), show that myosin II is immediately recruited to the wound edge, where it forms a ring that overlaps with actin throughout the repair process, and starts to dissipate immediately after wounding. Homozygous *sqh1* mutant embryos show severe developmental defects with few embryos completing development [120]. In embryos that do develop, the wound repair response is severely impaired: upon ablation, wounds expand normally but fail to contract. Actin accumulation is significantly reduced and any cable that forms appears disorganized and unstable; as a consequence the wounds reopen. Inhibition of myosin II phosphorylation by treatment with Y27632, a specific Rho kinase inhibitor [121], similarly disrupts cell wound repair. The myosin cytoskeleton is also disrupted in Lat B–treated embryos: both actin and myosin fail to be recruited to the wound edge. Together, this data suggest that myosin II is required for the stabilization of actin at the leading edge of wound [119] (Figure 23).

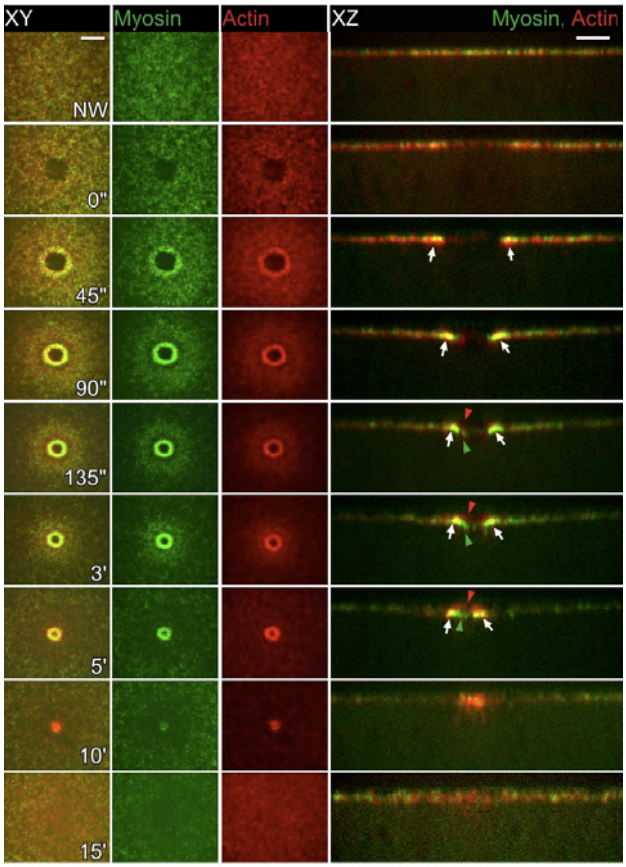
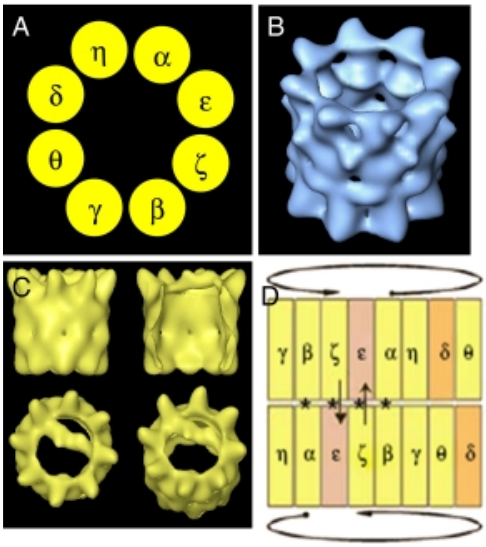


Figure 23. Contribution of myosin II to single-cell wound repair. Laser wounding of embryos coexpressing GFP-Spaghetti squash (myosin regulatory light chain, sqh-GFP) and actin (sChMCA) show that myosin II is immediately recruited to the wound edge (45 s), forms a ring that overlaps with actin during the repair process and starts to dissipate 10 min after wounding. Actin (red arrowhead); Myosin (green arrowheads) [119].

The TCP1 complex polypeptide

The folding and oligomeric assembly of newly synthesized proteins *in vivo* requires the function of certain accessory proteins, so-called 'molecular chaperones' [122] or 'polypeptide chain binding proteins' [123]. Molecular chaperones influence several cell components one of which is the cytoskeleton. The chaperones recognize proteins in non-native conformations, thereby preventing premature folding and aggregation, and mediate the acquisition of the native structure [124]. One such chaperone complex is the TCP1 complex, which is known to mediate folding of actin and tubulins. The TCP1 complex is an oligomeric particle (900 kDa) found in the eukaryotic cytosol that consists of eight distinct subunits, which are the products of

individual genes (α, β, γ, δ, ε, ζ, η and θ) [125] (Figure 24). The TCP1 complex acts as a chaperonin (as suggested by *in vitro* studies demonstrating the function of the complex in refolding urea-denatured tubulin and actin and the folding newly translated alpha and beta tubulin in rabbit reticulocyte lysates). The TCP1 complex is thus required for the production of native actin and tubulin and an undefined set of other proteins. Alterations in TCP1 components, therefore, have the potential to exert pleiotropic effects on cell biology [126], in particular participating in sustaining the integrity of the actin and tubulin-based cytoskeletal systems, essential in all cellular processes requiring cytoskeletal rearrangements, such as normal and metastatic cell migration, developmental morphogenesis and wound healing.



E

Mammalian	Yeast
CCTα	CCT1
CCTβ	CCT2
CCTγ	CCT3
CCTδ	CCT4
CCTε	CCT5
CCTζ-1	CCT6
CCTζ-2	-
CCTη	CCT7
CCTθ	CCT8

Figure 24. Subunit arrangement and three-dimensional analysis of TCP1. A) Cartoon diagram to illustrate the positioning of the eight TCP1 subunits within the chaperonin ring according to [127]. B) Cryoelectron single-particle reconstruction of the TCP1 oligomer [128] and C) TCP1–actin complexes with actin binding to TCP1 in an open conformation spanning the chaperonin cavity [129]. D) Inter- and intra-ring arrangement of TCP1 subunits as determined by immunolabelling and cryoelectron single-particle microscopy [130]. Circular arrows show the direction of intra-ring sequential changes [131]. Asterisks indicate subunits that may be involved in possible inter-ring signalling, with a basic signalling mechanism indicated by arrows [130]. E) Comparison of the nomenclature of TCP1 subunits in mammals and yeast.

The proteins interacting with TCP1 may fall into several categories: folding substrates such as actin and tubulins, which depend on TCP1 in order to reach their native structure; opportunistic proteins that may on occasion make use of the chaperone activity of TCP1 but do not usually require it in order to reach their native state; proteins that utilise TCP1 as a platform for complex assembly; and last, proteins that regulate TCP1 activity [125] (Figure 25).

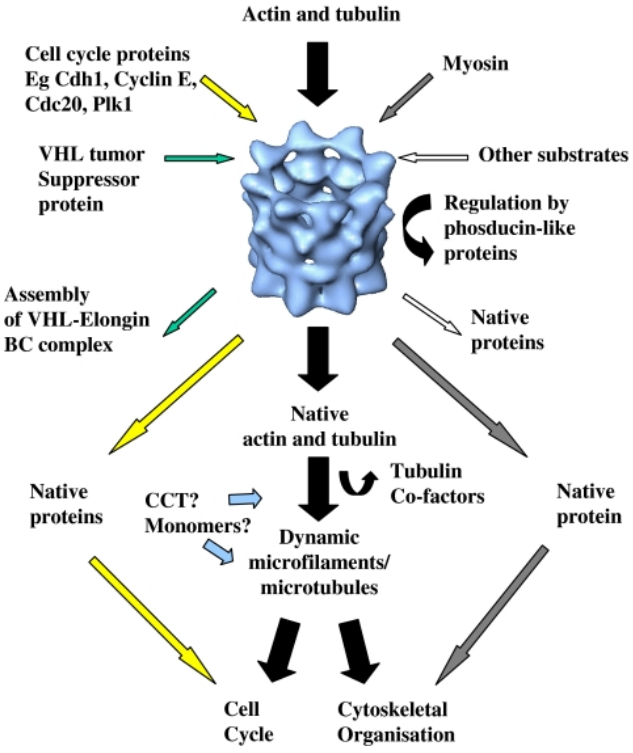
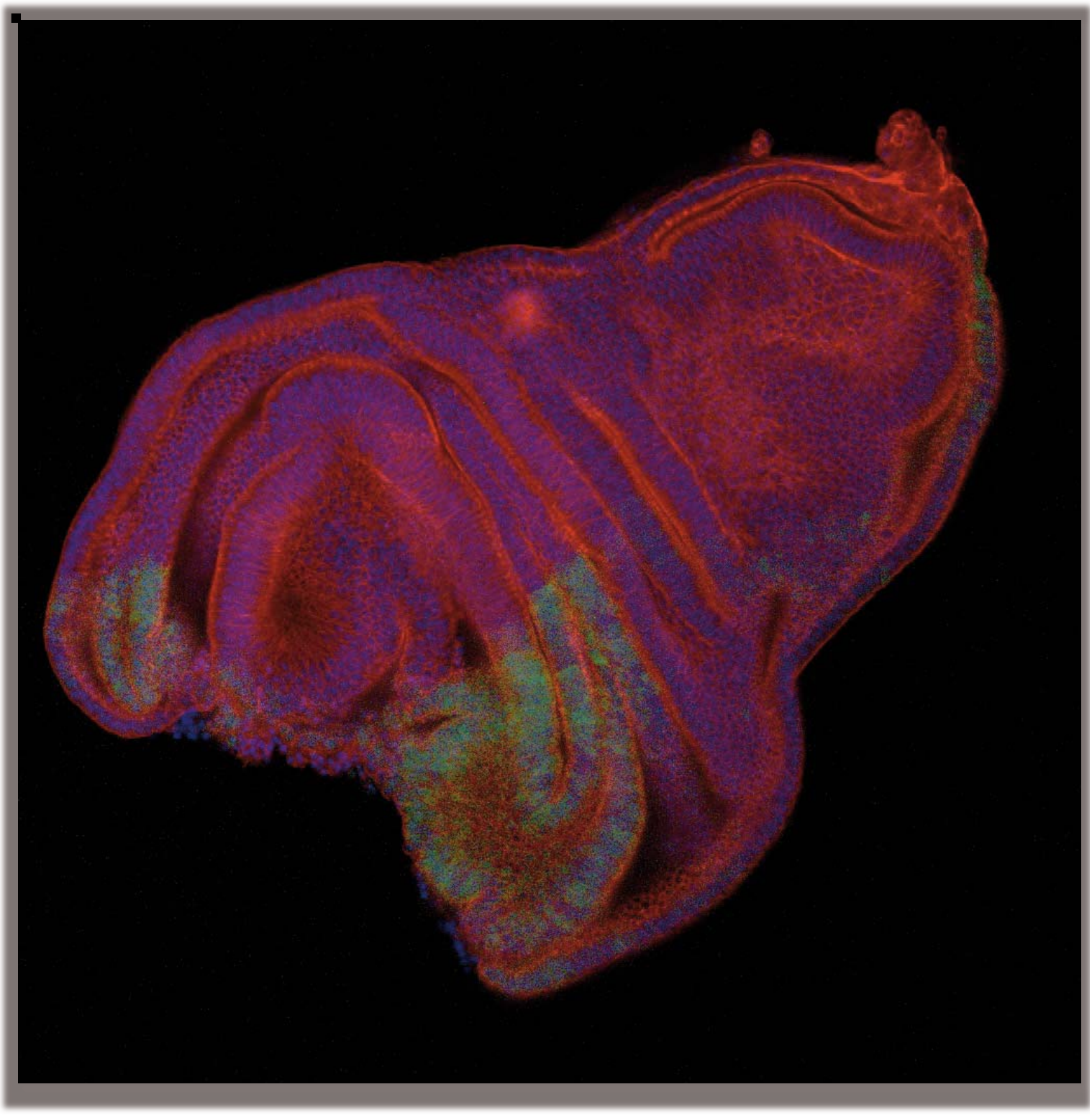


Figure 25. TCP1 coordinates an array of cellular functions via its folding substrates. TCP1 (shown here as a 3-dimensional reconstruction from single-particle electron microscopy [128], is able to accommodate the folding of its major substrates actin and tubulin with additional substrates, some of which are required during cell cycle progression. Coloured arrows track the interactions of proteins with TCP1 [130].

TCP1 α, γ, ζ and θ subunits behave selectively as microtubule-associated proteins *in vitro* [132] suggesting that TCP1 may have a role in tubulin assembly beyond production of correctly folded monomers. Further, TCP1 may also be involved in actin polymerization as suggested by immunofluorescence studies, which detected TCP1 subunits at ruffling edges of fibroblast like cells [132] and at the leading edge

of developing neurites [133]. Both are locations where constant modification of the actin cytoskeleton (microfilament assembly and disassembly) occurs. TCP1 might regulate the actin cytoskeleton dynamics beyond its already well-established role in producing new actin monomers [134]. It selectively binds F-actin, affects the elongation rates at actin filament ends, and reduction in TCP1 subunits levels by siRNA leads to disorganisation of the actin-based cytoskeleton promoting changes in cell shape in mammalian cells [135]. Disrupted levels of TCP1 subunits affected the re-growth of F-actin following treatment with Latrunculin A (LatA), a process that should be independent of new protein synthesis and, furthermore, cells lacking TCP1 ε fail to repolymerise their F-actin [136].

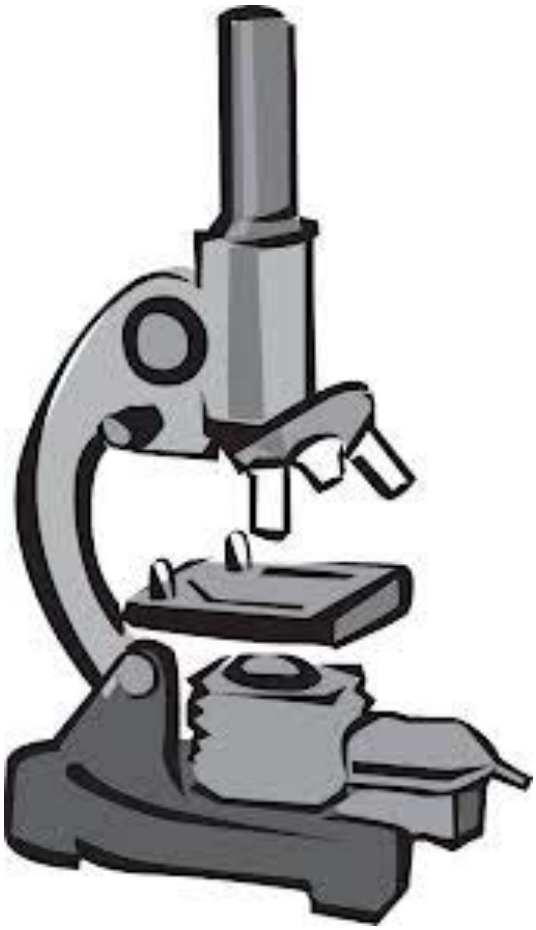
TCP1 is known to play a role during healing in mucosal wounds where mRNA levels of TCP1 ζ, δ, η, and ε are significantly increased [137].

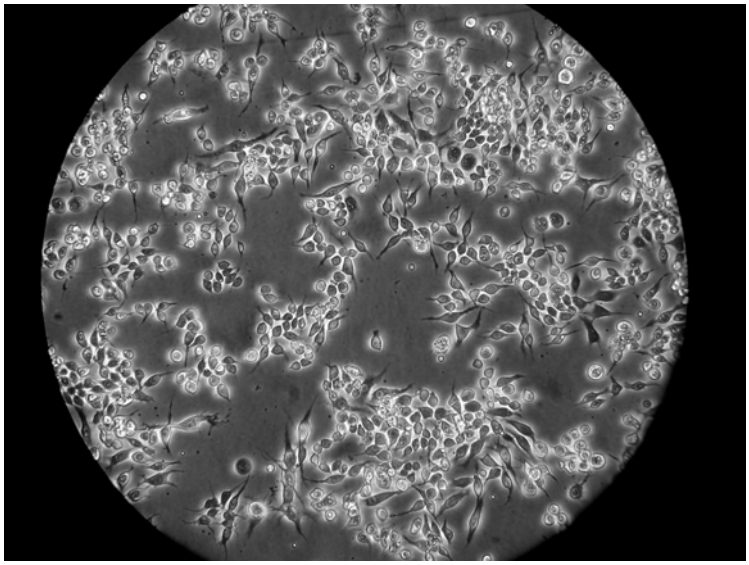


OBJECTIVES

The main aim of this Thesis was to identify and characterize the genes required for wound healing in *Drosophila* wing imaginal discs.

- Study and description of imaginal discs wound healing *in vitro*.
- Identification of genes whose expression changes in cells engaged in healing in imaginal discs
- Phenotypic characterization of healing defective genes (definition of phenotypic categories).
- Detailed characterization of selected healing defective genes.
- Identify healing regulators conserved through phylogeny
- Analysis of the role of the actin cytoskeleton in the healing process (the role of the TCP1 chaperonin complex).
- Exploration of the role of the TCP1 chaperone in the regulation of the actin and tubulin cytoskeleton.





MATERIALS AND METHODS

Drosophila Culture

All the *Drosophila melanogaster* lines were maintained on Standard culture media and according to standard procedures. Fly crosses were performed at 25ºC. Overexpression of UAS or RNAi lines were performed at 18ºC, 25ºC and 29ºC. Specific temperature shifts are indicated in the text.

Fly Stocks

Flies carrying dsRNAis under the control of UAS sequences (UAS-RNAi) were ordered from the VDRC, NIG-FLY and DRSC Stock centres and are enlisted in Supplementary Table 1. Other Stocks are displayed in Table 1.

Table 1

STOCK	GENE/GENOTYPE	LINK	REFERENCE
Pnr-Gal4	*yw; if/CyO; pnr-Gal4/TM6B*		[138]
Pnr-Gal4–Puc^{E69}	*yw; If/CyO; pnr-Gal4, puc^{E69}-LacZ/TM6B*		[14]
Jupiter-GFP	*y w; Jupiter^{ZCL0931}/TM3, Ser*		BDSC 6825
Life actin-GFP	*y w; UAS-Lifeact-GFP*		BDSC 35544
UAS-Tkv^{QD}	*If/CyO; UAS-Tkv^{QD}/TM6B*		[139]
Hh-Gal4; GFP	*yw; hh-Gal4; UAS-GFP*		[140]
Puc-Gal4; GFP	*yw; UAS-GFP; puc^{E69}-Gal4I/TM6B*		[14]
En-Gal4; GFP	*yw; en-Gal4; UAS-GFP*		[141]
UAS-Ap-2	*Ap2*	CG7807	[142]
UAS-Ara	*araucan*	CG10571	[143]
UAS-Bab2	*bab2*	CG9102	[144]
UAS-Bil080	*prickle*	CG11084	[145]
UAS-Bil082	*prickle*	CG11084	[145]
UAS-Bil083	*prickle*	CG11084	[145]
UAS-Broad	*broad*	CG11491	[146]
UAS-Caup	*caupolican*	CG10605	[147]

UAS-Dlg	*discs large 1*	CG1725	[148]
UAS-Dlg-GFP	*discs large 1*	CG1725	[149]
UAS-Grain 1	*grain*	CG9656	[150]
UAS-Grain 2	*grain*	CG9656	[150]
UAS-Head	*headcase*	CG15532	[151]
UAS-I4	*CG10631*	CG10631	Gift of K Venkatesha
UAS-Lamin	*Lamin*	CG6944	[152]
UAS-Mirror	*mirror*	CG10601	[153]
UAS-SP0	*BM-40-SPARC*	CG6378	Gift of F Prada
UAS-SP6	*BM-40-SPARC*	CG6378	Gift of F Prada
UAS-Spn43	*Serpin 43Aa*	CG12172	[154]
UAS-Sptr	*buttonhead*	CG12653	[155]
UAS-Sptr 2	*buttonhead*	CG12653	[155]

Overexpression System: Gal4/UAS

The Gal4/UAS system is an inducible expression system developed by Andrea Brand [156]. This system permits to express ectopically any gene in any part of the fly using specific drivers. The yeast Gal4 transcription factor is able to recognize UAS regulatory sequences. We used this property to specifically express in different tissues or patterns different target genes.

The Gal4 system relies on two components: (1) Gal4, a transcriptional activator from yeast, which is expressed in a tissue-specific manner and (2) a transgene under the control of the upstream activation sequence that is bound by Gal4 (UAS). The two components are brought together in a simple genetic cross. In the progeny of the cross, the transgene is only transcribed in those cells or tissues expressing the Gal4 protein (Figure 25).

All UAS-RNAi lines used for the screenings were expressed with Pnr-Gal4 for the thorax fusion screen and Pnr-Gal4 or En-Gal4 for wound healing assays.

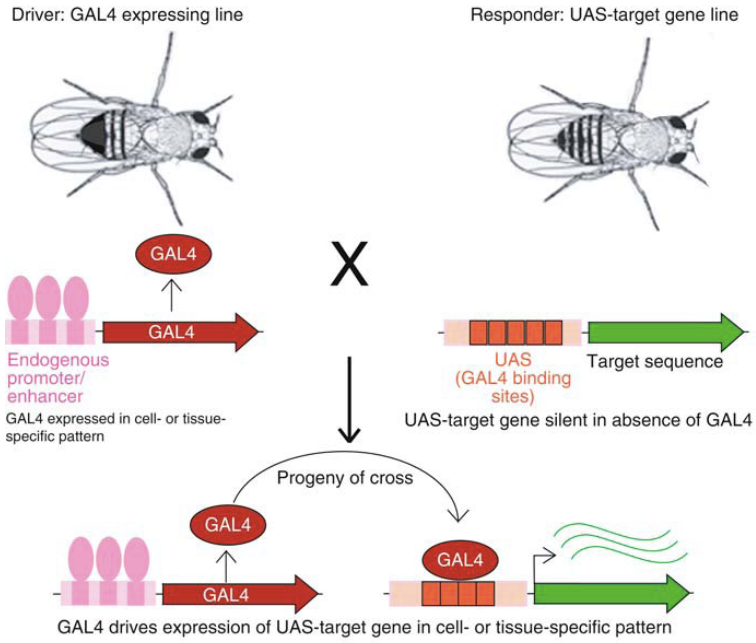


Figure 25. The Gal4/UAS System. The Gal4/UAS system is a bipartite system in which one transgenic line carries the Gal4 transcriptional activator under the control of a tissue-specific enhancer while the second transgenic line, the responder, carries the coding sequence for a gene of interest under the control of UAS sites. The UAS-transgene remains inactive in the absence of Gal4. When both lines are brought together in a cross, in the resulting progeny, Gal4 binds to the UAS activating the expression of the transgene [157].

Thorax Fusion Screen

The thorax fusion screen was carried out using a collection of transgenic fly lines carrying UAS-RNAi constructs. To drive the expression of the RNAis for each gene on the notum, males from each line were crossed with Pnr-Gal4 virgin females and tested at 3 different temperatures 18°C, 25°C and 29°C. Each phenotype was scored in a semi-quantitative manner based on the affected area within the Pnr-Gal4 expression domain. These phenotypes have been further classified based on the severity of phenotypes as described in the text.

Wound Healing Screen

Genes with strong fusion phenotypes were selected for a wound healing screening and their phenotypes further classified with regard to the cellular event affected. The

UAS-RNAi flies were crossed with En-Gal4 and maintained at 25°C. Third instar larval imaginal discs were wounded and cultured at 25°C as described in the *in vitro* culture system protocol and evaluated for healing defects.

In vitro Culture of imaginal discs

Third instar larvae wing imaginal discs were dissected and incubated in a MM3 medium (Shields and Sang´s) supplied with fly extract. The fly extract was prepared following the protocol suggested by the DGRC, Indiana University:

- Collect 300 flies/100 ml of extract
- Put 6.8 ml of medium/gr of flies and homogenize at 4ºC
- Centrifuge at 1500 g during 15 minutes at 4ºC.
- Discard the precipitate and collect the supernatant in a new tube.
- Incubate the supernatant during 5 minutes at 60ºC in a water bath.
- Centrifuge at 1500 g during 90 minutes at 4ºC. Discard the precipitate. Filtrate the supernatant with a 0.22 μm filter.

This modified media allows the culture of the wing imaginal discs for more than 24 hours

Live imaging of the healing process

Third instar larvae imaginal disc dissections were performed in cultured medium and incisions of precise sizes were carefully executed with a pair of tungsten needles. This was followed by culture in supplemented MM3 media for 16-18 hours. We found that imaginal discs in culture lose their morphology after 5 to 6 hours, becoming spherical in shape. To flatten the samples we mounted them within a silicon square in a "sandwich" enabling to perform time-lapse confocal imaging. At least 10 imaginal discs were placed into the silicone square area. When all discs were cut the slide corresponding field was refilled with media mixed with 1 ml of FM4-64 (9 μM) to label the imaginal disc membranes. The imaginal discs were covered with a coverslip and a bit of pressure was exerted over to minimize the free space between the slide and the coverslip avoiding imaginal discs spherical deformation (eversion). The ready-made chamber also prevents desiccation. Time-lapse recording was initiated

after 5 hours of incubation using inverted Leica SP5 and SP2 or Zeiss LSM700 confocal microscopes and 63X objectives. Images (Z-stacks of 1μm thickness) were captured each 10 minutes and in diferent positions to film at least 3 imaginal discs per slide. Laser intensity was kept at a minimum to avoid photo bleaching and to minimize photo toxicity. Image analysis was performed with Leica and Zeiss Confocal Software and ImageJ (NIH Image) was used for mounting time-lapse movies in AVI format.

Immunostaining of cultured imaginal discs

After 16-18 hours the imaginal discs were recovered using a 200 μl pipette humidified in MM3 media to avoid the imaginal discs to stack in the walls of the tips, followed by a fast wash in PBS 1X to eliminated the remnants of the MM3 media. The wash was followed by fixation in 4% paraformaldehyde for 20 minutes.

Antibody incubations were performed following standard procedures. Primary antibodies are detailed in Table 2 and secondary antibodies in Table 3. All steps were carried out at room temperature on a shaker.

- Fixation with 4 % Paraformaldehyde in PBS for 20 minutes at room temperature.
- Rinse once with PBS 1X.
- Wash thrice with PBT (PBS + 0.3 % Triton) (15 minutes each wash) at room temperature.
- Blocking is done for 1 hour with PBT-BSA (PBT + 1 % BSA) at room temperature.
- Incubate with primary antibody in specified dilution at 4°C overnight.
- Wash thrice with PBT 15 minutes each wash at room temperature.
- Incubate with secondary antibody at room temperature for 2 hrs.
- Wash thrice with PBT 15 minutes each wash at room temperature.
- Mount the sample in vectashield and store at 4°C until imaging

For nuclei staining, DAPI (Sigma) was used at a dilution of 1:1000 from a stock solution at 1 mg/ml. The actin cytoskeleton was labelled with phalloidin coupled with Rhodamine (Molecular Probes) 1:1000 (1 mg/ml). After several washes, imaginal

discs were mounted in Vectashield (Vector). Images were captured with Leica SP2 or SP5 or Zeiss LSM700 confocal microscopes.

Table 2

	PRIMARY ANTIBODIES		
ANTIBODY	**SOURCE**	**REFERENCE**	**WORKING DILUTION**
anti-Talin	Nicholas Brown	[158]	1:50
anti-Integrin (bPS)	Hybridoma Bank	CF6G11	1:50
anti-Pvr	Benny Shilo	[159]	1:500
anti-Sqh	Robert Ward	[160]	1:50
anti-Mmp1	Hybridoma Bank	5H7B11	1:20
anti-Beta-Gal	Cappel	8559761	1:200
anti-tubulin-FITC	Invitrogen tech	F2168.2ML	1:500
anti Tubulin (beta)	Hybridoma Bank	E7	1:500

Table 3

SECONDARY ANTIBODIES		
ANTIBODY	**REFERENCE**	**WORKING DILUTIONS**
Mouse Alexa-488	Invitrogen-A11029	1:500
Mouse Alexa-555	Invitrogen-A21424	1:500
Rabbit Alexa-488	Invitrogen-A11034	1:500
Rabbit-Alexa-555	Invitrogen-A21429	1:500
Mouse-Cy5	Jackson ImmunoResearch 115-175-205	1:500

Cell culture

Drosophila S2R+cells were cultured in Schneider's medium (GIBCO, Invitrogen) supplemented with 10% foetal bovine serum that is heat inactivated (GIBCO, Invitrogen), penicillin and streptomycin (100 units/ml and 100 μg/ml) (GIBCO, Invitrogen) and were grown in 25 ml cell culture flasks at 25°C. Cells were maintained in cultures by splitting (1:5) every 4 days (making sure that cells were at least 80 % confluent).

SiRNA treatment of S2R+ cells

Ds-RNAs were used to interfere with gene function in Schneider S2R+ cells. The DsRNAs were obtained from the DRSC stock centre and are listed in Table 4.

Approximately 200.000 cells/well were plated in 24 well plates a day prior to transfection in a final volume of 500 μl. They were treated with Effectene (Qiagen) following standard procedures. 2 μg/μl of control DNA (pMT-Act-GFP or pMT-Tub-GFP) to label the actin and tubulin cytoskeleton and 5μg/μl of siRNAs were transfected simultaneously. The next day, the transfection medium was replaced by fresh medium and the cells were incubated for additional 4 days at 25ºC and subjected afterwards to either live imaging or immunostaining.

Table 4

GENE NAME	AMPLICON ID
CG5525	DRSC02865
CG8977	DRSC16602
CG8351	DRSC16410
CG8231	DRSC20095
CG7033	DRSC18415
CG8416	DRSC07530
CG4027	DRSC17723

CG8439	DRSC07357
CG5374	DRSC16877
CG14801	DRSC17958
CG12007	DRSC12189
CG1725	DRSC18895
CG6831	DRSC11300
CG1703	DRSC19764
CG3595	DRSC18837
CG10200	DRSC05977
CG15027	DRSC19608
CG2275	DRSC07447
CG11066	DRSC04849
CG17158	DRSC29870

Live imaging of S2R+ cells

Transfected *Drosophila* S2R+ cells were cultured in 2 well chambered cover glass (2 ml capacity, NUNC 155380). Live imaging was performed with Leica SP2 or Zeiss LSM700 microscopes at 40X magnification, images (Z-stacks of 1µm thickness and number steps depending on the thickness of the sample) were acquired at intervals of 5 minutes.

S2R+ cells immunostaining

Immunostaining of transfected and/or DsRNA-treated cells was performed using a standard protocol modified as follows. All the steps were carried out at room temperature, except when specified, on a shaker.

- Cells were plated on Concanavalin A and allowed to adhere for about 4 hours
- Medium was replaced with PBS.

- The cells were washed with PBS for 5 minutes.
- Cells were fixed with 4% Paraformaldehyde in PBS for 20 minutes at room temperature.
- Wash once with PBS.
- Wash thrice with PBT (15 minutes each wash) at room temperature.
- Blocking is done for 1 hr. with PBT-BSA at room temperature.
- Incubate with primary antibody in specified dilution at 4°C overnight.
- Wash thrice with PBT 15 minutes each wash at room temperature.
- Incubate with secondary antibody at room temperature for 2 hrs.
- Wash thrice with PBT 15 minutes each wash at room temperature.
- Mount the sample in vectashield and store at 4°C until imaging.

Inhibitors treatment

The actin polymerization inhibitor Latrunculin A (LatA) was used to depolymerise F-actin in S2R+ cells.

Drosophila S2R+ cells were split at 200.000 cells/per well in 24 well plate a day prior to transfection in a final volume of 500 µl. The next day the cells were transfected with control DNA and RNAi and incubated for 4 days at 25°C. After 4 days the cells were treated with LatA (0.5 mg/ml) for 1 hour and let to recover for 48 hours. Last, they were immunostained with Phalloidin (to label F-actin) to check if actin polymerisation was affected in TCP1 knockdown conditions.

Reverse transcription PCR and cDNA synthesis

Reverse transcription PCR was performed to check the efficiency of RNAi knockdown. RNA was isolated from control and RNAi treated cells using Qiagen RNA extraction kit to prepare cDNA. cDNA synthesis and RT (reverse transcription) PCR was done according to the standard protocols.

Cellular Reporters

The cellular reporters employed are described in Table 5.

Table 5

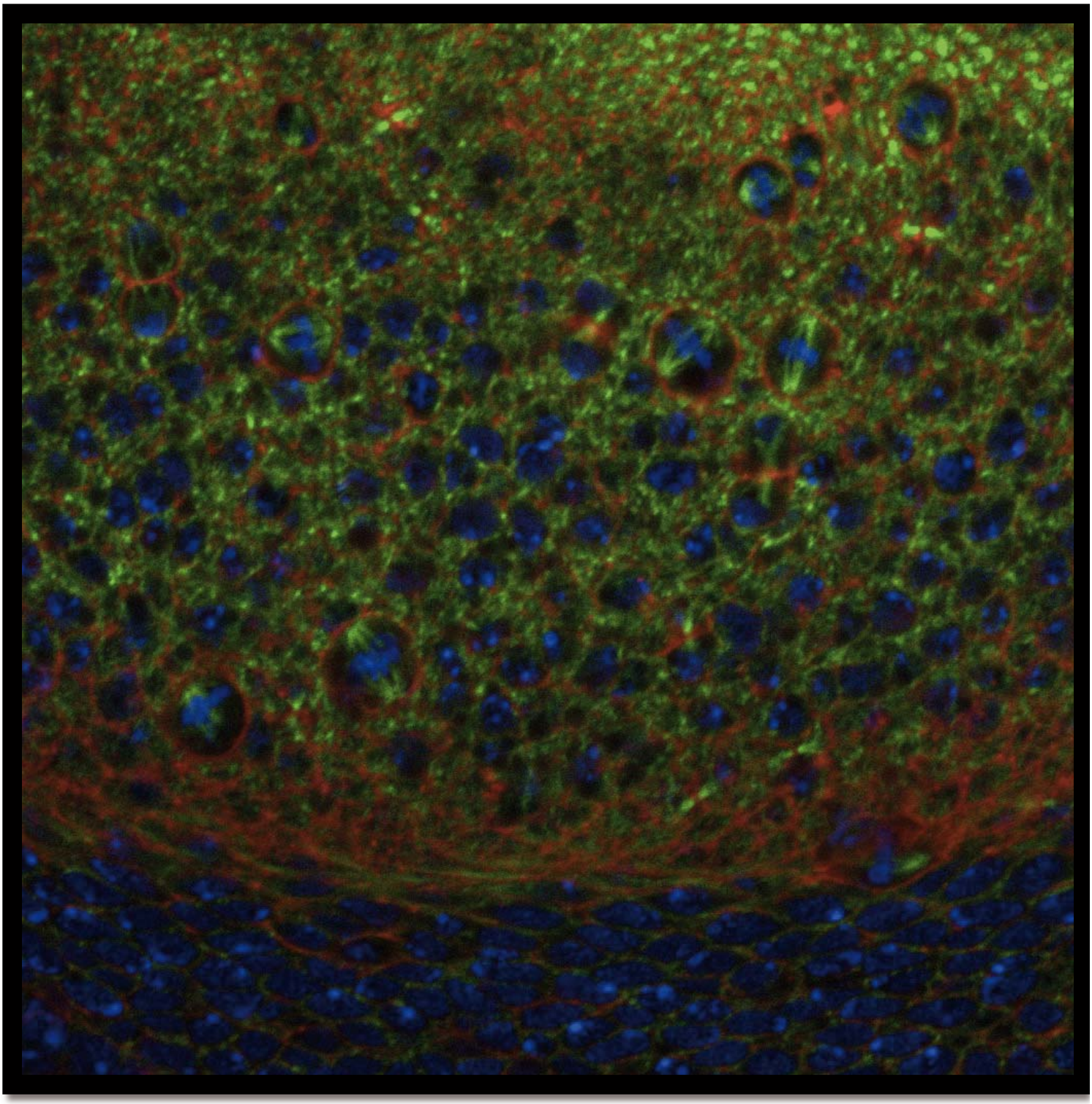
REPORTER	REFERENCE	SOURCE
pMT-act-GFP	[161]	Ron Vale
pMT-Tub84B-GFP	[162]	Ron Vale
pMT-H2B-GFP	[163]	Ody Sibon

We also generated a GFP-photoactivable cherry ($G^{PA}C$-actin) to monitor the levels of newly synthesized actin in TCP1 subunit knockdown experiments. The pUAST-$G^{PA}C$ plasmid was generated by recloning the sequences carrying $G^{PA}C$-actin from pArek1-$G^{PA}C$ [164] into p(UAST) in between the Bgl II and Xho1 restriction sites.

Photoactivation experiments were carried out by transfecting S2R+ cells with $G^{PA}C$ (2 μg/μl) and dsRNAs (5 μg/μl) (transfections were done according to the standard protocol described in detail in previous sections). Live imaging experiments (after 4 days of transfection) were done in an Andor confocal spinning disk using a 40X objective and following a series of sequential steps. Photoactivation was done by using a 405 nm laser at 14% power with no zoom and with pixel dwell of 60 μs. After photoactivation images were acquired in Z-stacks (1 μm thickness with 4 minutes interval in between stacks) with a 488 nm laser at 20 % power and a 561 nm laser at 20 % power with pixel resolution of 512 x 512.

G-actin determination

DNAse 1 Alexa 488 was used to detect monomeric G-actin in control and RNAi treated S2R+ cells. Four days after transfection, the cells were washed with PBS for 5 mins and fixed in 4 % paraformaldehyde for 20mins at room temperature. Cells were blocked with PBT-BSA for 30 mins and incubated with DNAse 1 conjugated to Alexa 488 (in PBS-BSA) for 1 hour at room temperature. Cells were washed with PBT 3 X 15 mins (each wash), mounted in Vectashield and store at 4°C until imaging.



RESULTS

1. Gene expression in *Drosophila* imaginal disc wound healing

Epithelia act as physical barriers protecting living organisms and their organs from the surrounding environment. They have evolved robust healing mechanisms to ensure their integrity upon injury [165]. Healing is essential to all organisms throughout the animal kingdom and occurs both in developing stages and in adults. Healing and regeneration recapitulate many morphogenetic processes including gastrulation, neural tube closure, palate formation, and eyelid fusion in vertebrates, and dorsal closure (DC) in *Drosophila* and ventral enclosure in *Caenorhabditis elegans* [88, 166, 167]. Although multiple studies have been carried out to understand the process of healing, it is still puzzling why some wounds fail to heal.

Among insects, the fruit fly *Drosophila melanogaster* has become the most commonly used model organism for genetic, cellular and molecular studies providing important contributions applicable to other models. Pioneer healing studies in *Drosophila* showed that injured larval imaginal discs transplanted into female abdomens were able to heal, regenerate and differentiate [3, 4] demonstrating the suitability of flies for healing analysis.

Although several genome wide expression analyses have been performed in genetically amenable organism aiming to identify genes involved in the control of healing processes, no thorough functional screenings have been employed before. *Drosophila* imaginal discs represents a good *in vivo* model to develop such functional approach. While imaginal discs healing differs from that of vertebrates as it invovles cells of a single type (epithelia) and does not retrieve an angiogenic response, the analysis of their healing and repair response can provide many cues like which are the signals promoting cell migration during healing, how actin cytoskeletal remodelling at the leading edge initiates and how the final sealing of the gap is established. To progress in answering these questions we have carried out a genome wide transcriptome analysis and develop functional assays for selected genes after setting up an *in vitro* model recapitulating healing amenable to molecular studies.

- **Culture of wing imaginal discs *in vitro***

To facilitate the transcriptomic analysis of wounded imaginal discs, we have developed a procedure to culture imaginal discs *in vitro* [168]. Employing a modified

healing-permissive medium, and this whole process of tissue repair could be monitored live. Discs healing *in vitro* fully resemble to the wounded discs that heal and regenerate *in vivo* in fly abdomens [92]. *puc* expression activation, actin accumulation, cell shape changes and final tissue sealing (see Introduction) proceeds as *in vivo* albeit at a slower kinetics.

- **Microarray analysis of wounded imaginal wing discs**

To define the transcriptome program and identify the pathways and genes critical for healing in imaginal discs, we carried out a genome-wide expression profiling of imaginal cells actively involved in healing of wounded discs (JNK-activity positive cells expressing a *puc*-GFP reporter combination) versus their non healing-engaged siblings [168].

The data obtained from the microarrays were mined in two different ways.

1) A direct comparison of the levels of expression of the genes expressed in JNK-activity positive cells and their siblings (JNK negative) from wounded discs. The expression data were represented by the log-expression values for each probe. The coefficients of the fitted models describe the differences between the RNA sources hybridized to the arrays. After fitting a model, the standard errors were moderated using a simple empirical Bayes model. A moderated t-statistic and log-odds of differential expression were computed to compare each gene. This analysis helped us to identify 302 genes whose expression increases more than 2 folds ($p<0.05$) in healing engaged cells. Correspondingly, 209 genes decrease their expression in the same condition (see Supplementary Table 2).

2) A dual comparison of JNK-activity positive cells and their negative siblings from wounded and non-wounded discs. This led to remove any bias into the analysis from cells endogenously expressing the *puc*-GFP reporter in wing disc (as those present in the disc stalk and the peripodial layer not involved in healing). The statistical significance of differential gene expression signals was assessed using a moderated t-statistic with the LIMMA Bioconductor package. The corresponding p-values were corrected for multiple hypotheses testing using a False Discovery Rate criterion. A relatively mild fold change cut-off (1,3) was applied and individual analysis and computation of the interaction terms amongst datasets was performed. Successive

intersectional computation allowed to identify two relevant gene subsets (Figure 26); 430 genes (293 upregulated and 137 downregulated) significant and differentially expressed between GFP+ and GFP- cells in wounded discs only (dw1_dnw0_dd1) and 634 genes differentially expressed between GFP+ and GFP- cells both in wounded (399 upregulated and 235 downregulated) and unwounded discs (431 upregulated and 203 downregulated) but significantly different in the two conditions (dw1_dnw1_dd1) (Supplementary Tables 3 and 4).

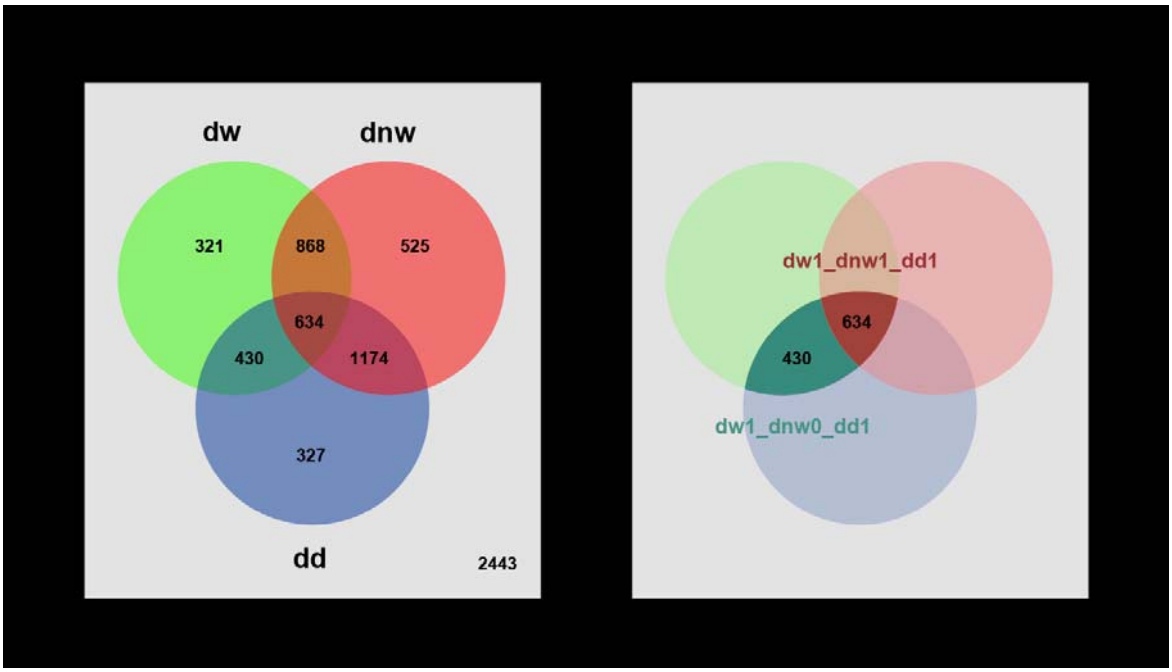


Figure 26. Venn diagrams

These diagrams display the dw, dnw and dd populations and their intersections. The dw1_dnw1_dd1 and dw1_dnw0_dd1 subpopulations are highlighted.

- **Ontology analysis of Healing genes**

The differential expression of a gene in healing-engaged cells does not ensure their significance as a regulator or structural element participating in healing. They will only show to be relevant after characterizing their activity in a healing model. To prioritize "healing" genes for functional assays we first performed a GO term enrichment analysis [169]. For the *dw1_dnw0_dd1* subpopulation, we identified 26 Biological Process (BPs), 22 Molecular Function (MFs) and 17 Cellular Component

(CCs) enriched terms with a p-value < 0,05; while 78 BPs, 34 MFs and 10 CCs were detected for *dw1_dnw1_dd1*. These figures decreases after multiple testing correction (FDR adjusted p-value < 0,2), 0 BPs, 0 MFs and 9 CCs for *dw1_dnw0_dd1* and 7 BPs, 13 MFs and 1 CCs for *dw1_dnw1_dd1*. Overall, these analyses gave us an outline of the potentially relevant gene categories differentially expressed during healing. The ontology analysis of the genes detected in the transcriptomic analysis has been previously described [168] and is presented as Annex 1.

- **Validation of Transcriptomic analyses**

The datasets obtained from the microarray analyses were validated by analysing the level of expression of selected genes by *in situ* hybridization and antibody stainings of wounded discs in the process of healing [168]. *paxillin* (+2.3X), *cheerio* (+3.12X) and *windbeutel* (+2.98X) were tested by antibody staining and showed specific upregulation in healing-engaged areas of wounded discs [168]. I have further extended this analysis and study the expression of three more genes potentially involved in the healing response, MMP1 (+7.43X), *spaghetti squash* (+1.73X) and *rhea*/Talin (+1.65X) in the same experimental conditions.

Matrix metalloproteinases (MMPs) are extracellular proteases normally involved in matrix removal and highly expressed at wound sites in vertebrates [170]. Knockout mice for each of four secreted MMPs (*MMP-3, MMP-8, MMP-9*, and *MMP-13*) display a mild delay in closing excisional wounds [171]. The *Drosophila* secreted Mmp, Mmp1, has been shown to be required in larval epidermis repair, remodelling the basement membrane, promoting cell elongation and actin cytoskeletal reorganization and eventually leading to re-epithelialization [90]. Interestingly, the jun N-terminal kinase (JNK) pathway upregulates *mmp1* expression after wounding, although in unwounded epidermis is expressed independently of the JNK pathway activity [90].

spaghetti squash (sqh) encodes the *Drosophila* homologue of the regulatory light chain of non-muscle myosin II. It is required in multiple morphogenetic process, including leg elongation, head involution or dorsal closure [172] and its participation in wound repair has been previously documented [119].

Talin is a FERM domain protein that links the cytoplasmic tail of integrins, which are

the main adhesion receptors of the extracellular matrix, to the actin cytoskeleton [173]. Similar to other FERM proteins, Talin undergoes intramolecular conformational changes that inhibit its own activity. When this is genetically disrupted in *Drosophila*, mutant embryos display morphogenetic defects during dorsal closure (DC), a process that resembles wound healing [174]

We found that wing discs cultured for 8hrs after wounding showed enhanced immunostaining for MMP1, Sqh and Talin in healing-engaged cells (Figure 27). These data further validated the transcriptomic analysis and gave a preliminary indication of the potential role in wound repair of those genes singularized in the study.

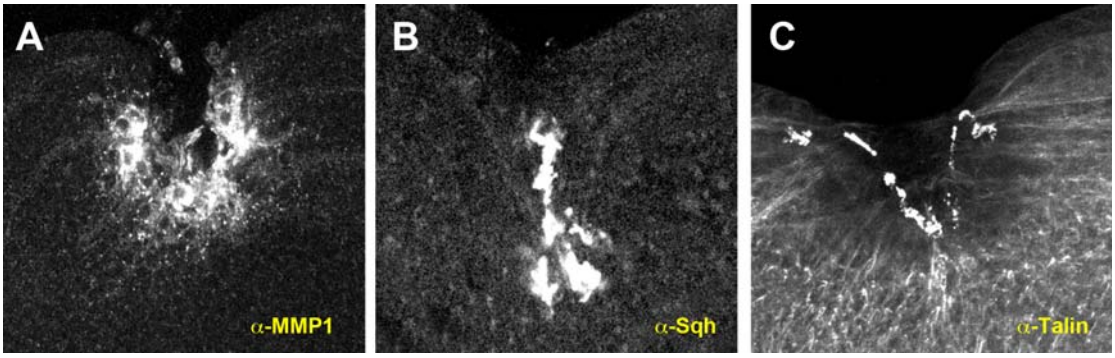


Figure 27 Expression of upregulated genes during healing.

A-C) *mmp1, sqh and talin* show an enhanced expression near the wound after 8 hours of *in vitro* culture.

2. Functional studies from wound healing transcriptomic analysis

To investigate the potential role of selected genes identified in transcriptomic analysis during wound healing, we employed the Gal4/UAS system. Different Gal4 drivers were used to overexpress two types of UAS-constructs: 1) UAS-RNAi lines (to interfere with the expression of those genes upregulated in "healing" cells), and 2) UAS lines to reload back those genes downregulated by overexpression. A first analysis studied the fusion of imaginal discs during metamorphosis and a secondary screen was performed for wound healing in cultured discs.

These tasks, initiated by a former graduate student in the lab, Carmen Álvarez-Fernández [168], are presented in the current document in full detail and totally

consolidated. Any previous data will be referenced.

We used three criteria to pick and choose particular genes for functional analysis during healing; (1) Robust changes of gene expression between different populations, (2) availability of functional tools, and (3) relevance of gene annotation (when known) in relation to JNK signalling and healing in *Drosophila* or other model systems. We gathered 2 non-saturating collections of 363 UAS-RNAi lines, designed to specifically interfere with the expression of 249 upregulated genes and of 12 UAS overexpressing lines designed to direct the expression of 18 downregulated genes (see Supplementary Table 1 and Table 1) (see also [168]).

- **Primary screening for thorax fusion**

Firstly we analysed the functionality of RNAi and overexpression lines during the process of fusion of imaginal discs during metamorphosis. As described in the Introduction, about 6 hours after pupariation, the proximal regions of wing discs detach from the larval epidermis and expand upon it, replacing it gradually. The stretching of the imaginal epithelia is driven by filopodia and actin bridges that protrude from their leading edge. It requires the activation of the JNK signalling cascade, which leads to the expression of *puc* at the front edge cells (Figure 28). Wing imaginal discs mutant for the JNK pathway or downstream genes do not spread, or fail to reach the midline and to form a continuous epithelium. Similar abnormalities are observed after overexpressing Puc or dominant-negative JNK transgenes with Pnr-Gal4.

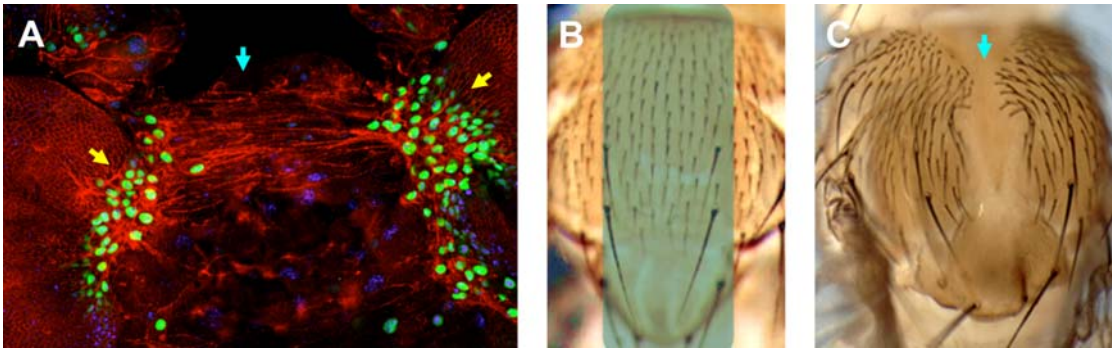


Figure 28. Puc expression and JNK mutant phenotypes during imaginal discs fusion

A) Puc expression in green and actin (phalloidin) in red are highlighted in a dissected thorax. B) wild type thorax of an adult fly. Green shadow depicts the pannier expressing area. C) Thoracic defects of and adult fly expressing a dominant negative form of basket (JNK) showing a thorax cleft.

Transgenic UAS-RNAi (363) and UAS-overexpressing (21) lines corresponding to 249 genes and 15 genes respectively were crossed with Pnr-Gal4 flies and their progeny scored for imaginal disc fusion defects. The driver Pnr-Gal4 is expressed in a broad domain in the presumptive notum region of the wing disc reflecting the expression pattern of the *pannier* (*pnr*) gene [138] (Figure 29 A).

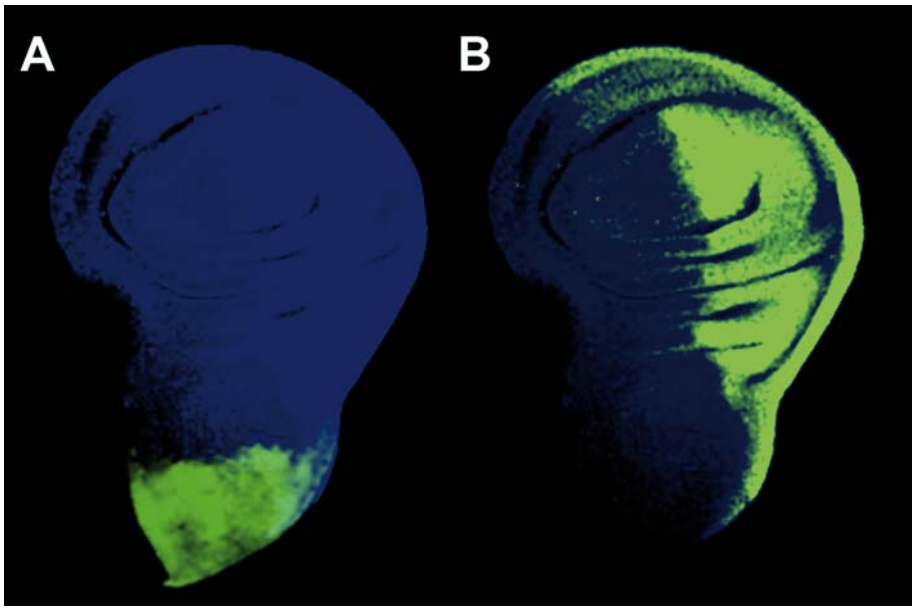


Figure 29 Expression domains of pnr-GAL4 and en-GAL4 in third instar larval imaginal disc

A) Wildtype imaginal disc expressing GFP (UAS) in the notum (pannier domain) driven by Pnr-Gal4. B) Wildtype imaginal disc expressing GFP (UAS) in the posterior compartment (engrailed domain) driven by En-Gal4. (GFP in green; DAPI in blue).

The phenotypes obtained from this screen were grouped according to their defects (embryonic lethal – Class 1TC, pupal lethal – Class 2TC, strong notum clefts – Class 3TC, medium notum cleft – Class 4TC, weak notum clefts – Class 5TC, excess of fusion – Class 6TC and bristle polarity defects – Class 7TC). Resulting phenotypes are displayed in Supplementary Table 5. A total of 159 RNAi lines (43.8% of those tested) corresponding to 134 genes (53.8%) of those tested) displayed thorax fusion phenotypes ranging from mild fusion problems to early lethality (Figure 30). It is important to note that these figures might not correspond to the actual number of

positive regulators since many of these lines might not efficiently knockdown their targets. Further, 16 overexpression constructs (76 % of those tested) corresponding to 11 genes (73 % of those tested) also lead to thorax fusion defects [168].

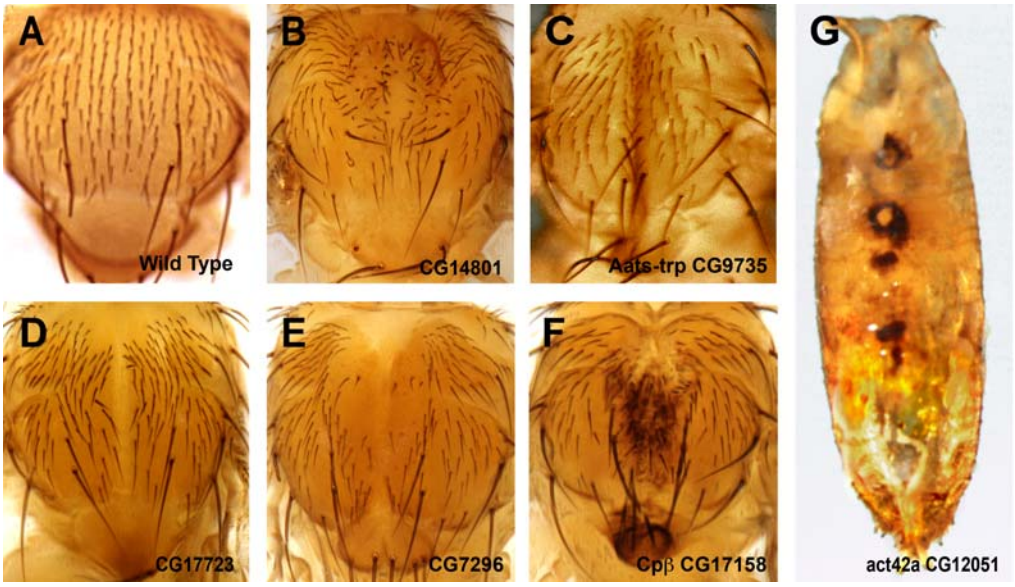


Figure 30. Thorax fusion phenotypes of different upregulated genes. (RNAi's tested with pnr-GAL4)

A) Wild Type. B) class 7TC. C) class 6TC. D) class 4TC. E) class 3TC. F) class 2TC. G) class 2TC.

A subset of genes with strong fusion defects was selected to be tested in a wound healing screen.

- **Cellular analysis of imaginal discs wound healing**

A secondary screen targeting imaginal discs healing was performed for those lines that showed significant imaginal disc fusion defects. To interpret mutant (defective healing) phenotypes from the screening, we first performed a thorough analysis of normal healing in wild type discs after incisive wounding. We focused at three different times of the healing process/stages (6 hours, 12 hours and 18 hours – when wound closure is completed under these *in vitro* conditions).

At 6 hours of *in vitro* culture the wounded imaginal discs have already initiated the healing process. Both epithelia (the CE and PE) curl towards each other reducing the wound surface and establish heterotypic contacts. This contractile property, as

determined in *in vivo* cultures, seems to be carried out by the action of microfilaments underlying the CE epithelia [175]. The CE epithelia initiates the zipping of the wound from basal to apical by filopodial extensions, while actin accumulation is observed at the wounded edges of the PE. This accumulation of F-actin has been earlier reported during healing of imaginal discs *in vivo* [92, 97]. The cells at the wound vertex in CE change their shape (Figure 31). This has also been observed at the leading edge during embryonic wound closure [85]. JNK signalling activity, monitored through the expression of its downstream target *puc* is also detected at this time [168]. Requirement of JNK for wound healing as been earlier reported in wing imaginal discs, larval and adult cuticle wound healing [81, 92, 98]. Absence of JNK activity brought by mutants of *hep* or *bsk* results in impaired healing [74, 92, 97].

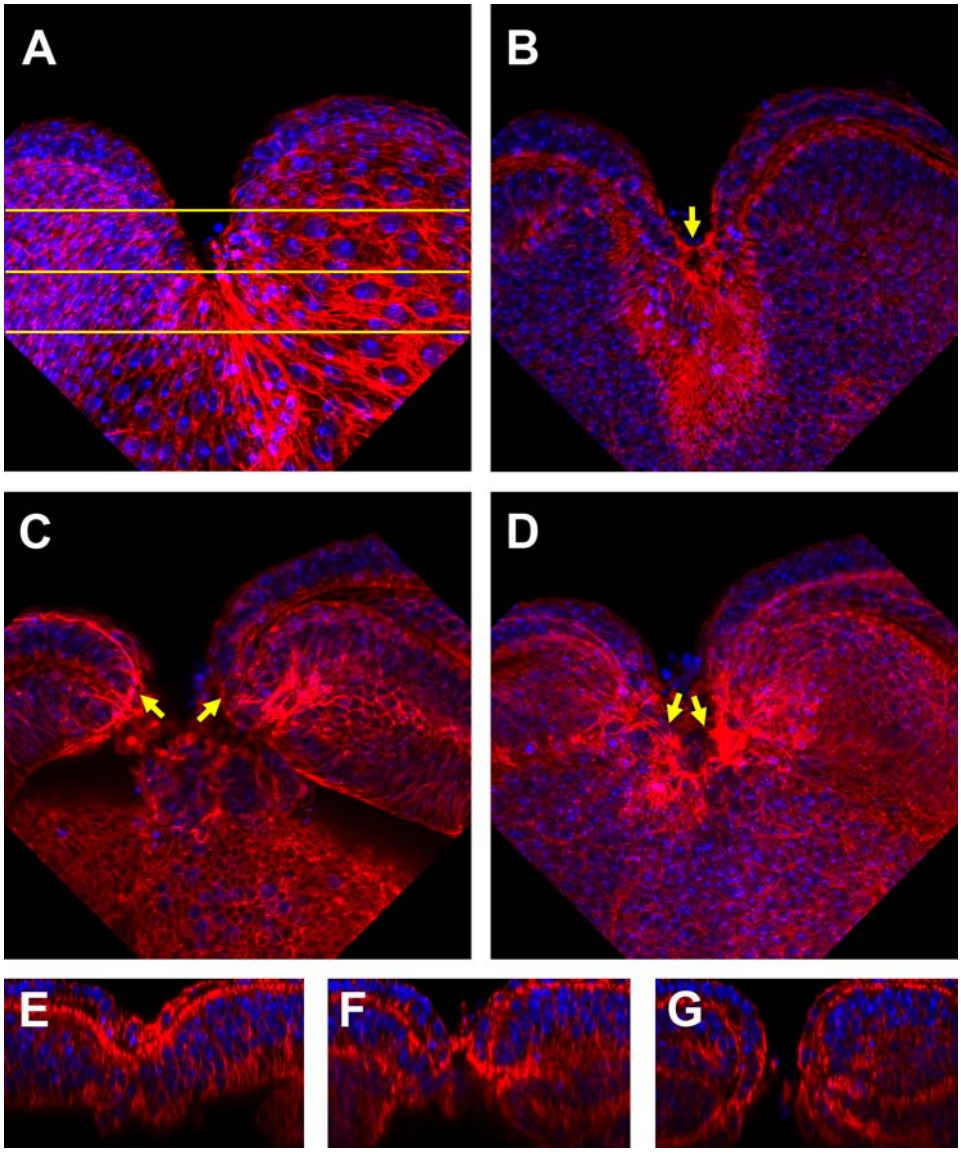


Figure 31. Cellular events occurring during 6 hours of *in vitro* culture.

A) Peripodial view displaying a wide wound gap. B) Columnar view, showing zippering of the epithelia and elongated cells at the leading. C) Heterotypic contacts (arrows)Wound tips (columnar) show contraction towards the wound vertex D) Filopodia at the zipping columnar epithelia. E to G) Orthogonal sections (yellow lines in A). Phalloidin (actin) is shown in red; DAPI (nuclei) in blue.

At 12 hours of *in vitro* culture, the elongated peripodial epithelial cells starts closing the wound leading to a remarkable reduction in the wound surface (Figure 32). By this time, formation of homotypic contacts within the CE and PE epithelia are observed and the CE epithelium completes baso-lateral zippering. This has also been earlier reported in healing of imaginal disc *in vivo* at 12 hours [92, 175].

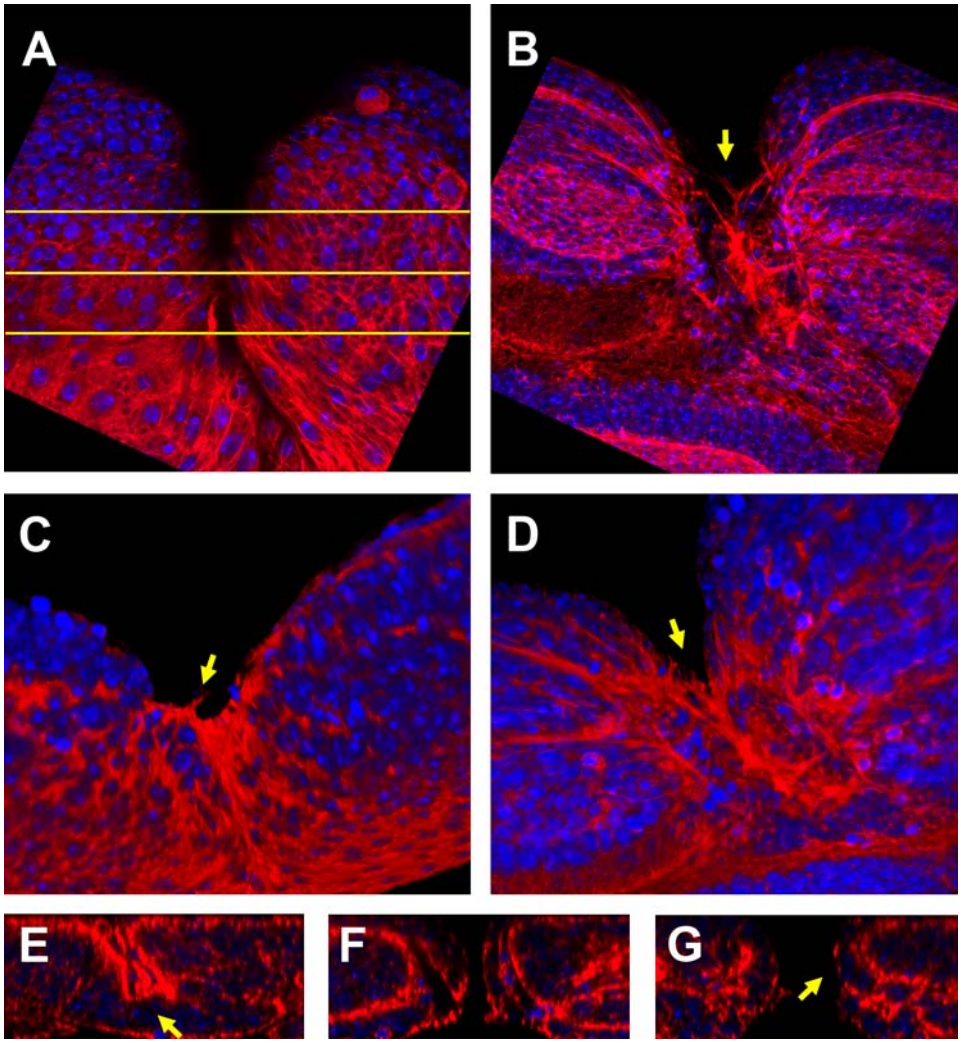


Figure 32 Wounded imaginal discs after 12 hours of *in vitro* culture

A) Peripodial view (PE). C) Columnar view (CE). There is a remarkable reduction in wound size (arrows). C and D) 3D High magnification images of A and B. E to F) Orthogonal sections showing the basolateral zippering of the columnar epithelia (arrows). Phalloidin (actin) is shown in red; DAPI (nuclei) in blue.

Filopodia and zipping progression have also been observed during embryonic dorsal closure and embryonic healing [85, 176]. In the embryo, the formation of these filopodia depends on the activity of Cdc42, which is required for wound suturing [85, 119].

The wound closure is completed after 18 hours of *in vitro* culture. The PE and CE epithelia meet and fuse respectively to completely close the wound. Both the columnar and peripodial epithelia initiate tissue relaxation leaving no scar behind (Figure 33).

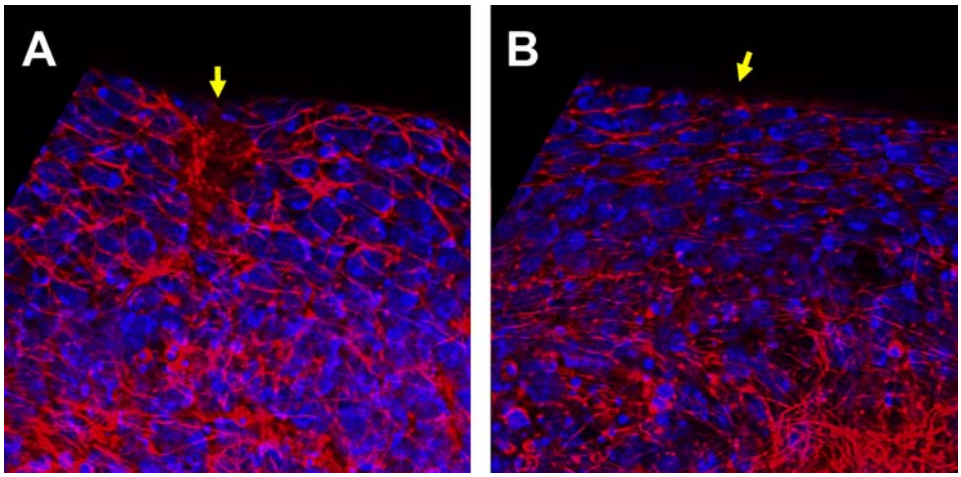


Figure 33. Wounded imaginal discs after 18 hours of *in vitro* culture

Complete wound closure is observed in both the peripodial (A) and columnar (B) epithelia (arrows). Phalloidin (actin) is shown in red; DAPI (nuclei) in blue.

In Table 6 is shown a temporal description of the main cellular events in place during the healing of incisional wounds in the imaginal discs *in vitro*.

Table 6

6 hours of culture
1. Accumulation of actin in the apical side of both epithelia
2. Cell shape changes at the wound vertex in columnar epithelia.
3. Formation of heterotypic contacts between the CE and the PE epithelia
4. Initiation of sealing by zippering in columnar epithelia.
12 hours of culture
1. Homotypic contacts of CE and PE
2. CE basolateral zippering
3. Partial PE sealing
18 hours of culture
1. Complete closure of CE
2. Complete closure of PE
3. Tissue relaxation

- **Wound healing functional analysis**

53 UAS RNAi corresponding to 46 genes and 3 UAS-overexpressing lines for 3 genes were tested in total (42 UAS RNAi lines corresponding to 39 genes and 2 UAS-overexpression lines in the present work) (Supplementary Table 6). The criteria used to select these genes were, their significant upregulation in the microarrays, the magnitude of the thorax fusion defects after RNA interference, and considerations from Gene Ontology about their potential as healing regulators.

UAS constructs were screened using two different Gal4 lines: Pnr-Gal4 or En-Gal4. Pnr-Gal4 has been described above. En-Gal4 is a posterior compartment specific driver reflecting the expression pattern of the *engrailed* (*en*) gene [141]. The phenotypes induced by transgenes expressed with this line are confined to the posterior region of the disc. The anterior compartment of the same discs can be used as internal control (Figure 29B). All crosses were set at 25°C and third instar larval imaginal discs dissected out, wounded in a specific region depending on which Gal4

was used, cultured in modified medium for about 20-24 hours and screened for healing defects.

3. Wound Healing phenotypes

RNA interference for 30 genes (65.2%) showed healing defects, while all the 3 genes tested by overexpression showed a healing phenotype. Their phenotypes (both for interfered and overexpressed genes) were grouped according to the cellular events affected during healing.

Classes 1, 2, 3: Interference with the expression of genes belonging to these groups impaired the early stages of healing (6 hours), indicating a potential role in healing initiation. Interference in Class 1 genes blocked the very first step of the healing process. Interference in Class 2 genes resulted in wounded discs displaying actin accumulation and also cell shape changes at the leading cells in the CE epithelia but not forming heterotypic contacts. Wound discs lacking Class 3 genes function complete all initial steps but cannot undergo the formation of homotypic contacts.

- ***Class 1***: In this class we find: *act42a-CG12051, verprolin-CG13503, CG12007, rhea/*Talin-*CG6831, TCP1 η-CG8351* and *TCP1 ζ-CG8531.*
- ***Class 2:*** In this class we find: *jra-Jun related antigen-CG2275* and *aats-val-CG4062*.
- ***Class 3:*** In this class we find: *rho1-CG8416* and *PDGF- and VEGF-receptor related-CG8222.*

Classes 4, 5: Interference in these groups of genes blocked healing midway (12 hours) and showed incomplete closure. Interference in genes belonging to Class 4 resulted in healing impairment beyond the PE homotypic contacts and partial peripodial sealing. Interference in Class 5 genes blocked both CE and PE closure although both epithelia are able to establish homotypic contacts.

- ***Class 4:*** In this class we find: *Capping protein α-CG10540, Capping protein β-CG17158, CG15027* and *cytC-p-CG17903.*
- **Class 5:** In this class we find: *CG7296, scab-CG8095, scarface-CG11066, CG15611* and *sqh-CG3595* by interference and *mirror-CG1943* and *lamin-CG6944* by overexpression.

Class 6: In this class we find *fimbrin-CG8649* which cannot achieve complete columnar closure.

Class 7: Genes belonging to this group show complete healing but are defective for tissue relaxation. In this class we find: *arc1-CG12505* and *CG10200*.

- ***Class 1***

Act42a. Actin is one of the most abundant and highly conserved proteins in eukaryotes. As a major cytoskeletal component it is essential for a variety of cellular activities including cell shape control, cell motility, cytokinesis, intracellular transport and contractility. Actin proteins differ in only a few amino acids within species even at high evolutionary distances (e.g. fly and mouse) [177].

The *act42a* gene showed an upregulation in direct comparisons in healing-engaged cells in wing imaginal discs of 3.42 fold. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *act42a* by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in pupal lethality. Healing assays with En-Gal4 yielded a fully open wound phenotype. Healing was blocked as early as 6 hours after wounding. Indeed, the wounded tissue showed no signs of actin accumulation or filopodia formation at the leading edge and extensive apoptotic nuclei can be observed (Figure 34 shows in detail the loss of function healing phenotype upon interference on Act42a, as a prototype of the Phenotypic Class 1).

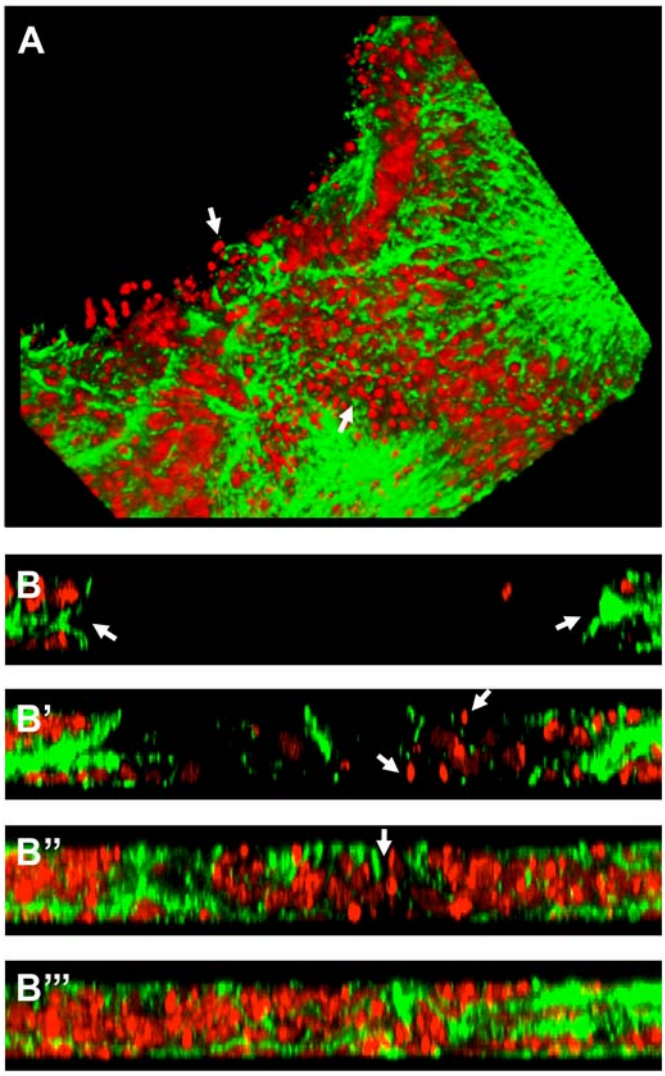


Figure 34. Act 42A healing phenotype (Class 1)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. It shows a large open wound and extensive cell death (arrows). Bs) sequential orthogonal sections. No heterotypic contacts, absence of actin cytoskeleton and occasional picnotic nulei are depicted with arrows). Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

***reptin*.** Reptin is a DNA helicase that is a component of the PRC1 Polycomb complex involved in chromatin silencing. Several studies have reported their overexpression in cancer, including hepatocellular carcinoma and colorectal cancer. It interacts with the oncogenes c-myc and β-catenin, and modulates their transcriptional activities. It is also required for the biogenesis of telomerase [178]. In a developmental context, it functionally interacts with Myc in the control of cell proliferation and growth in Drosophila [179, 180]. Reptin also serves to regulate heart growth during

development, at least in part via the beta-catenin pathway [181] and is essential for cilia motility in zebrafish [182].

reptin expression showed an upregulation of 1.64 fold in direct comparisons in healing-engaged cells in wing imaginal discs. In dual comparisons (dw1_dnw0_ dd1), it shows an upregulation of 1.69 fold in healing-engaged cells in wounded discs, while was increased a negligible 1.07 times in unwounded. The comparative fold difference between the two populations was 1.57.

Downregulation of *reptin* by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in embryonic lethality. Healing assays with En-Gal4 yielded a fully open wound phenotype. Healing was blocked as early as 6 hours after wounding.

verprolin. *vrp*, originally identified in yeast, encodes for a predicted WH2-containing actin binding protein needed for actin polymerisation during polarised growth and during endocytosis [183]. Vertebrate Verprolins regulate actin dynamics either by binding directly to actin, binding to proteins of the WASP family or binding to other regulatory proteins [184]. Interestingly, Verprolins have also been implicated in endocytosis [185]. In *Drosophila*, *vrp*, also known as *verprolin1* (*vrp1*), DWIP, *solitary* and *solas*, appears to be implicated in recruiting the actin-polymerization machinery to sites of myoblast attachment and fusion [186].

verprolin expression showed an upregulation of 2.97 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *verprolin* by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in notum malformations. Healing assays with En-Gal4 yielded a fully open wound phenotype. Healing was blocked as early as 6 hours after wounding.

CG12007. This unknown gene is predicted to act as a Rab geranyl-geranyl-transferase. Certain experimental evidence shows that this gene regulates cell shape changes and cell adhesion [187].

CG12007 expression showed, in dual comparisons (dw1_dnw1_dd1), an upregulation of 2.70 fold in healing-engaged cells in wounded discs, while was increased 1.96 times in unwounded. The comparative fold difference between the two populations

was 1.38. No difference was observed in the global comparison.

Downregulation of CG12007 by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in notum malformations. Healing assays with En-Gal4 yielded a fully open wound phenotype. Healing was blocked as early as 6 hours after wounding.

***rhea*/Talin.** Talin is a cytoskeletal protein which links integrins to cytoskeleton [188]. In *Drosophila*, Talin is encoded by the gene *rhea* [158]. Here, Talin is necessary for the assembly of focal-adhesion like structures in the imaginal disc epithelia and for the formation of junctions between muscle and tendon cells.

rhea expression showed an up-regulation of 1.65 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *rhea* by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in pupal lethality. Healing assays with En-Gal4 yielded a fully open wound phenotype. Healing was blocked as early as 6 hours after wounding.

TCP1 η. *TCP1 η* codes for one subunit of the TCP chaperonin complex, which has a conserved function in all eukaryotes, promoting folding of actin and tubulins [189, 190]. In *Drosophila*, this function is unexplored.

TCP-1eta expression showed an upregulation of 1.64 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *TCP-1 η* by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in pupal lethality. Healing assays with En-Gal4 yielded a fully open wound phenotype. Healing was blocked as early as 6 hours after wounding.

- ***Class 2***

aats-val. This gene encodes for the *Drosophila* Valyl-tRNA synthetase. In mammals it is known to be associated with the elongation factor eEF1 [191, 192]. In yeast, the VAS1 gene was shown to encode both mitochondrial and cytoplasmic ValRSs [193]. In *Drosophila*, the *aats-val* gene has been found upregulated in screenings related to

the age response to oxidative stress [194].

aats-val expression showed an upregulation of 3.26 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *aats-val* by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in pupal lethality. Healing assays with En-Gal4 yielded an open wound phenotype. Healing was blocked as early as 6 hours after wounding. Indeed, the leading edge of the wounded tissue shows unstructured actin accumulation and no filopodia formation. The cells of the CE align along the edge and change shape (Figure 35 shows in detail the loss of function healing phenotype upon interference on *aats-val*, as a prototype of the Phenotypic Class 2).

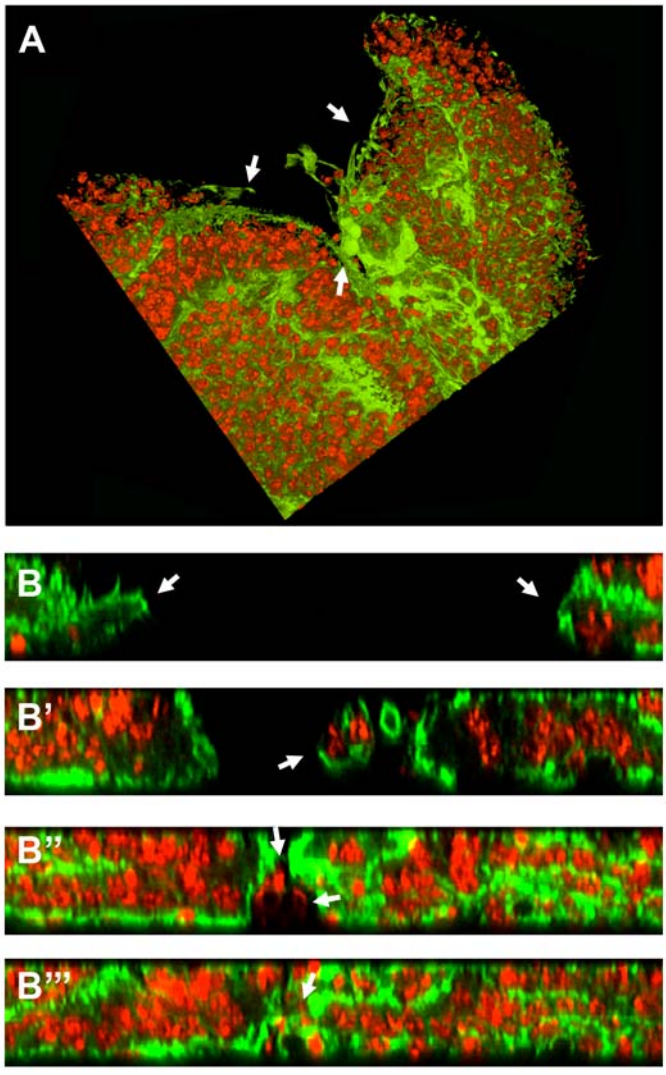


Figure 35. Aats-Val healing phenotype (Class 2)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. It shows an extensive open wound but the presence of actin at the leading edge (arrows). Bs) sequential orthogonal sections. Early approximation of the leading edges but failure on fusion. Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

Jra-Jun Related Antigen. *Djun* is the homolog of the mammalian proto-oncogene C-Jun in *Drosophila*. It encodes a component of the AP-1 transcription factor, a key transcriptional mediator of the JNK signalling cascade participating in the control of multiple cell behaviours conserved in evolution. In *Drosophila*, the JNK signalling has been implicated in developmentally programmed epithelial migratory events, including dorsal closure (DC), thorax closure and border cell migration [89]. Loss of *DJun/Jra* affects the progress of wound closure in the *Drosophila* embryo and larvae [89, 165]

jun expression showed an upregulation of 1.64 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *jra* by UAS-RNAi in the dorsal thorax with Pnr-Gal4 resulted in pupal lethality. Healing assays with En-Gal4 yielded an open wound phenotype. Healing was blocked at 6 hours after wounding. The leading edge shows unstructured actin accumulation and no filopodia formation. At the CE cells align along the edge and change shape.

- ***Class 3***

PDGF- and VEGF-receptor related (pvr). *CG8222* encodes Pvr, a receptor tyrosine kinase. The *Drosophila* receptor tyrosine kinase *Pvr* shows considerable similarity to members of the mammalian PDGF and VEGF receptor families and *Pvr* is considered an evolutionary ancestor of PDGF/VEGF receptors [195]. *pvr* has known functions in the guidance of cell migration [196] and has been implicated in the control of migration and maintenance of macrophage-like hemocytes in *Drosophila* embryos [197]. Pvr is also known to activate the ERK (MAPK) pathway in Schneider cells [195]. This receptor tyrosine kinase has been shown to be essential for wound closure in the larval epidermis where it regulates healing mediating the formation of actin

based protrusions towards the wound [198].

pvr expression showed an upregulation of 2.30 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

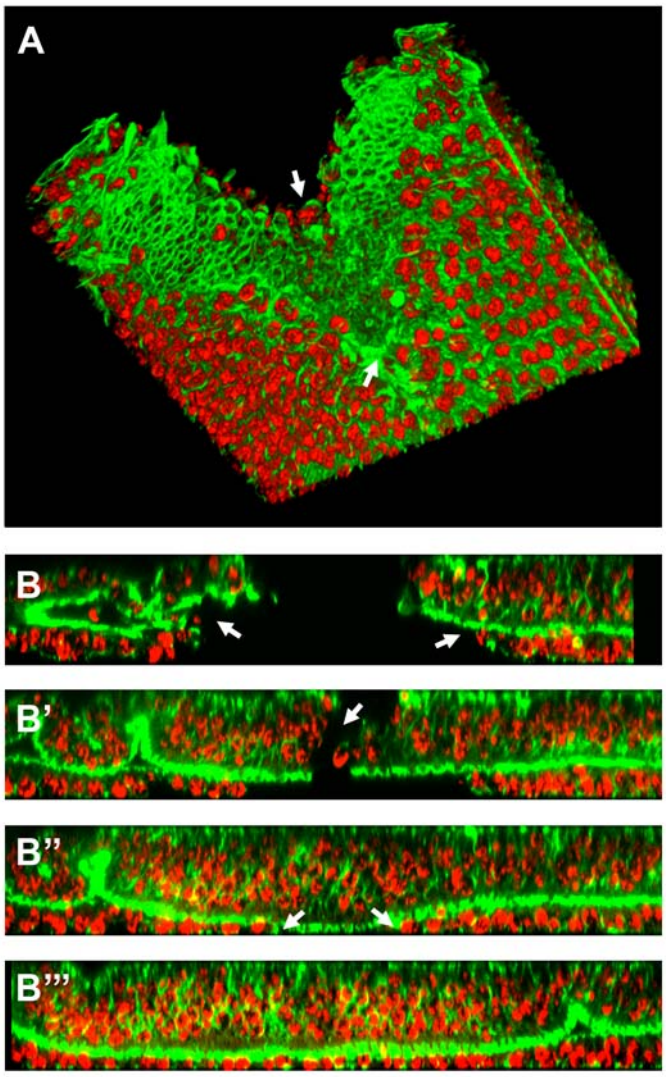


Figure 36. pvr healing phenotype (Class 3)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. It shows a large open wound. The columnar epithelia has initiated healing but the peripodial epithelia fails to heal (arrows). Bs) sequential orthogonal sections. Arrows point to the delay on healing of the peripodial epithelia. Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

Downregulation of *pvr* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing with large wounds. These imaginal discs complete the initial steps of of healing (6 hours)

like actin accumulation, heterotypic contacts between two membranes and initiation of zippering of the CE but showed no peripodial sealing or obvious filopodia at the leading edge (they are essentially defective for formation of homotypic contacts): healing is blocked as early as 6 hours. (Figure 36 shows in detail the loss of function healing phenotype upon interference on *pvr*, as a prototype of the Phenotypic Class 3)

***rho1*.** Rho1 is a small GTP-binding protein of the Rho superfamily. Rho GTPases play an important role in diverse basic cellular processes including actin cytoskeleton organization, gene transcription, cell cycle progression and adhesion [199]. During early *Drosophila* development Rho1 is required in different morphogenetic processes, including dorsal closure and head involution [200]. Rho1 also has a role in embryonic wound healing [85].

rho1 expression showed an upregulation of 2.26 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *Rho1* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in pupal lethality. Healing assays with Pnr-Gal4 yielded incomplete healing with large wounds. These imaginal discs complete the initial steps of healing (6 hours) but showed no peripodial sealing, neither signs of filopodia formation at the leading edge, as a result healing is incomplete.

- ***Class 4***

Capping protein β (Cpβ). Capping protein beta is a regulator of actin filament growth [201]. Capping proteins cap the barbed end of actin filaments inhibiting loss and addition of actin monomers. Knockdown of *Cpβ* in *Drosophila* cells resulted in actin accumulation [187]. Loss of *Cpβ* affects border cell migration reducing the length of actin protrusions. It also causes abnormal actin aggregates on the inner side of nurse cell membranes [202].

Cpβ expression showed an upregulation of 3.26 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *Cpβ* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in

notum malformation. Healing assays with En-Gal4 yielded incomplete healing although heterotypic contacts were present and partial peripodial sealing achieved. In the CE, filopodia do not form properly and a great accumulation of actin can be observed; CE zippering was affected (Figure 37 shows in detail the loss of function healing phenotype upon interference on *Cpβ*, as a prototype of the Phenotypic Class 4).

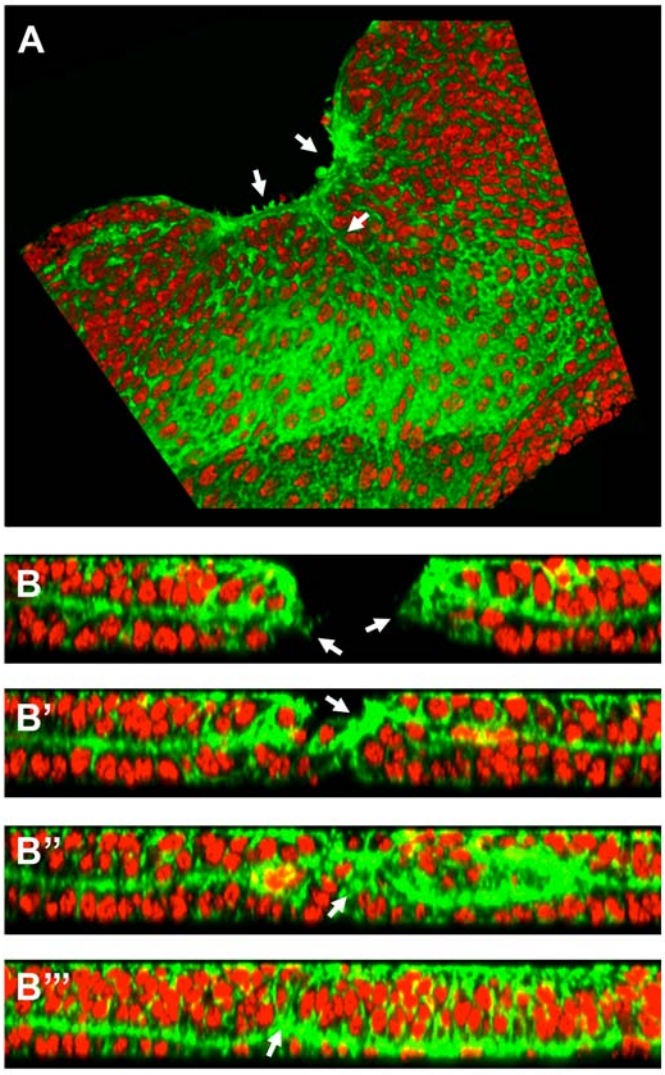


Figure 37. Cp β healing phenotype (Class 4)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. It shows a large open wound and abnormal accumulation of actin (arrows). Bs) sequential orthogonal sections. Actin accumulation impedes the normal fusion of the epithelia (arrows). Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

Capping protein α (Cpα). Capping protein alpha is a regulator of actin filament

growth *in vitro* [201]. Capping proteins cap the barbed ends of actin filaments with high affinity preventing the addition or release of actin subunits [203]. Decreased expression of *Cpα* in the *Drosophila* eye imaginal disc causes actin filament accumulation [204]. Cpα is also required for the proper development of the wing blade primordium and its loss results in cells extrusion and apoptosis [205, 206]. Recently, it has also been shown that the knockdown of *Cpα* results in the accumulation of actin and myosin in wing discs [207]. Further, loss of both capping proteins results in abnormal bristle morphology [205, 208].

Cpα expression showed an upregulation of 1.80 fold in direct comparisons in healing-engaged cells in wing imaginal discs. In dual comparisons (dw1_dnw0_ dd1), it shows an upregulation of 1.84 fold in healing-engaged cells in wounded discs, while was increased a negligible 1.22 times in unwounded. The comparative fold difference between the two populations was 1.51.

Downregulation of *Cpα* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing. Heterotypic contacts were present and partial peripodial sealing achieved. In the CE, filopodia are aberrant, actin accumulates and zippering was affected.

Downregulation of both *Cpα* and *Cpβ* produce the same healing phenotype indicating that they might be involved in similar processes during healing (see Discussion).

CG15027. *CG15027* is an uncharacterized translation machinery-associated protein 16 homologue, with predicted nuclear localization and unknown molecular and biological function.

CG15027 expression showed an upregulation of 1.68 fold in direct comparisons in healing-engaged cells in wing imaginal discs. In dual comparisons (dw1_dnw0_dd1), it shows an upregulation of 1.71 fold in healing-engaged cells in wounded discs, while was increased a negligible 1.15 times in unwounded. The comparative fold difference between the two populations was 1.49.

Downregulation of *CG15027* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing. Heterotypic contacts were present and partial peripodial sealing achieved. In the CE, filopodia are aberrant, actin accumulates and zippering was affected.

CytC-p. Cytochrome C proximal is an electron carrier involved in oxidative phosphorylation [209]. The *CytC-p* has been shown to activate caspases in *Drosophila* cells [210].

CytC-p expression showed an upregulation of 3.05 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

Downregulation of *CytC-p* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing. Heterotypic contacts were present and partial peripodial sealing achieved. In the CE, filopodia are aberrant, actin accumulates and zippering was affected.

- ***Class 5***

scab. *scab*, also known as *αPS3*, codes for an alpha integrin subunit [211]. Integrins are heterodimeric transmembrane receptors for the extracellular matrix or for surface molecules on other cells [212]. Through their cytoplasmic domains, they can interact with cytoskeletal elements and cause changes in cell shape in response to ligand binding. The *Drosophila* gene *scb* is transcribed in tissues undergoing morphogenetic movements [211] and in its absence, dorsal closure fails [213].

scab expression showed, in dual comparisons (dw1_dnw1_dd1), an upregulation of 2.04 fold in healing-engaged cells in wounded discs, while was increased 5.00 times in unwounded. The comparative fold difference between the two populations was -1.59. No difference was observed in the global comparison.

Downregulation of *scab* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia proceed through closure but do not complete it. Heterotypic contacts are abolished and gaps are found between both epithelia (Figure 38 shows in detail the loss of function healing phenotype upon interference on *scab*, as a prototype of the Phenotypic Class 5).

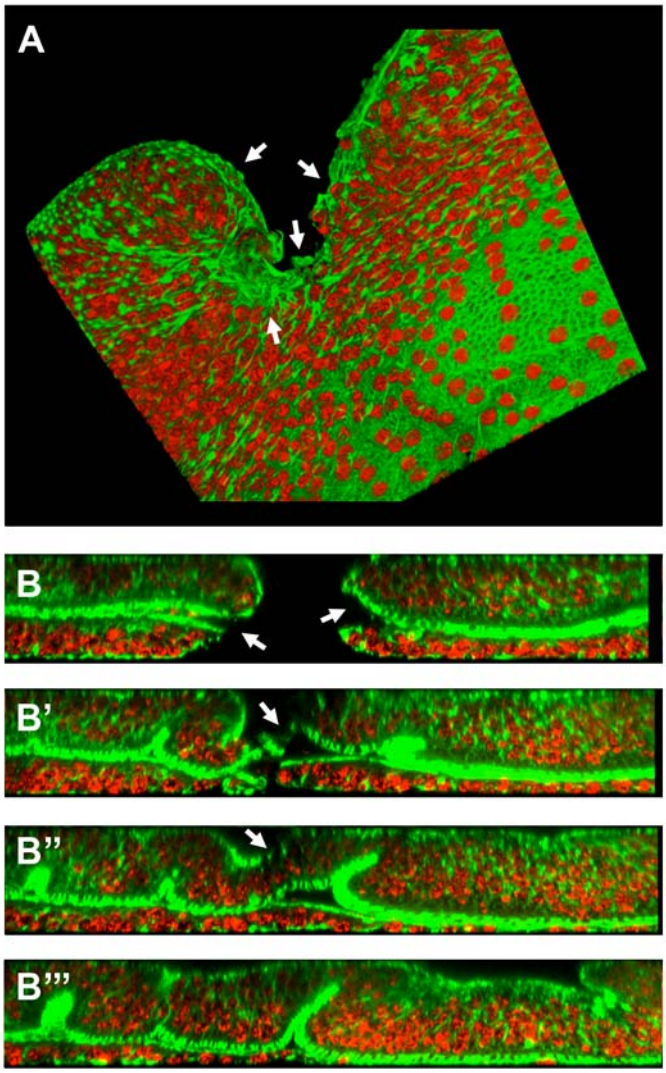


Figure 38. scab healing phenotype (Class 5)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. It shows a large open wound and a gap between the peripodial and the columnar epithelia (arrows). Bs) sequential orthogonal sections. The gaps at the edge between layers is more evident. The columnar epithelia initiates sealing apically (arrows), Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

CG7296. *CG7296* is an unknown gene with no known molecular function. *CG7296* is upregulated in response to septic shock and this upregulation is abolished in *mekk1* loss of function conditions [214], thus probably responding to P38 activation by stress.

CG7296 expression showed an upregulation of 2.60 in global comparisons of healing engaged cells in wing imaginal discs. In dual comparisons (dw1_dnw1_ dd1), it shows an upregulation of 3.66 fold in healing-engaged cells in wounded discs, while

was increased 2.19 times in unwounded. The comparative fold difference between the two populations was 1.67.
Downregulation of *CG7296* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia proceed through closure but do not complete it. Heterotypic contacts are abolished and gaps are found between both epithelia.

scarface. Scarface is a member of a subgroup of serine protease homologues (SPH) whose functions are poorly understood. SPs are endopeptidases that use a serine for their catalytic activity [215]. *scarface* is a general transcriptional target of the JNK pathway and loss of *scarface* in *Drosophila* provokes defects in dorsal closure [216].
scarface expression showed an upregulation of 3.05 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.
Downregulation of *scarface* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia proceed through closure but do not complete it. Heterotypic contacts are abolished and gaps are found between both epithelia.

CG15611. *CG15611* is annotated as a Rho guanyl-nucleotide exchange factor, potentially implicated in the regulation of Rho signal transduction. *CG15611* appears to be involved in *Drosophila* leg morphogenesis [217].
CG15611 expression showed an upregulation of 4.94 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.
Downregulation of *CG15611* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia proceed through closure but do not complete it. Heterotypic contacts are abolished and gaps are found between both epithelia.

***sqh*.** The Drosophila *spaghetti squash* (*sqh*) gene encodes for the regulatory myosin light chain (RMLC) of non-muscle myosin II [218]. In *Drosophila*, *sqh* is required for wound healing, cytokinesis, morphogenesis of interfollicular stalks, border cell migration and nurse cell cytoplasm dumping into the oocyte [219, 220]
sqh expression showed, in dual comparisons (dw1_dnw1_dd1), an upregulation of 1.61 fold in healing-engaged cells in wounded discs, while was increased 2.36 times in unwounded. The comparative fold difference between the two populations was -1.32. No difference was observed in the global comparison.
Downregulation of *sqh* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia proceed through closure but do not complete it. Heterotypic contacts are abolished and gaps are found between both epithelia.

lamin. Lamins are intermediate filaments that line the inner surface of the nuclear envelope, providing structural support and making contacts with chromatin [221, 222]. In *Drosophila*, lamin (lam^{p}) null mutants result in prepupal lethality [221] and hypomorphs result in severe phenotypes like delayed development, sterility and impaired locomotion [223, 224]. It has been reported that ubiquitous overexpression of *Drosophila* Lam results in lethality, while when expressed in a tissue specific manner (GMR-Gal4) resulted in severe eyes malformations (deformed, small and lack pigmentation and ommatidia) and lethality [225, 226]
lamin expression showed a downregulation of 1.59 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this decrease was not observed in the dual microarray analysis.
Expression of UAS-Lamin in the dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia proceed through closure but do not complete it. Heterotypic contacts are abolished and gaps are found between both epithelia.

***mirror*.** *mirror* is a member of the *Iroquois Complex* (*Iro-C*). *Iro-C* genes encode conserved homeodomain transcription factors [227]. All the members of the

Drosophila Iroquois gene family are implicated in the development of the peripheral nervous system and the regionalization of the wing and eye imaginal discs [228]

mirror expression showed a downregulation of 2.55 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this decrease was not observed in the dual microarray analysis.

Expression of UAS-*Mirror* in the dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia fail to complete closure (Figure 39 shows in detail the gain of function healing phenotype upon overexpression of *Mirror*, as a prototype of the Phenotypic Class 5).

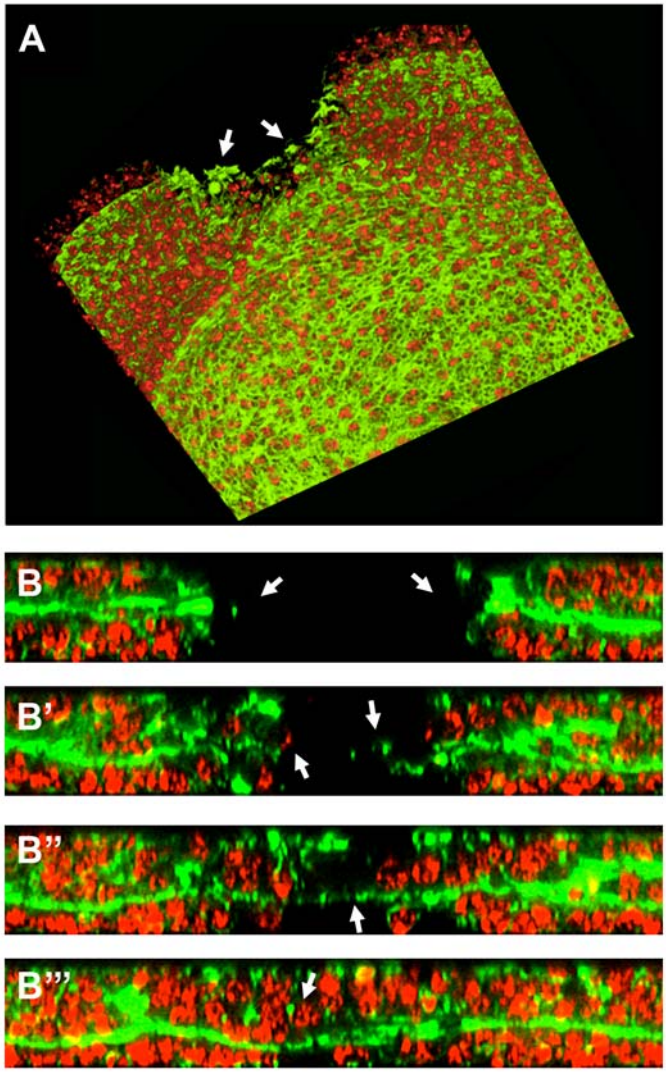


Figure 39. UAS-Mirror healing phenotype (Class 55)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. It shows partial closure and an abnormal distribution of actin (arrows). Bs) sequential orthogonal sections.

Apical sealing precedes basal attachments that seem disorganized (arrows). Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

- ***Class 6***

***fimbrin*:** Fimbrin is a cytoskeletal protein associated with microfilaments in microvilli, microspikes, stereocilia, membrane ruffles, and cell-substratum attachment sites [229]. It directs the formation of tightly bundled F-actin assemblies [230]. In *Drosophila* it has been shown to participate in female meiosis chromosome segregation [231].

fimbrin expression showed an upregulation of 3.03 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.

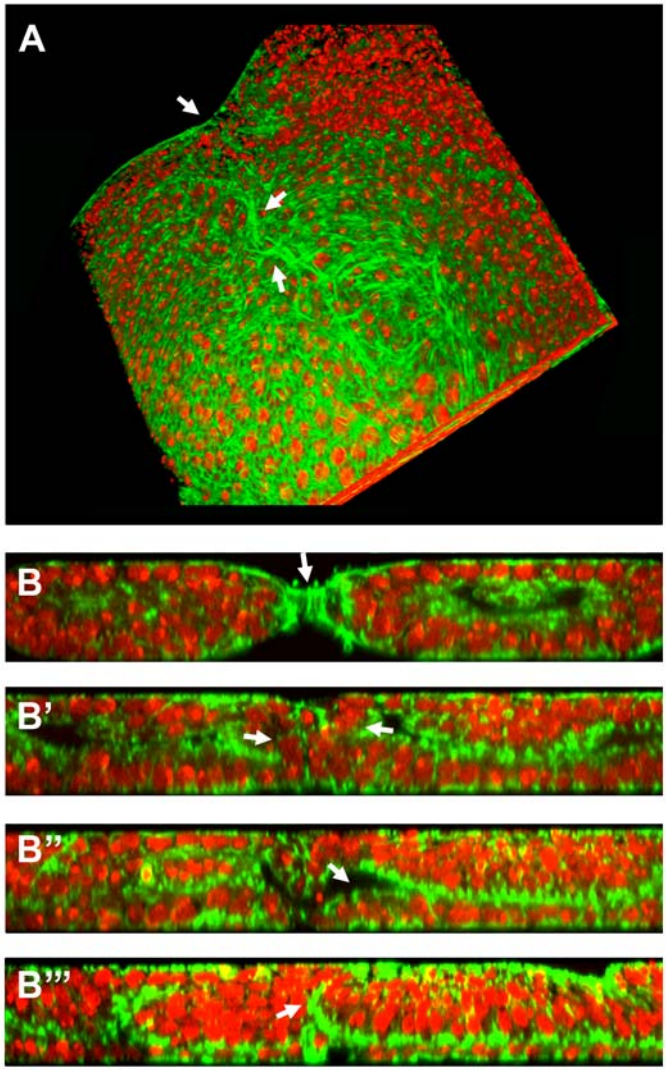


Figure 37. fimbrin healing phenotype (Class 6)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. Advance closure but disorganized actin cytoskeleton in the peripodial epithelia (arrows). Bs) sequential orthogonal sections. Actin accumulation at the junction is evident. The peripodial epithelia remains disorganized at the fusion point (arrows). Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

Downregulation of *fimbrin* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded incomplete healing after 18 hours. PE and CE fail to complete a tight closure. The tissue remains loose and disorganized with frequent apical gaps (Figure 40 shows in detail the loss of function healing phenotype upon interference on *fimbrin*, as a prototype of the Phenotypic Class 6).

- ***Class 7***

arc1. *darc1* is one of three *Drosophila* homologs of the mammalian activity-regulated cytoskeleton-associated protein (ARC) [232]. *ARC* knockdown affects wound closure and cytoskeletal organisation in PC3 cells [233]. In *Drosophila*, *darc1* is expressed in neurosecretory cells and *darc1* mutants are starvation resistant [232].

arc1 expression showed an upregulation of 4.19 fold in direct comparisons in healing-engaged cells in wing imaginal discs. *arc1* expression also showed, in dual comparisons (dw1_dnw1_dd1), an upregulation of 1.70 fold in healing-engaged cells in wounded discs, while was increased 8.91 times in unwounded. The comparative fold difference between the two populations was -1.81.

Downregulation of *arc1* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded complete healing in both CE and PE epithelia. However, they were defective in tissue relaxation (Figure 41 shows in detail the loss of function healing phenotype upon interference on *arc1*, as a prototype of the Phenotypic Class 7).

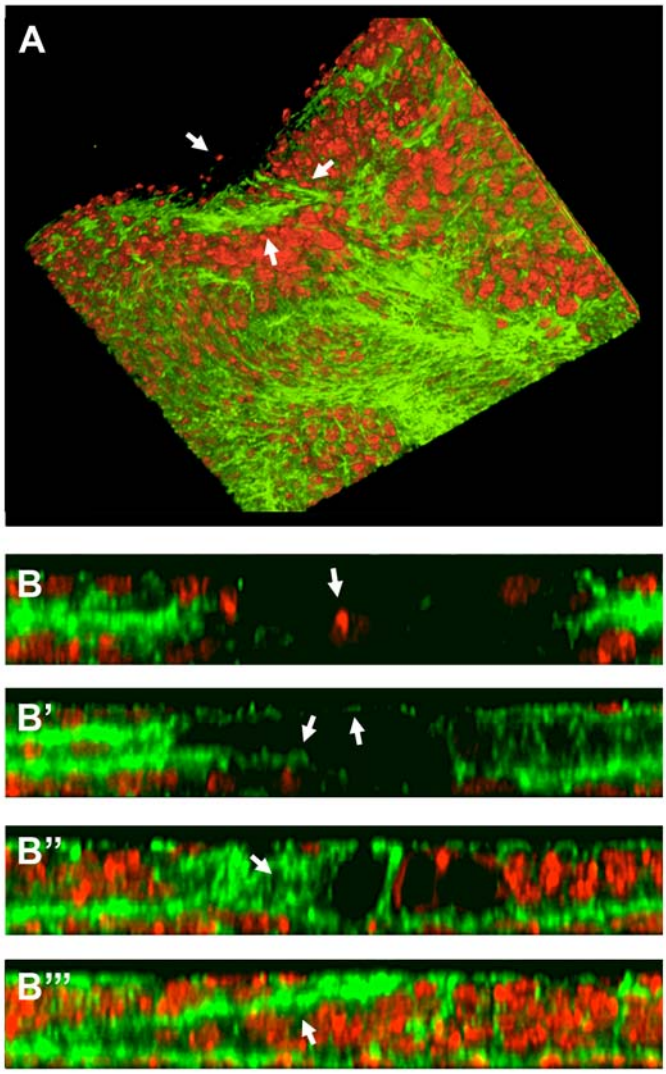


Figure 41. arc1 healing phenotype (Class 7)

A) 3D reconstruction of the peripodial and columnar epithelia at 18 hours after wounding. It shows a full closure but shape abnormalities at the junction and adjacent territories (arrows). Bs) sequential orthogonal sections. Apical gaps are observed at the fusion area and surrounding. Tissue layers are shifted (arrows). Phalloidin (actin) is shown in green; DAPI (nuclei) in red.

CG10200. *CG10200* is an unknown gene with no known molecular function. In RNAi screenings it has been found to participate in the immune response and notum formation in *Drosophila* [234, 235]. Further, its product has been shown to interact with GST1, the homologue of the Glutathione-S-Transferase D1 [236]. CG10200 has been also identified as an EGFR responsive gene in transcriptional analyses [237]. Recently, it has also been reported as a novel gene required for wing development regulated by hedgehog signalling [238].

CG10200 expression showed an upregulation of 3.07 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.
Downregulation of *CG10200* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in notum malformation. Healing assays with En-Gal4 yielded complete healing in both CE and PE epithelia. However, is defective in tissue relaxation.

Aats-Trp. Aminoacyl-tRNA synthetases catalyze the first committed step of protein synthesis. Tryptophanyl-tRNA synthetases are crucial for survival affecting the cellular metabolism and are highly conserved. Interestingly, this protein has also been linked to angiogenesis [239], and in fact, tryptophanyl-tRNA synthetase-derived-polypeptides are in trial as angiogenic inhibitors in oncology. In *Drosophila* Aats-Trp has been implicated in the terminal arborization of both dendrites and axons during development [240].

Aats-trp expression showed an upregulation of 2.43 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis.
Downregulation of *Aats-trp* by UAS-RNAi in dorsal thorax with Pnr-Gal4 resulted in pupal lethality. Healing assays with En-Gal4 yielded complete healing in both CE and PE epithelia. However, they were defective in tissue relaxation.

- **Other healing regulators (Álvarez-Fernández, PhD Thesis, 2013) [168]**

The gene coding for the PDGF and VEGF related factor 1 (*Pvf1*) was upregulated 5.20 fold in direct comparisons in healing-engaged cells in wing imaginal discs. In dual comparisons (dw1_dnw1_dd1), this gene showed an upregulation of 4.85 fold in healing-engaged cells in wounded discs, while was increased 9.04 times in unwounded. The comparative fold difference between the two populations was -1.46. The downregulation of the Pvf1 with specific RNAi constructs using Pnr-Gal4 caused a failure of imaginal discs fusion and a big thorax cleft. When this downregulation was performed in the En-Gal4 domain, healing completion failed. Actin did not accumulate at the leading edge and filopodia were absent (it can be grouped in the phenotypic class 1).

The gene coding for the chaperonin TCP1 ζ was upregulated 2.54 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis. The downregulation of the TCP1 ζ with specific RNAi constructs in the dorsal area of the adult thorax with Pnr-Gal4 caused a failure of imaginal discs fusion and a big thorax cleft. When this downregulation was performed in the En-Gal4 domain, healing was blocked as early as 6 hours after wounding. Indeed, the wounded tissue showed no signs of actin accumulation or filopodia formation at the leading edge (it can be grouped in the phenotypic class 1).

The gene coding for Serpin 55B (*spn55B*) was upregulated 2.42 fold in direct comparisons. However, this enhancement was not observed in the dual microarray analysis. Its downregulation caused a failure of imaginal discs fusion and a thorax cleft. A single cut in the Pnr area of cultured discs, showed that healing progressed almost normally although the distal completion of closure and tissue relaxation of the sealed area (downregulation of actin accumulation) was not achieved (it can be grouped in the phenotypic class 7).

4. Functional characterization of wound healing phenotypes

For functionally characterizing the different observed wound healing phenotypes, we analysed the expression of key markers upon wounding, both in wild type discs and after interfering in selected genes whose absence leads to healing phenotypes. This, non-fully comprehensive analysis mainly focused in regulators of the cell cytoskeleton, cell attachments and signal transduction elements. A summary of all expression analyses described below is presented in Table 7.

Table 7

	CG12007	*capping protein α-CG10540*	*capping protein β-CG17158*	*scarface-CG11066*	*scab-CG8095*	*CG15611*	*CG7296*	*sqh-CG3595*	*spn55b-CG10913*	*verprolin-CG13503*	*TCP1-η-CG8351*	*TCP1-ζ-CG8231*
puc	X	√	√	X	X			√	√			
MMP1	√	~	~	√			~					
Scab	~	√	√	√	X	X				X		
Talin	√	√	√	X	~	X		~				
Tubulin	√	~	~	√	√		~				~	~
Phenotypic Class	1	4	4	5	5	5	5	5	7	1	1	1

- **JNK activity during wound healing *in vitro***

In unwound third instar larval wing imaginal discs, JNK signalling activity (*puc* expression) is limited to the stalk region and to the PE. In regenerating discs, this WT pattern is maintained, but *puc* is also expressed at the wound edges. Such expression is observed in both the CE and the PE epithelia. In the PE, the increase of *puc* expression is circumscribed to imaginal cells elongating towards the wound edge and is coincident with the contraction of their distal tips (see Figure 19) [92]. The high level of *puc* expression, and hence JNK activity, on healing-engaged cells, together with the fact that in the absence of the JNK upstream regulator *hep* or other members of the JNK signalling cascade, wound healing is impaired strongly indicates that the JNK signal has an instrumental role in the healing process. But which is this role? Is the activation of the JNK cascade causally involved in the remodelling of the actin cytoskeleton dynamics? Or it can participate in the modulation of cells adhesivity? To

explore these possibilities we monitored the expression of *puc* in wounded imaginal disc when interfering in the expression of some of the genes previously identified in the functional screening.

Previous analyses of selected upregulated genes (Table 8) displaying thorax fusion phenotypes on the process of fusion of imaginal discs during metamorphosis failed to detect any alteration on the expression pattern of *puc* [168]. In the absence of *CG1703* - strong notum cleft (3TC), *l(1)1Bi-CG6189* - strong notum cleft (3TC), *Tg-CG7356* - medium notum cleft (4TC) or *Aats-trp-CG9735* – excess of fusion (6TC), *puc* pattern remained unaffected indicating that their activities do not impinge on JNK signalling and are downstream of it or related to unconnected cellular functions.

Table 8

GENE	PHENOTYPE	FUNCTION	*puc*
CG1703	3TC	ABC-F protein – translation (?)	+
CG6189	3TC	DNA polymerase V (?)	+
CG7356	4TC	γ-glutamyltransferase - hemolymph coagulation (?)	+
CG9735	6TC	Aminoacyl tRNA synthetase – neurogenesis (?)	+

We performed an equivalent analysis during *in vitro* imaginal discs healing. To do so, we used a *puc-LacZ* enhancer trap chromosome recombined with the Gal4 lines employed for the gene expression interference analysis (see Materials and Methods). We chose a set of genes belonging to different phenotypic classes (Table 9).

Table 9

GENE	PHENOTYPE	FUNCTION	*puc*
Cpα	4WH	F-actin-capping protein subunit - blocks actin filaments	+
Cpβ	4WH	F-actin-capping protein subunit - blocks actin filaments	+
sqh	5WH	Non muscle myosin II regulatory light chain	+
spn55b	7WH	Serine-type endopeptidase inhibitor	+
CG12007	1WH	Rab geranyl-geranyl-transferase - cell shape; cell adhesion	-
scarface	5WH	Secreted Serine protease homologue (inactive)	-
scab	5WH	Integrin alpha subunit - ECM attachment; migration	-

Interference in four genes expression (*Cpα, Cpβ, sqh* and *spn55b*) do not affect the expression of *puc* at the leading edge, suggesting that they act downstream of the JNK cascade or in independent pathways. However, interference in three other genes (*CG12007, scarface and scab*) resulted in a strong reduction of *puc* expression in the healing area (Figure 42). They seem to act, at least in part, upstream of the JNK cascade during healing, may be sensing and/or transmitting wound signals.

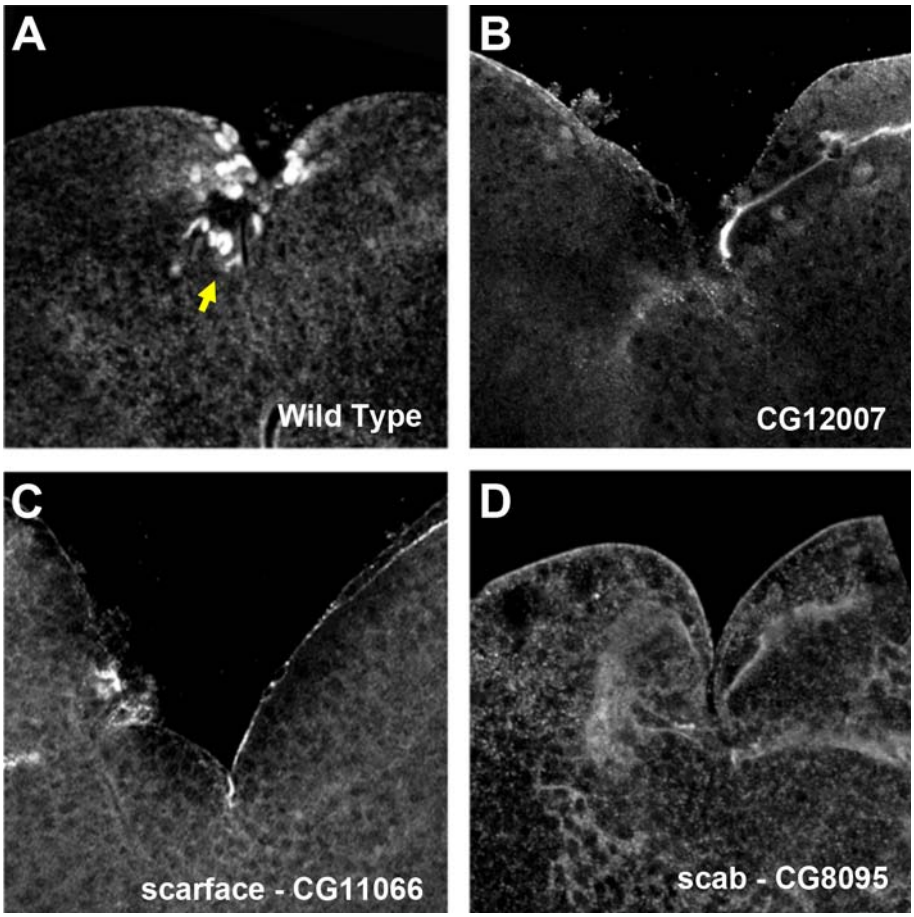


Figure 42. *puc* expression in healing defective genes.

A) Control wounded imaginal discs exhibit enhanced *puc(lacZ)* expression near the wound. B to D) Interference with the expression of different genes as labelled. Interference results in reduced *puc (lacZ)* expression near the wound. Anti-βGal in grey.

- **Expression of MMP1 in healing defective genes**

Matrix metalloproteinases (MMPs) are extracellular proteases involved in basement membrane degradation highly expressed at wound sites [241, 242]. MMPs are regulated by JNK signalling during healing and loss of MMPs result in impaired repair [90]. In *Drosophila* there are two MMPs – MMP1 and MMP2 [241, 243]. The

secreted MMP1 is known to be highly upregulated during healing in embryos and larval tissues where it is required for reepithelialisation (cell elongation, reorganization of the actin cytoskeleton and repair of basement membrane [90, 244].

mmp1 is upregulated 7.43 fold in the healing global transcriptome comparisons and in dual comparisons (dw1_dnw1_dd1) showed an upregulation of 2.22 fold in *puc* positive cells in wounded discs and 3.03 times in unwounded. The comparative fold difference between the two populations was -1 27. *mmp2* expression changes were not detected in the healing transcriptome. Wild type imaginal discs immunostained with an anti-MMP1 antibody showed increased expression in culture as early as 8 hours after wounding. This expression was restricted to areas close to the wound in both apical and basal surfaces, with stronger expression on the CE epithelium. The expression was seen all around the wound in cell membranes and within cells (Figure 43). We performed a limited survey to screen, amongst those genes essential for healing, which could be involved in activating *mmp1* expression and, hence, which could be necessary for basal membrane remodelling (Table 10).

Table 10

GENE	PHENOTYPE	FUNCTION	*MMP1*
CG12007	1WH	Rab geranyl-geranyl-transferase - cell shape; cell adhesion	+
scarface	5WH	Secreted Serine protease homologue (inactive)	+
Cpα	4WH	F-actin-capping protein subunit - blocks actin filaments	+/-
Cpβ	4WH	F-actin-capping protein subunit - blocks actin filaments	+/-
CG7296	5WH	Unknown	+/-

While abolishing *scarface* or *CG12007* expression do not affect MMP1, interference in three other genes (*Cp α, Cpβ* and *CG7296*) affect its localization. Limiting *Cp α* and *Cp β* expression leads to a redistribution of MMP1, which becomes exclusively extracellular around cell membranes, and limited to the apical surface. On the other hand, interference in *CG7296* results in the restriction of MMP1 to the basal surface of leading edge cells, showing strongly reduced levels. In all cases, the reduction in the levels of MMP1 may result in insufficient removal of the basal membrane.

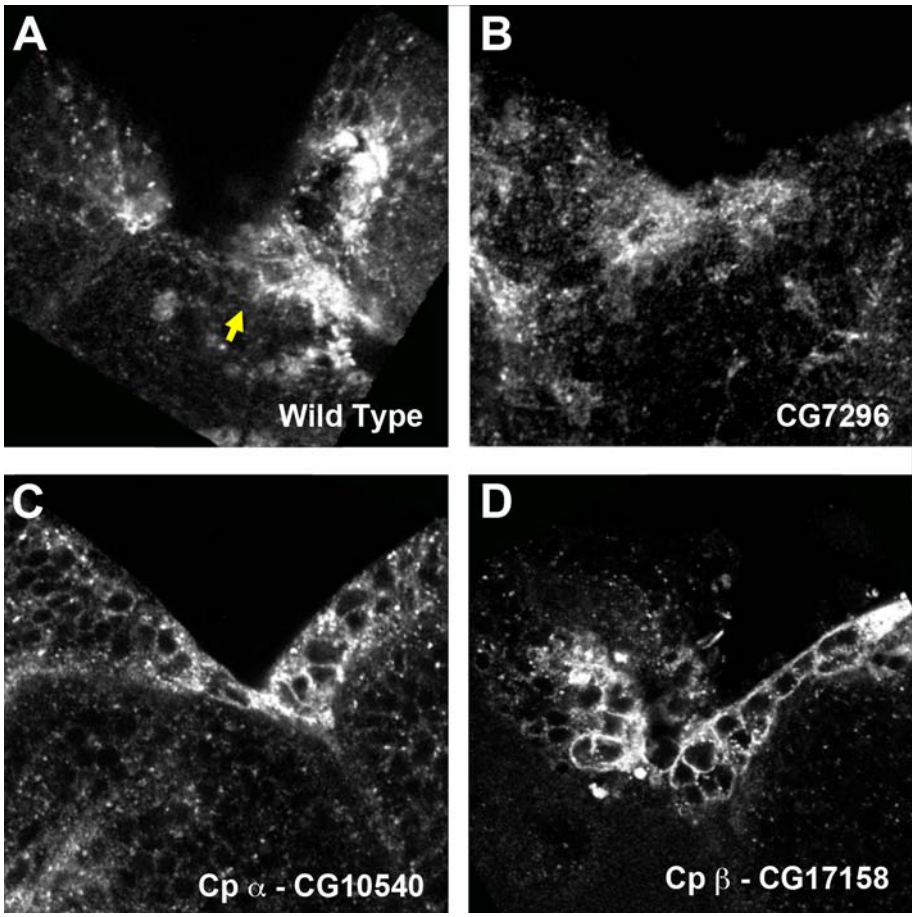


Figure 43. MMP1 expression in healing defective genes.

A) Control wounded imaginal discs exhibit enhanced MMP1 expression near the wound. B to D) Interference with the expression of different genes as labelled. Interference results in abnormal expression near the wound (phenotypes described in the text). Anti-MMP1 in grey.

- **Expression of Scab in healing defective genes**

Integrins are a superfamily of heterodimeric cell adhesion receptors (α and β subunits) that bind to extracellular matrix components [245]. In mice, loss of β1 integrin in keratinocytes cause severe defects in wound healing due to reduced production of new ECM and a failure of growth of the connective tissue [246]. *Drosophila* has five integrins [212] required for the assembly of the ECM, which are key elements participating in different developmental processes such as dorsal closure [247].

We found that the expression of the α-integrin *scab* is upregulated during imaginal disc healing. *scab* is upregulated 2.04 fold in healing-engaged cells in wounded discs, while was increased 5.00 times in unwounded [dual comparisons (dw1_dnw1_dd1)].

The comparative fold difference between the two populations was -1.59. No difference was observed in the global comparison. Antibody stainings showed an increased expression of Scab in wound leading edges for both the CE and PE epithelia (Figure 44). To check if this expression, and then ECM attachment, could be affected in healing defective conditions, we interfered in the expression of a set of genes belonging to different phenotypic healing classes, including some involved in actin cytoskeleton control (Table 11).

Table 11

GENE	PHENOTYPE	FUNCTION	*SCAB*
Cpα	4WH	F-actin-capping protein subunit - blocks actin filaments	+
Cpβ	4WH	F-actin-capping protein subunit - blocks actin filaments	+
scarface	5WH	Secreted Serine protease homologue (inactive)	+
CG12007	1WH	Rab geranyl-geranyl-transferase - cell shape; cell adhesion	+/-
CG15611	5WH	Rho guanyl-nucleotide exchange factor	-
verprolin	1WH	WASp Interacting Protein - actin filament; cell shape	-
scab	5WH	Integrin alpha subunit - ECM attachment; migration	-

While interfering in the expression of *Cpα, Cpβ* or *scarface* do not affect Scab expression, interference in four other genes (*CG12007, CG15611*, *verprolin* and *scab* itself) resulted in a strong Scab expression reduction in the healing area (Figure 44). Downregulation of *CG12007* resulted in a loss of uniformity in the expression of Scab, which becomes clumpy, along the leading edges. The downregulation of *CG15611, verprolin* or *scab*, however, resulted in the complete loss of Scab expression at the leading edge, both on the CE and PE epithelia (Figure 44).

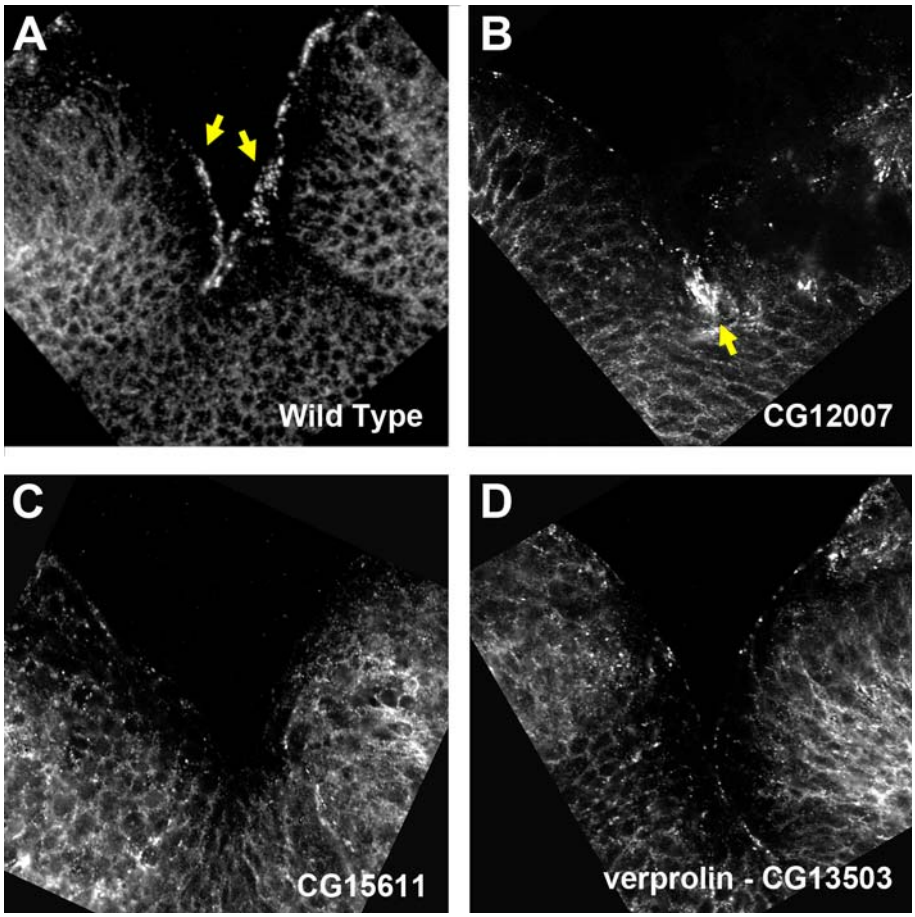


Figure 44. Scab expression in healing defective genes.

A) Control wounded imaginal discs exhibit enhanced MMP1 expression near the wound. B to D) Interference with the expression of different genes as labelled. Interference results in abnormal expression near the wound (phenotypes described in the text). Anti-Scab in grey.

- **Expression of Talin in healing defective genes**

Talin is an actin-binding protein that helps to form a direct link between cell-matrix adhesion receptors (integrins) and the internal cytoskeleton [248]. The protein structure of Talin is modulated by force, which allows Talin to sense and feel the mechanical properties of the extracellular environment [249, 250]. In many processes, loss of Talin mimics loss of integrins [158]. In the human epidermis, Talin concentrates at the basement membrane, but is absent from the leading edge during wound healing [251]. Its function during wound healing is unknown. In *Drosophila*, embryos deficient for *Talin* have very similar phenotypes to integrin *(βPS)* null embryos, including failure in germ band retraction and muscle detachment amongst other processes [158]. Talin is also required for the formation of focal adhesion-like

clusters of integrins on the basal surface of the imaginal disc epithelia [158].

We observed that *Talin* expression is upregulated during imaginal disc wound healing. It showed an upregulation of 1.65 fold in direct comparisons in healing-engaged cells in wing imaginal discs. However, this enhancement was not observed in the dual microarray analysis. Wounded control imaginal discs after 8 hours of culture showed an increased expression of Talin in leading edge cells membranes of both the CE and PE epithelia (Figure 45). Further, its downregulation results in defective healing. These results resemble those reported during dorsal closure where *talin* is expressed along the leading edge and its loss results in dorsal open phenotypes [158].

To explore which function during wound healing the links between the actin cytoskeleton and matrix attachments may have, we analysed the expression of Talin upon downregulating a set of healing defective genes including integrins, myosin and actin cytoskeleton regulators (Table 12). In particular, *scab* is known to be a binding partner of talin. In *Drosophila* embryos, both interfering in α or β-integrins results in altered expression of Talin [158, 252]. Talin has also been shown to be a direct binding partner of myosin in several models [253].

Table 12

GENE	PHENOTYPE	FUNCTION	*TAL*
CG12007	1WH	Rab geranyl-geranyl-transferase - cell shape; cell adhesion	+
Cpα	4WH	F-actin-capping protein subunit - blocks actin filaments	+
Cpβ	4WH	F-actin-capping protein subunit - blocks actin filaments	+
CG15611	5WH	Rho guanyl-nucleotide exchange factor	+/-
scab	5WH	Integrin alpha subunit - ECM attachment; migration	+/-
sqh	5WH	Non muscle myosin II regulatory light chain	+/-
scarface	5WH	Secreted Serine protease homologue (inactive)	-

While interference in three genes (*CG12007, Cpα* and *Cpβ*) do not affect Talin, in four other genes (*CG15611, scab, sqh* and *scarface*) it resulted in strong Talin expression alterations (Figure 45). RNAi interference in *CG15611*, *sqh* and *scab* expression lead to an anomalous accumulation of *Talin* near the leading edge. In the absence of *scab* this accumulation was associated to a characteristic change of the

shape of the leading cells (Figure 45). *scarface* loss of function results in the elimination of Talin expression near the leading edge, while normal expression was still present in the rest of the disc.

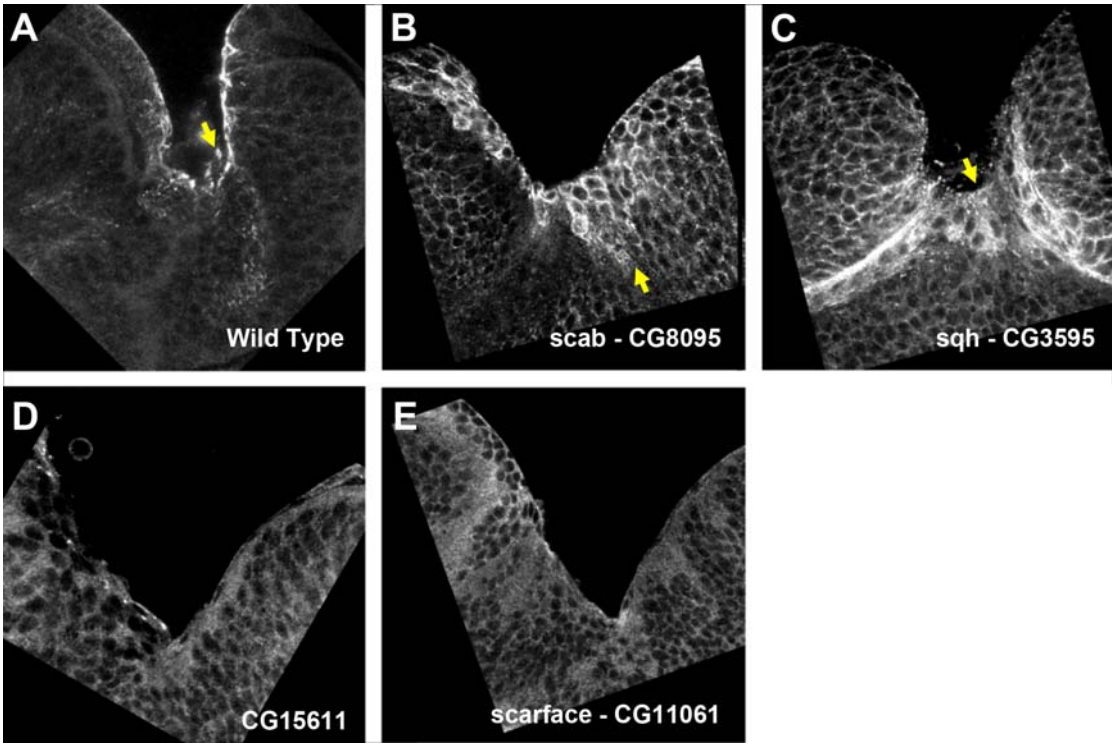


Figure 45. Talin expression in healing defective genes.

A) Control wounded imaginal discs exhibit enhanced MMP1 expression near the wound. B to D) Interference with the expression of different genes as labelled. Interference results in abnormal or absence of expression near the wound (phenotypes described in the text). Anti-Talin in grey.

- **Behaviour of tubulin filaments during healing**

Microtubules are an important component of the cell cytoskeleton, and the interaction between microtubules and actin cytoskeleton is fundamental in many cell processes [115]. Scratch assays in cultured epithelial cells show that tubulin filaments reorient towards wound edges. This has been claimed to aid cell migration towards the wound, constraining the cell movements to specific directions. In *Xenopus* oocytes, wounding leads to a dramatic reorganization of the microtubule network, which rapidly relocalises to the region of cortex adjacent to the wound site [254]. Microtubules are also known to play an essential role during dorsal closure in *Drosophila*. Although the elements directing microtubule reorganization in this model are unknown, it is thought to aid structurally the zippering process. In *Drosophila* embryos microtubules

transport regulatory factors such as Rho GTPase activating proteins (GAPs) and exchange factors (GEFs) fulfilling instructive activities. In this way they can regulate the actin machinery and affect cell shape [118] or cortex contractility (e.g. delivery of Rho GEF2 to the apical cortex to induce Rho1-dependent myosin II function during *Drosophila* gastrulation) [255]. So, far its role in wound healing as not been reported in *Drosophila.*

Wild type wounded imaginal discs show reorientation of microtubules towards wound edges. This reorganization is more prominent in the CE epithelial side while a dense network of microtubules is seen on the PE side of the disc. Tubulin shows co-localization with actin, probably aiding wound contraction and subsequent closure (Figure 46).

A set of healing defective genes belonging to different phenotypic classes including two subunits of the *TCP1* complex polypeptide known to be required for tubulin folding were analysed to test if the reorientation of microtubules could be affected as a result of their downregulation (Table 13).

Table 13

GENE	PHENOTYPE	FUNCTION	*TUB*
CG12007	1WH	Rab geranyl-geranyl-transferase - cell shape; cell adhesion	+
scarface	5WH	Secreted Serine protease homologue (inactive)	+
scab	5WH	Integrin alpha subunit - ECM attachment; migration	+
Cpα	4WH	F-actin-capping protein subunit - blocks actin filaments	+/-
Cpβ	4WH	F-actin-capping protein subunit - blocks actin filaments	+/-
CG7296	5WH	Unknown	+/-
TCP1 η	1WH	Chaperonin Subunit – Protein synthesis and folding	+/-
TCP1 ζ	1WH	Chaperonin Subunit – Protein synthesis and folding	+/-

While interference in three genes (*CG12007, scarface* and *scab*) do not affect microtubules organization, interference in five genes (*Cpα, Cpβ*, *CG7296, TCP1 η* and *TCP1 ζ*) resulted in defects in microtubules organization during healing at the wound edge (Figure 46).

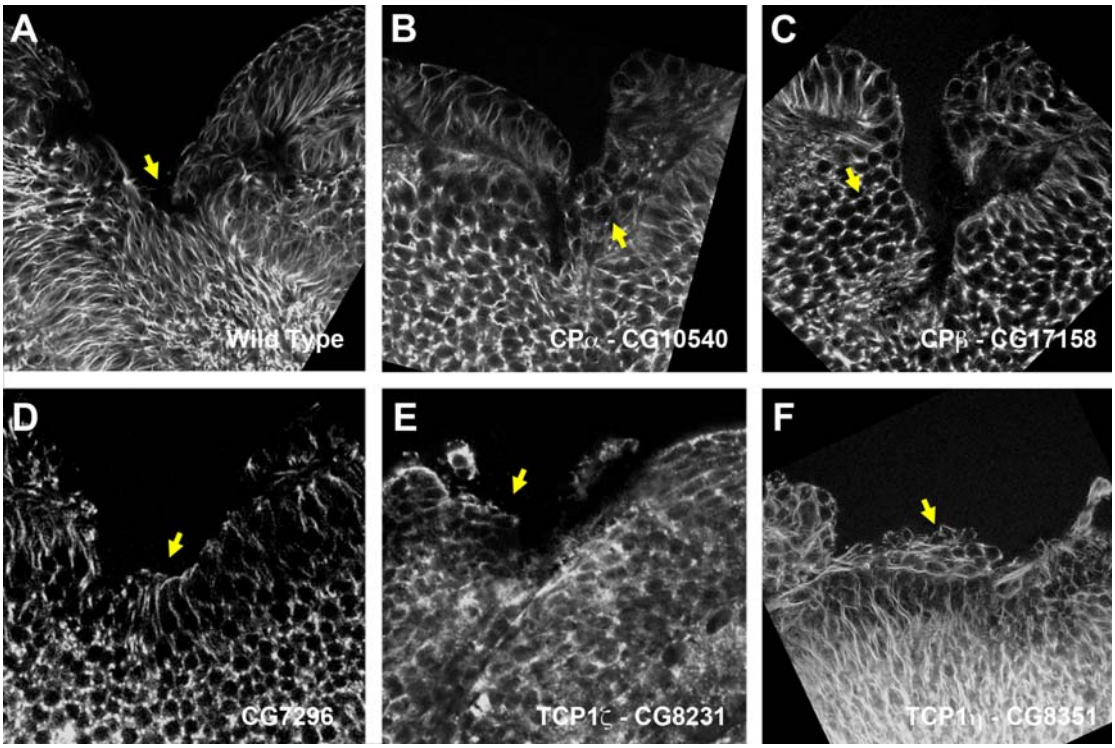


Figure 46. Tubulin expression in healing defective genes.

A) Control wounded imaginal discs exhibit enhanced MMP1 expression near the wound. B to D) Interference with the expression of different genes as labelled. Interference results in abnormal expression near the wound (phenotypes described in the text). Tubulin GFP in grey.

5. The TCP1 Chaperonin complex

The TCP1 functions as a cytosolic chaperonin promoting the folding of non-native actins and tubulins, amongst other substrates, in eukaryotic cells *in vivo* [135]. It is composed of eight different subunits (α, β, γ, δ, ε, ζ, η, θ) that assemble into a double ring hexadecameric structure, creating an internal cavity that serves as a folding chamber [256]. Importantly, TCP1 not only regulates protein folding but also regulates actin and tubulin synthesis by binding to ribosomes during translation [257].

- **Knockdown of TCP1 complex subunits affects thorax fusion**

Two subunits of the TCP1 complex, eta and zeta showed significant upregulation in our healing transcriptome. TCP1 η and TCP1 ζ expression increased 1.64 and 2.54 fold respectively in direct comparisons in healing-engaged cells in wing imaginal discs. These enhancement, however, were not observed in the dual microarray analysis.

The downregulation by UAS-RNAi (expressed with Pnr-Gal4) of TCP1 complex subunits during dorsal thorax closure resulted in strong imaginal disc fusion defects. Importantly, not just the knockdown of the η and ζ subunits but RNAi silencing of all subunits result in strong thoracic clefts for all of them except for TCP1 γ which is embryonic lethal (Figure 47). This suggests that the fusion of the imaginal discs is dependent of one systemic function implemented by the TCP1 complex as a whole, i.e., the loss of any one subunit will interfere with the functionality of the whole complex in this process. In this scenario, the transcriptional upregulation of the eta and zeta subunits observed in the healing transcriptome may be the result of a need for an additional supplement of these subunits levels on stress conditions (in order to coop for a major requirement on the levels of properly folded substrates. Considering that the recognition of specific substrates maps to different subunits of the complex [258], the function of TCP1 during imaginal fusion must then be related to its basic functionality as a chaperonin or linked to general substrates of the complex such as actin or tubulin.

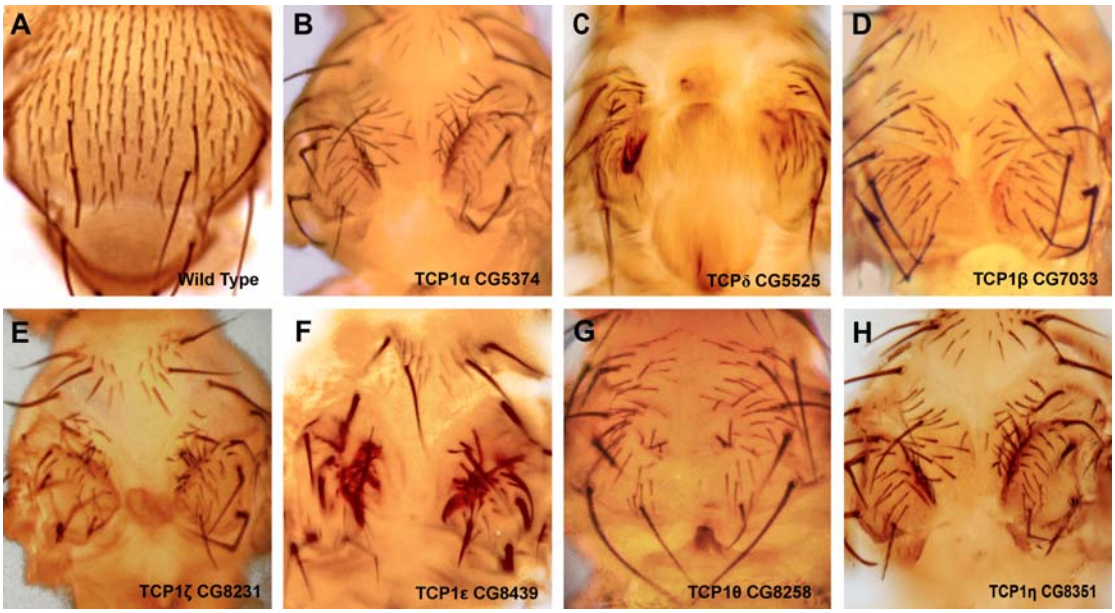


Figure 47. Thorax fusion phenotypes of TCP1 subunits. (RNAi's tested with pnr-GAL4)

A. Wild Type. B to H) Severe thorax malformations observed after interference with the expression of the different subunits as labelled.

- **TCP1 complex subunits knockdowns result in impaired wound healing**

Healing assays with En-Gal4 and specific RNAi lines for the different TCP1 subunits in the posterior domain of third instar wing imaginal discs yielded an open wound phenotype after 20-24 hours of culture. Healing was blocked as early as 6 hours after wounding. Both epithelia, CE and PE, failed to close the wound. Indeed, the wounded tissue showed disorganized actin accumulation while filopodia formation at the leading edge is abolished (Figure 48).

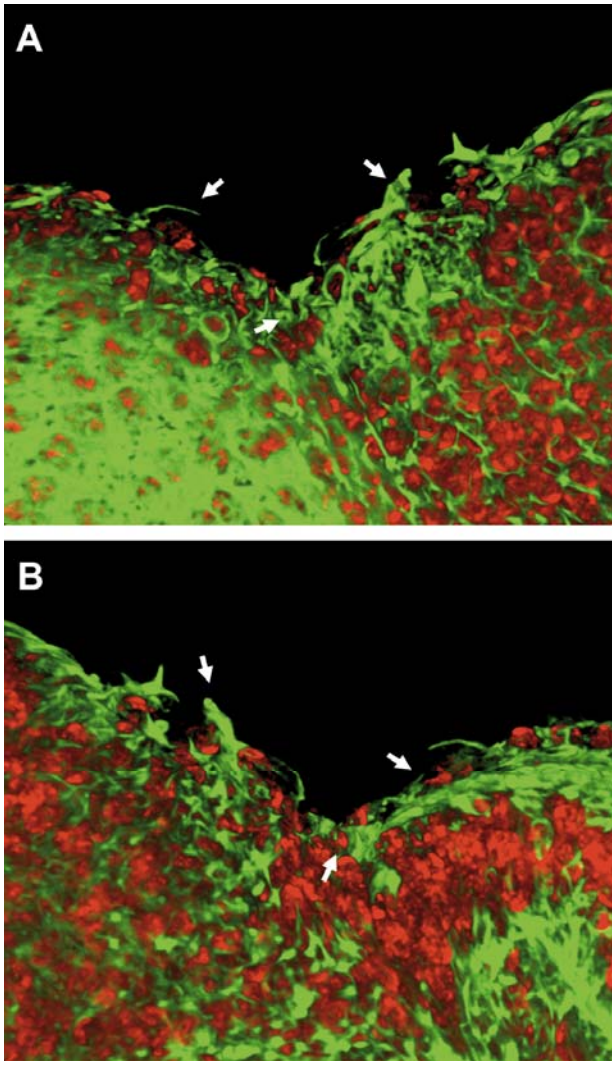


Figure 48. Impaired healing in TCP-1 RNAi imaginal discs.

A) Peripodial view. B) Columnar view. TCP-1η (CG8351) (RNAi) knockdown results in impaired healing after 20-24 hrs of culture *in vitro*. Arrows point to abnormal actin-rich structures. Phalloidin (actin) is shown in green; DAPI (nuclei) is shown in red.

This early phenotype was observed for all subunits except TCP1 δ, whose inhibition leads to embryonic lethality and TCP1 α that has not been tested. As for imaginal fusion, all subunits are apparently required for proper wound closure. Since both processes are actin-dependent and the actin cytoskeleton seems deranged in TCP1 complex subunits loss of function conditions during healing, it is relevant to hypothesize that TCP1 could be essential in *Drosophila*, as in other organisms, regulating actin synthesis and promoting its proper folding. These two events could be indispensable for proper epithelial tissue migration.

- **TCP1 regulates actin dynamics in *Drosophila* cells in culture**

To analyse the potential role of *Drosophila* TCP1 on the regulation of actin dynamics, we performed RNAi mediated knockdowns of TCP1 subunits in *Drosophila* S2R+ cells. S2R+ were transiently transfected with subunit specific TCP1 dsRNAs and an actin reporter construct, pMT-Act-GFP, carrying an actin-GFP fusion under the control of a metal activated promoter (see Materials and Methods). Four days after transfection, cells were subjected to live imaging, monitoring actin dynamics. For any TCP1 subunit tested (η and ζ) when compared to control cells, dsRNA treated S2R+ cells changed their morphology, became rounded, and displayed no filopodial protrusions. The actin cytoskeleton dramatically reorganized (Figure 49, see also Supplementary Movie 1).

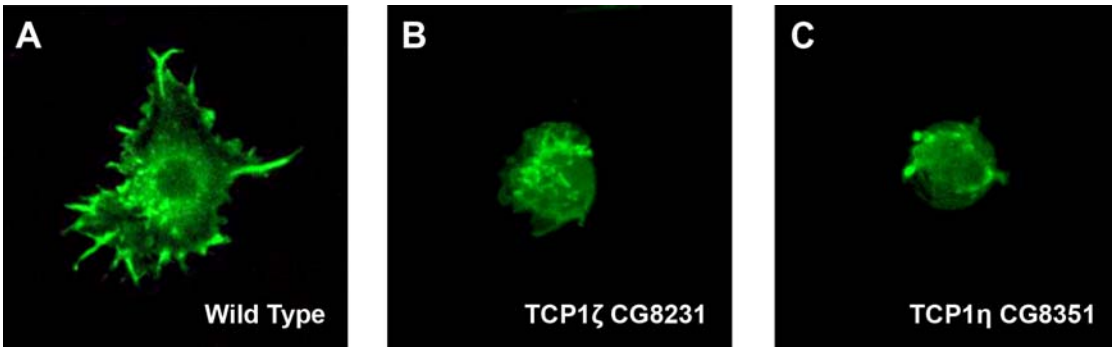


Figure 49. TCP1 knockdown affects the actin cytoskeleton of S2R+ cells.

A) Control cells transfected with pMT-act-GFP displaying normal actin cytoskeleton and filopodia. B and C) Cells transfected with TCP1 dsRNAs (subunit specific) exhibit defective actin cytoskeleton, rounded morphology and lack filopodia. pMT-act-GFP in green. (See supplementary movie 1).

On the other hand, when dsRNA treated cells were stained with phalloidin (to label F-actin), they did not show any significant difference in signal levels (Figure 50) indicating that actin polymerization seems to be unaffected in the absence of functional TCP1. These results are consistent with published data in other models showing that the levels of F-actin are not affected in *TCP1* mutant conditions [136]. Thus, TCP1, in S2R+ cells, is most probably involved in acquiring proper levels of G-actin (monomeric actin).

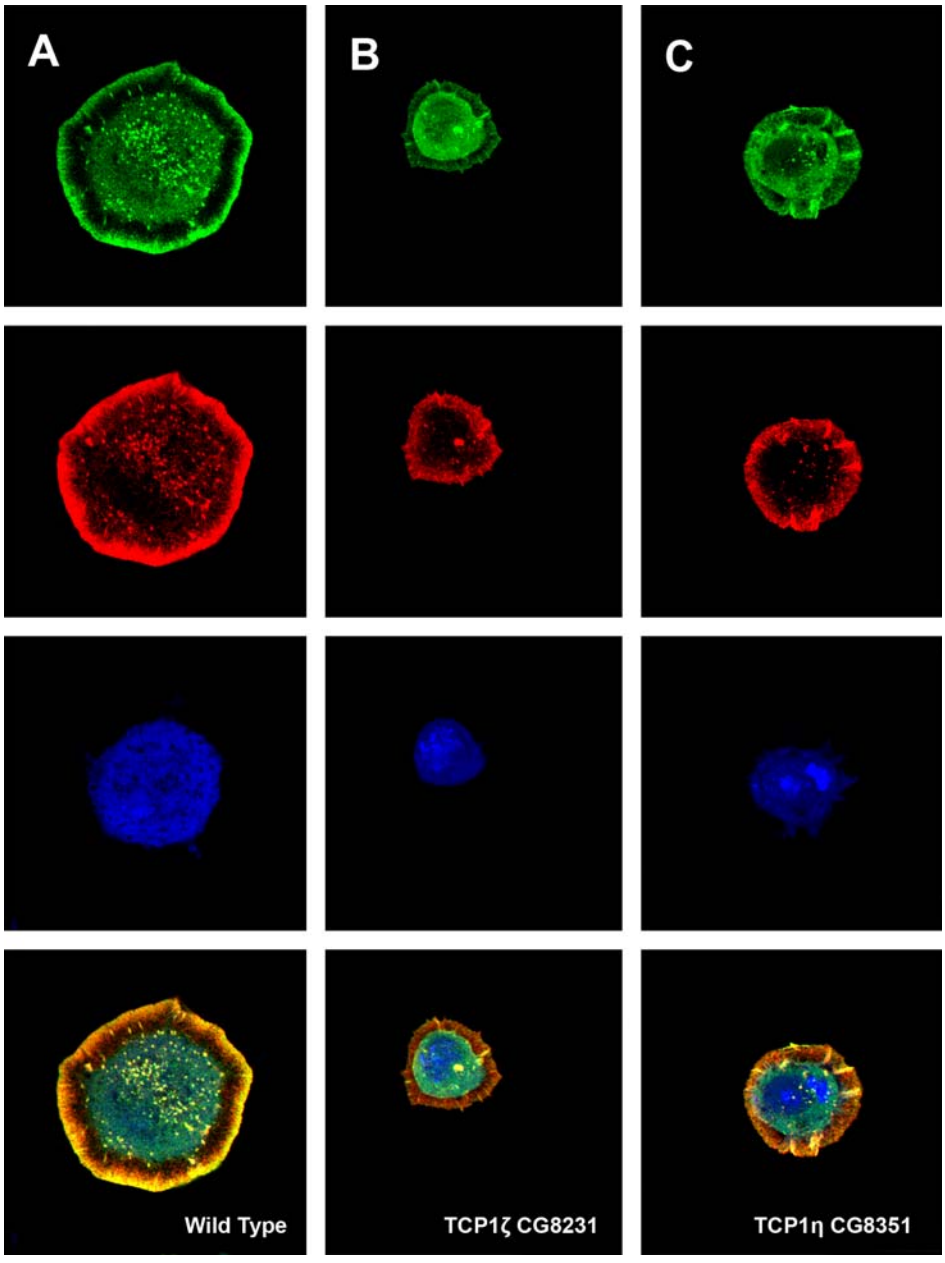


Figure 50. TCP1 knockdowns do not affect F-actin levels.

A) Control cells transfected with pMT-act-GFP and plated in Concanavalin A coated plates (flat morphology) displaying normal actin cytoskeleton and filopodia. B and C) Cells transfected with TCP1

dsRNAs (subunit specific) show normal levels and distribution of F-Actin. pMT-act-GFP in green; phalloidin (F-actin) in red; DAPI (nuclei) in blue.

- **TCP1 control of synthesis and folding of actin in *Drosophila***

TCP1 subunits knockdown in imaginal discs prevents proper healing and proper actin dynamics. However, a substantial amount of unstructured F-actin is still present in these conditions (see above). To investigate the role of TCP1 as a regulator of actin dynamics, we analysed its ability to influence actin filaments polymerization. Latrunculin A (LatA) was used to depolymerise F-actin [259] in TCP1 dsRNA treated S2R+ cells (see Materials and Methods).

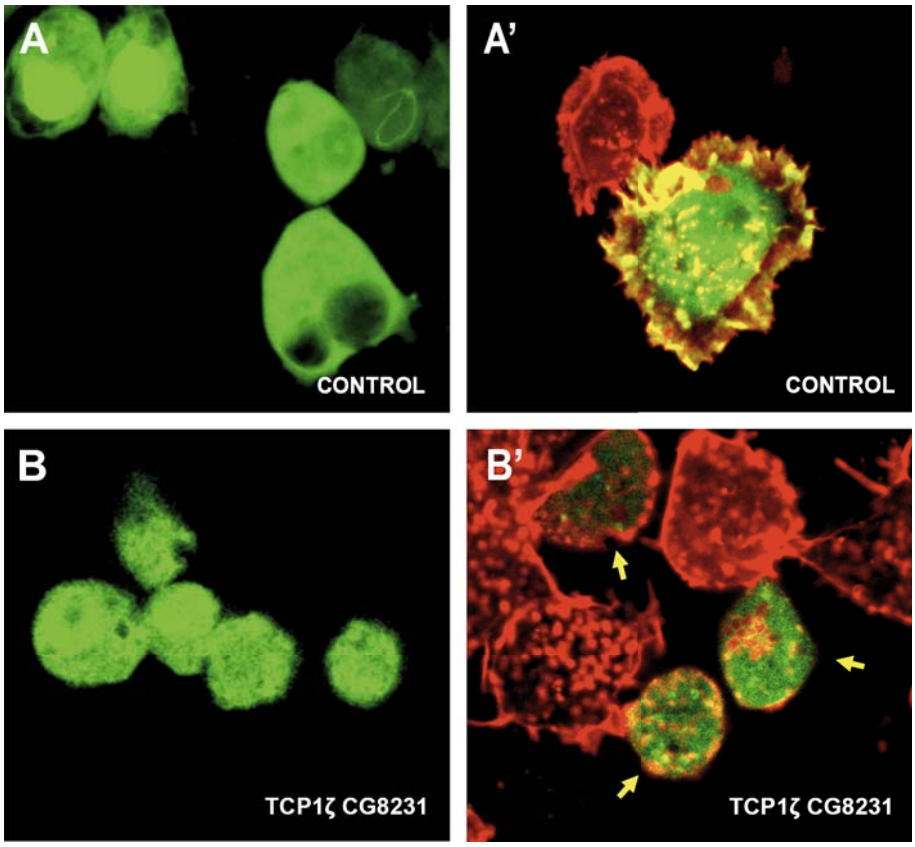


Figure 51. Latrunculin A mediated depolymerisation of F-actin and recovery in S2R+ cells.

A) Control S2R+ cells after treatment with Latrunculin A. A') Control cells after 48 hours of drug recovery. B) TCP1z-CG8231 RNAi treated cells after adding Lat A. B') TCP1z-CG8231 RNAi treated cells after 48hrs of drug recovery. RNAi treated cells (green) fail to reorganize their actin cytoskeleton. Phalloidin (actin) is shown in red.

Both, control and dsRNA (η subunit) treated cells become rounded immediately after LatA treatment displaying no F-actin (phalloidin) staining. Remarkably, after a recovery period of 48 hours in the absence of the drug, control cells restore their normal morphology and F-actin levels, while dsRNA treated cells remained rounded with no signs of F-actin recovery (Figure 51).

To address if the failure of cells lacking TCP1 η to re-build their actin filaments may be related to a deficiency on the synthesis and/or the folding of G-actin monomers, dsRNAs transfected S2R+ cells were treated with DNAse-1 conjugated to Alexa 488, which binds and sequesters G-actin monomers with high affinity [260] (see Materials and Methods). We observed that G-actin levels became higher in dsRNA treated cells compared to controls indicating that loss of TCP1 indeed affects the synthesis and folding of G-actin monomers (data not shown). Further, to monitor if TCP1 was involved in the synthesis of new actin polypeptides in *Drosophila* we generated a construct constitutively expressing a chimeric actin fused to a photoactivatable GFP and a cherry fluorescent protein (G^{PA}C-actin) (see Materials and Methods for details about the construct) [164]. We transfected S2R+ cells with G^{PA}C- actin +/- dsRNAs against TCP1 η and after four days of transfection subjected them to GFP photoactivation. Transfected cells were monitored live and scored for enhancement of the ratio of cherry signal versus GFP. Preliminary results indicate that, indeed, there is a reduction in the production of new actin molecules in TCP1 η knockdown conditions (data not shown). These preliminary results suggest that in *Drosophila* TCP1 participates in the modulation of actin production at the translational level. However, although an effect of TCP1 in the folding of the N-terminal fluorophores is highly improbable (in the employed construct, actin is placed at the C-terminal end), this cannot fully discarded.

- **TCP1 regulates microtubule dynamics in *Drosophila* cells in culture**

An equivalent analysis to that performed on actin dynamics was put in place to monitor the tubulin cytoskeleton. S2R+ cells were transiently transfected with subunit specific dsRNAs (η or ζ) and a construct, pMT-tub-GFP, carrying a tub-GFP fusion under the control of a ubiquitous promoter (see Materials and Methods). Four days after transfection, cells were subjected to live imaging, monitoring microtubule

dynamics. For any TCP1 subunit tested when compared to control cells, dsRNA treated S2R+ cells changed their morphology, became small and rounded, and showed a disorganized tubulin cytoskeleton (Figure 52, see also Supplementary Movie 2) indicating that, indeed, TCP1 might also be instrumental for tubulin folding in *Drosophila* cells.

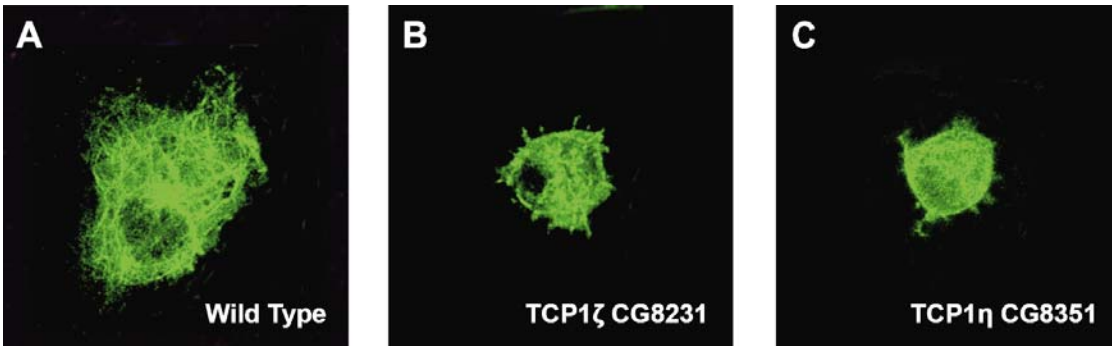
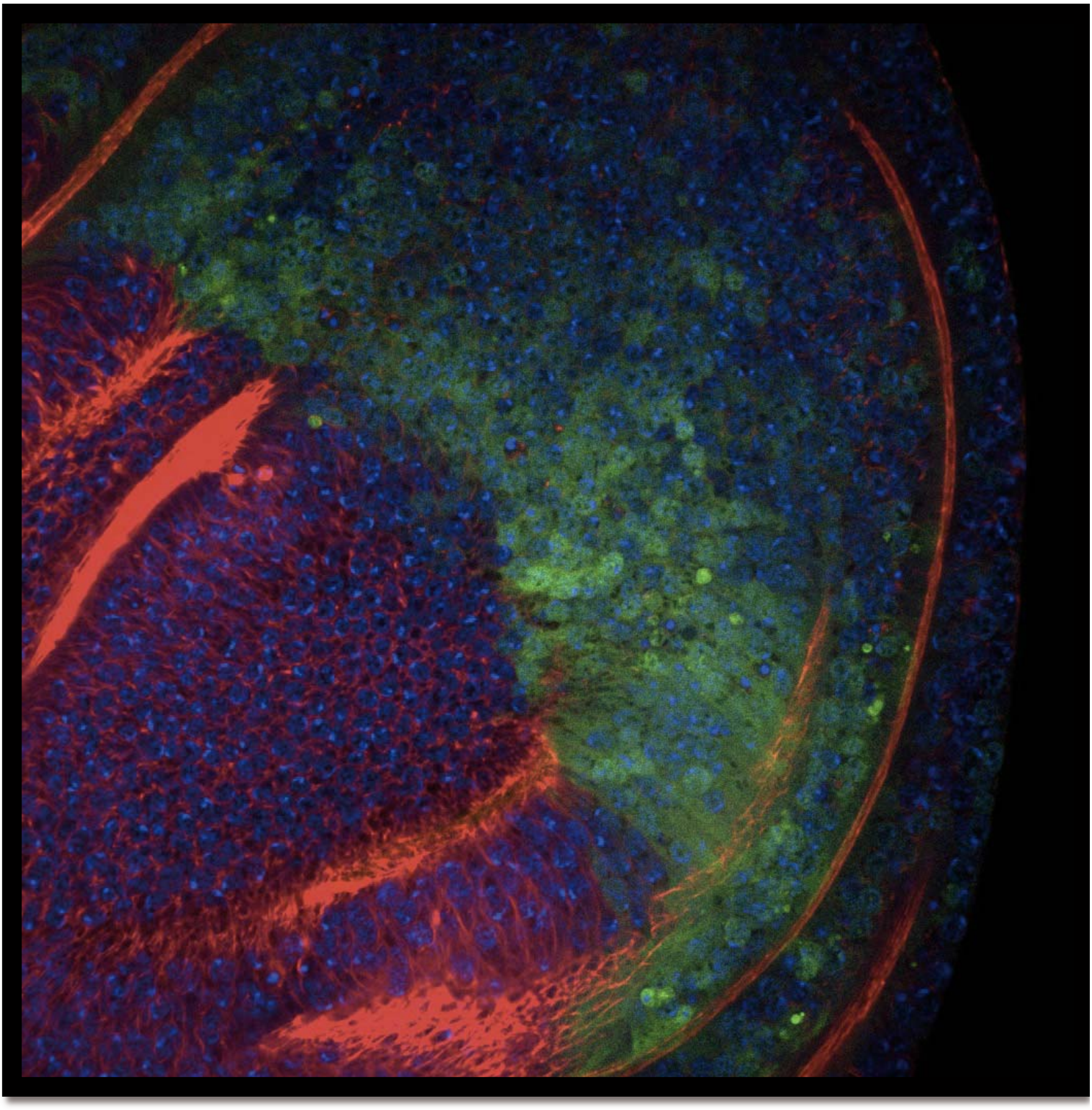


Figure 52. TCP1 knockdown affects microtubules of S2R+ cells.

A) Control cells transfected with pMT-tub-GFP displaying normal tubulin cytoskeleton and morphology. B and C) Cells transfected with TCP1 dsRNAs (subunit specific) exhibit defective microtubules androunded morphology. pMT-tub-GFP in green. (See supplementary movie 2).



DISCUSSION

1. Wound repair in imaginal discs

Wound healing problems are very common, affecting millions of people worldwide every year. Some wounds cause widespread morbidity, as they over-heal with extensive scarring or develop into keloids, others, as they fail to heal, become chronic, which may lead to their oncogenic transformation. Thus, understanding the molecular basis of wound healing and regeneration is of outmost clinical importance, not just to help improving physiological healing but for the development of successful treatments of healing disorders.

Drosophila has emerged as a valuable model for studying wound healing as the genetic networks and cytoskeletal machinery involved in epithelial movements occurring during embryonic dorsal closure, larval imaginal disc fusion/regeneration, and epithelial repair are similar to those acting during wound healing and regeneration in vertebrates. All stages of *Drosophila* development are suitable to wound healing analyses: embryo, larvae, imaginal discs and adults [86, 89, 92, 98]. Furthermore, the availability of powerful genetic tools makes possible to perform screens allowing genome-wide analyses to address novel biological questions including those relevant to human health. Importantly, the application of the yeast Gal4/UAS system in *Drosophila* allows the study of the effects of overexpression or inactivation of any gene in a selected tissue and developmental stage.

The imaginal discs due to their inherent capacity to heal and regenerate stand out as the most versatile amongst the diverse models of healing in *Drosophila*. Healing in imaginal discs is initiated immediately after wounding when both the peripodial (PE) and columnar (CE) epithelia curl towards each other emitting filopodia extensions to zip up the wound edges. The activation of JNK pathway is essential for this cytoskeletal remodelling. They eventually undergo tissue regeneration when needed switching off this same key cascade [92].

The main aim of this Thesis was to get a better understanding of the healing process while identifying the early responses triggered during healing. To achieve this goal we have set up a reliable culture system for wounded discs *in vitro*. We then pursued an exhaustive transcriptomic analysis for those cells engaged in the initial steps of healing identifying a set of genes whose expression is specifically modulated during the healing process. Finally, we functionally characterized these genes to extract

meaningful information about their roles by interfering with their expression and studying the cellular and phenotypical consequences of their misexpression during healing *in vivo* and *in vitro*.

2. Wing discs explants as a wound-healing model

We have developed a new *in vitro* model of wound healing for wing imaginal discs. Isolated imaginal discs were injured or cut and cultured. This *in vitro* model is healing permissive and recapitulates all the healing stages previously described. Specifically, cell rearrangements, heterotypic and homotypic contacts, the presence of an actin cable and filopodia, and the activation of the JNK signalling cascade at the wound edges. The only differences to *in vivo* classical imaginal disc healing and regeneration after implantation in the abdomen of adult flies were a slight delay of the whole process and the absence of the removal of cell debris. These delays could be due the need for adaptation to the new culture environment or, alternatively, due to lack of external signals provided in the abdominal "milieu" or the absence of haemocytes (macrophages) that could provide a healing facilitating function. The absence of macrophages might also be the cause for lack of removal of cellular debris.

3. Wing discs Healing Transcriptome

In injured imaginal discs, *puc* expression is detected after just a few hours pointing to the activation of the JNK pathway as one of the earliest responses to wounding. JNK is required for healing and early regeneration as *puc*-expressing cells do not die [92, 261-264] and cell lineage studies show that these cells contribute to regenerated tissues [261, 264]. A similar situation is found in fly tumours, where mutations in polarity genes activate JNK signalling and downregulate the E-Cadherin/β-Catenin adhesion complex, conditions necessary and sufficient to cause oncogenic RasV12-induced tumours [100]. Thus, it is possible that de-repression of JNK signalling and alterations in cell polarity induced in the wound edge cells after injury or massive death, are related. Actually, there is evidence that apoptosis and the pro-apoptotic genes *reaper* and *hid* can also activate the JNK pathway [265, 266].

How is the JNK pathway activated? In other words, how epithelial perturbation may be associated with unrestrained JNK activation? Growth factors, mechanical

stretching of the wound edge cells, loss of cell polarity or necrotic signals are all candidates for upstream activators [98-100]. Further, a gene network controlling JNK phosphorylation has been proposed following a screen to identify regulators of the JNK cascade in *Drosophila* [267]. Some of these regulators are genes encoding components of apicobasal polarity complexes that act as JNK suppressors. Indeed, several small GTPases of the Rho family are upstream activators of JNK signalling during healing in mammals and flies [53, 101-105], regulating the actin cytoskeleton, cell migration and proliferation [268, 269].

On the other hand, information is minimal on which are the primary responses triggered by the initial activation of JNK following injury. Matrix metalloproteinase-1 (MMP-1), which is important for basement membrane degradation, is regulated by JNK and Fos [270] and localizes near wounds in regenerating discs [271]. Other effectors may include the cell polarity factor Bazooka and the cytoskeleton adaptor D-Paxilin, through which JNK modulates cell–cell contacts and maintains sheet migration [272]. Additionally, a major class of genes whose expression increases during early regeneration have AP1 transcription factor binding sites in their regulatory regions [273]. These sequences are likely targeted by the Fos and Jun dimer that forms the AP1 transcription factor. Another potentially involved target is the JAK/STAT signalling cascade, which promotes cell proliferation in different cellular contexts [274]. After wounding, *unpaired* (*upd*), which encodes a JAK/STAT-activating cytokine related to interleukin-6, is elevated in a JNK dependent manner [275]. JAK/STAT might be involved in the propagation of JNK activity after wounding [276].

Applying the *in vitro* wound-healing model, we aimed to identify the relevant "healing transcriptome" through microarray analysis. Our analysis was based on puc^{E69}-Gal4I/UAS-GFP imaginal discs, which express GFP in *puckered* (healing-engaged; JNK active) expressing cells and respond both to JNK signalling agonists and antagonists and to incisive and ablating wounding. The conditions necessary for cell dissociation and sorting by flow cytometry were as mild as possible and allowed the isolation of homogeneous populations of intact GFP as well as non-GFP cells. To distinguish between the endogenous transcriptional response of JNK active cells [14] and that of the cells activating JNK in response to wounding, we performed quadruplicated assays for wounded and unwounded discs. It is relevant to point that

those genes potentially activated or inhibited by wounding in the JNK active cells do not necessarily respond to the JNK signalling and can be activated or inhibited by parallel mechanisms. JNK activity (GFP-expression) was just employed as a marker of healing-engaged cells. It is also important to state that the discs were suffering incisive wounding, thus no proliferation was required for repair. Our transcriptomic analyses thus tackle the early events after wounding, mostly cell rearrangements, cell adhesion and cytoskeleton dynamics.

Two previous screens, performed in *Drosophila*, aimed to explore the transcriptomic make up of healing tissues. Comparison of the RNA profiles of laser wounded vs. unwounded wild type and macrophage-deficient *serpent* (*srp*) mutant embryos [277] led to the identification of wound-activated haemocyte genes more related to the immune response associated to healing than to healing itself. More relevant microarray analyses were performed in regenerating wing disc after being manually fragmented and implanted into adult females at several time frames [273]. These assays allowed the identification of early- and late expressing genes potentially involved in regeneration, including members of the JNK and Notch pathways and chromatin regulators. Still, lack of discrimination between healing engaged and passive discs constituents reduces the informative capacity of this study providing a very partial account of the pathways and genes potentially participating in the healing of the *Drosophila* epithelia.

Initially, to obtain a global genomic description of the process we compared the expression level for each *Drosophila* gene in cells involved in healing (*puc*-GFP) versus neighbouring cells not involved in healing (non *puc*-GFP) in wounded discs by one to one comparisons. Further, dual comparisons allowed us to define two relevant subpopulations: genes modified in *puc*-GFP cells in wounded discs and unwounded disc but with differential expression in between both (*dw1_dnw1_dd1*) and genes only modified in *puc*-GFP cells of wounded discs (*dw1_dnw0_dd1*). Modified genes that belong to the *dw1_dnw0_dd1* subsets will be specific to wound healing but not for discs eversion and fusion. Those genes included in the *dw1_dnw1_dd1* subsets must be important for both.

Functional annotations helped to classify the genes with differential expression pattern during healing according to their GO terms. Indeed, specific GO terms are

differentially overrepresented or underrepresented in the individual subpopulations (*dw1_dnw0_dd1* or *dw1_dnw1_dd1*), while others are relevant in both groups.

The different processes identified in our transcriptome analyses, potentially relevant for imaginal discs healing are briefly discussed below in reference to the different subpopulations in which they are integrated.

- ***dw1_dnw1_dd1 subpopulation***

GOs overrepresented in the *dw1_dnw1_dd1* subpopulation of upregulated genes include genes involved in the regulation of cell motility and cell adhesion properties. Amongst the genes represented in the *dw1_dnw1_dd1* subpopulation of upregulated genes involved in intracellular communication we found *pvf1* and *pvf2*, which code for PDGF and VEGF related factors. By analysing dual comparison graphical representations for individual experiments we observed that for both genes their level of expression in GFP cells are similarly high in wounded and unwounded discs but much lower for non-GFP cells in unwounded discs. This suggest that these factors are important in cells actively involved in both fusion processes (healing and thorax) and they are non-autonomously upregulated during healing in neighbouring cells. Finally, in terms of downregulated genes, we found that GOs related to organ development, cell metabolism regulation and structural constituents of cuticle were overrepresented. These results suggest that the downregulation of these groups of genes is important both for thorax closure and wound healing. In this last case the normal activity of these genes may need to be interrupted for proper repair after dissection and injury.

- ***dw1_dnw0_dd1 subpopulation***

Interestingly, in the *dw1_dnw0_dd1* subpopulation, GOs for biological processes related to protein and cellular biosynthesis and ribosome activity are overrepresented amongst upregulated genes. Other overrepresented GO categories are those related to mitochondria activity and ATP synthesis. Altogether, this suggests that, upon injury, new proteins synthesis is required and the biosynthetic machinery of the cells needs to be immediately activated. In close relationship, the same applies to chromatin remodelling, which was previously reported to be essential for imaginal disc healing [92]. Cellular localization at the membrane and upregulation of genes related to the

secretory pathway were also overrepresented GO terms. Membranes must be presumably remodelled to accommodate cell shape changes. Further, after injury, many proteins might need to be secreted to remodel cell communication and/or to modify extracellular matrix attachments. In vertebrates it has been extensively demonstrated the importance of these secretory factors in the repair of wounded areas [278]. In flies, secreted factors like MMP1 (Matrix Metalloprotease 1) or Timp (Tissue inhibitor of matrix metalloproteases), regulated by the JNK signalling pathway, have been identified as necessary elements for matrix remodelling [90, 279]. These and other extracellular matrix proteins are specifically upregulated during healing. Interestingly, peptidases and endopeptidases are also overrepresented amongst those downregulated genes.

- ***dw1_dnw1_dd1 and dw1_dnw0_dd1 subpopulations***

Some relevant GOs overrepresented in both subpopulations are described below. Genes related to cytoskeleton or GTPase activity are enriched amongst upregulated genes. The upregulation of GTPases may be linked to their function as upstream activators of JNK signalling and regulators of the actin cytoskeleton and cell migration [53, 101-105]. Genes related to the defence response or responses to stress, are also significantly enriched. Other overrepresented GOs are apoptosis and induction and regulation of cell death and there is evidence that apoptosis and pro-apoptotic genes can activate the JNK pathway [265, 266]. A further enriched GO term includes JAK/STAT signalling elements. This cascade promotes cell proliferation in a JNK dependent manner, and might be involved in JNK activity propagation [274-276].

When comparing these results to the expression profile of imaginal discs during regeneration [273], several similitudes such as the upregulation of genes involved in stress and immune response and regulation of transcription, or the downregulation of genes related to RNA processing are highlighted. However, the downregulation of genes involved in cytoskeleton organization, biogenesis and cells proliferation or the upregulation of helix-loop-helix transcription factors [HLHm5, HLHm7 and E (spl)] during the first steps of regeneration [273] contrast with the overrepresentation of

these categories amongst upregulated and downregulated genes respectively in *puc*-GFP cells. These observed differences are most probably due to the fact that either study tackles a different paradigm. While our experiments were done on an *in vitro* cultured model and we defined an early wound healing expressing profile by sorting away cells actively involved in healing from those siblings not involved, Blanco et al analysed late regeneration *in vivo* by implanting wounded imaginal discs in the abdomen of adult flies analysing the expression response of whole discs without distinguishing healing/regenerating-engaged from unengaged cells.

4. Functional analysis of "healing" genes

Differential expression in healing-engaged cells does not ensure genes significance as regulators or structural elements participating in the healing process. They become relevant only after characterizing their activity. Genetic screens undertook by various laboratories have identified genes that drive wound healing and/or the wound inflammatory response in the *Drosophila* embryo and larvae [89, 165, 280]. A forward genetic screen on the basis of insertional mutagenesis identified 30 lethal mutants with defects in embryonic epithelia repair [165]. This set of genes includes most of those previously described to have a role in dorsal closure (DC). Further, analysing wound-responsive markers, a set of deficiencies and single mutations have been screened for their role in the response to laser cuts [280]. From this large unbiased screen, several genes were identified as regulators of the signals that activate wound response transcription near puncture wounds. At the cellular level, a correlation between genetic functions required for localizing wound-induced gene activation, and cellular functions required for endocytosis and/or apical-basal polarity were identified. Last, the overexpression of UAS-RNAi transgenes in larvae has been employed to identify genes involved in the control and coordination of epithelial migration during postembryonic wounds [89]. Interestingly, this study highlighted genes involved in JNK and Stress-Activated Protein Kinases (SAPK) signalling and actin cytoskeleton remodelling. On the other hand, genetic screens to identify regulators of regenerative growth have also been performed analysing several deficiencies and loss of function conditions upon activation of the apoptosis program in wing imaginal discs [96]. These screens are recent, and so the subsequent follow-

up work has been limited, but altogether they show that several aspects of the mammalian wound response are conserved in *Drosophila*.

We developed reverse functional screens based on the Gal4/UAS system for those genes with altered expression in our transcriptomic analysis. First, we interfered in the natural process of thorax closure. This screen identified 145 genes (approximately 55% of tested ones) whose upregulation or downregulation yielded a thorax closure phenotype. These results probably underscore the real number of potential positive regulators as many RNAi lines might not efficiently knock down gene expression. The scored phenotypes were segregated into different classes: embryonic lethal, pupal lethal, weak notum clefts, medium notum clefts, strong notum clefts and bristle polarity defects. Those lines producing reproducible stronger phenotypes were tested in a healing assay in imaginal discs *in vitro*. Several differences between the two test models are obvious and a full correlation was not expected. Thus, the cells from the peripodial epithelia are in charge of sheet movement and fusion in thorax closure, while during healing they create heterotypic contacts with the columnar epithelia and mainly support the imaginal epithelia fusion. Further, thorax closure occurs through spreading of contralateral edges over the larval epidermis, while during healing, edges fusion requires active pulling and zippering by an actin-rich purse string without any sliding over a substrate. Despite these differences, we found, using two different Gal4 lines, that interfering in the expression of 72,7 % of the monitored genes lead to wound healing defects.

Different phenotypic categories (both for interfered and overexpressed genes) are represented amongst the genes whose knockdown result in strong closure defects and, eventually, wing discs healing failures. They also display the specific enrichment of different GO terms. The most significant set of identified functionally relevant genes includes, as expected, factors involved in the activation or transduction of JNK signalling. Transcriptional activators or repressors constitute a second category of genes highly represented amongst those whose misregulation resulted in strong closure and/or healing phenotypes. Regulators of actin cytoskeleton dynamics under the control (or not) of the JNK signalling pathway are also enriched. This class constitutes a highly overrepresented category in the GO analysis. Matrix attachment components are also highlighted and in their absence healing does not proceed. Further, interference in genes involved in intracellular communication, as cytokines

and growth factors or Rho signalling regulators also resulted in healing defects. A final class of genes we found functionally relevant for closure and/or healing is that including proteins involved in the immune and inflammatory response.

- ***JNK signalling***

The most significant set of identified functionally relevant genes includes, as expected, factors involved in the activation or transduction of JNK signalling. Interference in the expression of *Jra (Jun Related Antigen) – DJun,* GTPase activity regulators such as *loco* or JAK/STAT signalling elements (*upd*) [23, 281] show extreme closure and healing defects.

DJun encodes a component of the AP-1 transcription factor and is the homolog of the mammalian proto-oncogene C-Jun in *Drosophila*. Loss of *DJun* affects the progress of wound closure in the embryo and larvae [89, 165]. Downregulation of *Jra* by UAS-RNAi in the dorsal thorax resulted in pupal lethality, while disc healing assays yielded an open wound phenotype (Phenotypic Class 2). Healing was blocked as early as 6 hours after wounding and, although some signs of actin accumulation were observed, filopodia formation at the leading edge did not proceed although the CE epithelia manifested evident cell shape changes.

Interference in *loco* expression leads to scutellum closure defects, while impairing *upd* expression results in a cleft phenotype during thorax closure.

More interestingly, non-previously identified regulators or mediators of JNK activity were also identified through interference as mediators of closure or healing. An element of this class is *CG1703*, a member of the ABC-F subfamily of ABC proteins [282]. ABC-F proteins are ATPases, which recently have been shown to regulate translation [283]. The downregulation of *CG1703* results in thorax closure failure as a result of the detachment and rounding of leading edge cells, a phenotype highly reminiscent of that observed upon upregulation of the JNK pathway during thorax closure [14]. Considering that the release of intracellular ATP or Ca2+ by damage or signals secreted by inflammatory cells may participate in JNK activation [106] is tempting to speculate that *CG1703* may somehow regulate the activity of the JNK signalling cascade at the level of translation.

- ***Transcriptional activators***

Transcriptional activators (*AP-2*, *mirror*, *ara*, or *capu*) or repressors (*Ssb-c31a* or *rept*) constitute a second category of genes highly represented amongst those whose misregulation resulted in strong closure and healing phenotypes. It is worth noticing that *mirror*, *ara* and *capu*, whose expression is downregulated during healing, belong to the Iroquois complex, which has been linked to the activity of different signalling cascades like those mediated by Notch, Wg or TGFβ and amongst other functions to the regulation of proneural genes and the development of sensory bristles [284-286]. *mirror* or *caup* expression restoration in the wing imaginal discs leads to incomplete healing. Both, the PE and CE established homotypic contacts but they fail to complete closure (Phenotypic Class 5). The activity of the Iroquois complex seems to prevent healing activity in imaginal discs.

- ***Cell signalling***

Interference in genes involved in intracellular communication, as cytokines and growth factors and signal transducers as Rho GTPases or their effectors, previously reported as mediators of healing completion [77, 278, 287], also resulted in healing defects.

Downregulation of *pvf1*, which codes for a PDGF and VEGF related factor, or *pvr1*, coding for a PDGF- and VEGF-receptor related by UAS-RNAi in dorsal thorax resulted in notum malformation interference. Blocking their expression caused incomplete healing with large wounds. Imaginal discs showed proper actin accumulation and heterotypic contacts but no peripodial sealing, neither signs of filopodia formation at the leading edge or zippering (Phenotypic Class 3). Recently, Pvf1 and Pvr have been shown to be required for larval epidermal wound closure [198]. In larvae, Pvf1 and Pvr activate the intracellular actin polymerization machinery allowing the extension of actin-rich processes in the direction of the wound gap. Pvf and Pvr are necessary for the apical assembly of the actin cytoskeleton [159] and are also required for other diverse developmentally programmed processes, including thorax closure during metamorphosis and border cell migration during oogenesis [288-290].

Rho1 small GTPases are required for many morphogenetic events during *Drosophila*

oogenesis and embryogenesis [199]. During the wound response in *Drosophila* embryos, Rho1 mediates actin cable formation, which is critical at the initial stages of wound contraction [105]. We found that, as in embryos, loss of Rho1 blocks the early stages of healing in imaginal discs. Interestingly, interference in *CG15611* [a Rho guanine nucleotide-exchange factors (*RhoGEF*)] and *CG12007* (an uncharacterised Rab geranyl-geranyl-transferase) also lead to equivalent phenotypes. It is thus tempting to speculate that these genes may have linked functions during healing.

- ***Cytoskeleton dynamics***

Regulators of actin cytoskeleton dynamics under the control (or not) of the JNK signalling pathway are also enriched amongst those genes whose function is essential for closure and healing. This class constitutes a highly overrepresented category in the GO analysis. Emission of filopodia and actin accumulation in the leading edge cells is indeed necessary to complete healing in all *Drosophila* models [23, 81, 92, 291]. Relevant genes identified in our analyses include *Act42a, verprolin, TCP1*η, *TCP1*ζ, *Capping protein* α*, Capping protein* β*, fimbrin*, *sqh*, *Arc1* or *l(1)1Bi* (CG6189).

Downregulation of *act42a* by UAS-RNAi in the dorsal thorax resulted in pupal lethality. Healing assays yielded an open wound phenotype. Healing was blocked as early as 6 hours after wounding. Indeed, the wounded tissue showed no signs of actin accumulation or filopodia formation at the leading edge (Phenotypic Class 1). We also found that downregulation of *Act5c* results in severe thorax fusion defects leading to lethality at pupal stage. Although the loss of *Act5c* results in reduction in actin levels in healing assays it shows no affect on healing itself, suggesting that its loss is probably compensated by other actins such as *Act42a*.

vrp, originally identified in yeast, encodes for a predicted WH2-containing actin binding protein needed for actin polymerisation during polarised growth and during endocytosis [183]. In *Drosophila*, Vrp participates in recruiting the actin-polymerization machinery to sites of cell-cell attachments [186]. Downregulation of *vrp* by UAS-RNAi in the dorsal thorax resulted in notum malformations. Healing assays yielded an open wound phenotype by blocking filopodia formation, consistent with its role as an actin regulator. Indeed, this phenotype is indistinguishable of that resulting from interfering on Act42a expression (Phenotypic Class 1).

The TCP1 complex functions as a cytosolic chaperonin for the folding of non-native actins and tubulins, amongst other substrates [135]. Interference with the expression of *TCP1η* or *TCP1ζ,* subunits of the TCP1 chaperonin results in both thorax closure and healing phenotypes, eventually affecting cytoskeleton reorganization. Indeed, the wounded tissue showed no signs of actin accumulation or filopodia formation at the leading edge (Phenotypic Class 1). Interestingly, interference in the expression of any TCP subunits show similar defects in thorax fusion and healing, indicating that wound healing requires the concerted action of all the subunits of the complex, since individual losses cannot be compensated (see further discussion below).

Cpα and Cpβ are regulators of actin filament growth [201]. Capping proteins cap the barbed ends of actin filaments with high affinity preventing the addition or release of actin subunits [292]. Decreased expression of *Cpα* in the *Drosophila* eye imaginal disc [204] or knockdown of *Cpβ* in *Drosophila* cells in culture [187] resulted in actin accumulation. *Cpα* is also required for the proper development of the wing blade primordium and its loss results in cells extrusion and apoptosis [205]. Indeed, the capping protein αβ heterodimer limits Src-induced apoptosis or tissue overgrowth by restricting JNK activation in imaginal discs [293]. Recently, it has also been shown that the knockdown of *Cpα* results in the accumulation of actin and myosin in wing discs. On the other hand, loss of *Cpβ* affects border cell migration reducing the length of actin protrusions and also causes abnormal actin aggregates on the inner side of nurse cell membranes [202]. Downregulation of *Cpα* or *Cpβ* in the dorsal thorax by UAS-RNAi resulted in notum malformation. Healing assays yielded incomplete healing although heterotypic contacts were present and partial peripodial sealing achieved. In the CE, filopodia do not form and epithelial zippering was affected (Phenotypic Class 4). Capping proteins are unstable as individual subunits but together they form a stable functional heterodimer [294]. Remarkably, the downregulation of either each subunit leads to the same phenotype indicating that the heterodimer is required for efficient healing.

Fimbrin is a cytoskeletal protein associated with microfilaments in microvilli, microspikes, stereocilia, membrane ruffles, and cell-substratum attachment sites [229]. It directs the formation of tightly bundled F-actin assemblies [230]. In *Drosophila* it has been shown to participate in female meiosis chromosome

segregation [231]. Downregulation of *fimbrin* by UAS-RNAi in dorsal thorax resulted in notum malformation. Healing assays yielded incomplete healing after 18 hours. The actin cable formation is not affected and PE shows complete sealing, but the CE epithelium fails to complete closure (Phenotypic Class 6).

Loss of functional non-muscle myosin is known to cause defects in dorsal closure affecting cell shape and cell sheet movements [219]. In laser wounded embryos myosin II is immediately recruited to the wound edge and in *sqh1* mutants the wound repair response is severely impaired. Actin accumulation is significantly reduced [119]. Interestingly, downregulation of *sqh* also produce severe wound healing phenotypes in imaginal discs leading to increased accumulation of (disorganised) actin and abnormal cell shapes in the affected area resulting in incomplete sealing of the epithelium. Somehow, the early steps of healing proceed in this condition suggesting that the myosin motor activity just plays a role in the late healing events. Alternatively, an early role for *sqh* may have been overlooked in our experiments as a consequence of a potential partial of efficiency in our interference protocol.

Arc1, the *Drosophila* homologue of mammalian activity regulated cytoskeleton associated protein acts as an immediate stress response gene in the nervous system [232]. Interference in Arc1 expression affects cytoskeletal organization both in *Drosophila* and mammalian cells [233]. Our analysis indicates that the loss of Arc1 results in a failure of tissue relaxation at the end of the healing process in imaginal discs. This suggests that its function may be related to the late suppression of the healing response by contact inhibition.

Finally, the downregulation of *l(1)1Bi* (CG6189) during thorax closure results in actin hyperaccumulation and extreme changes in the shape of leading edge cells that detach from the epithelia. It does affect both cell migration and cells adhesion. Remarkably, its vertebrate homologue, MYBBP1A, has been linked to the control of cell migration in squamous carcinoma cells [295].

- ***Cell matrix attachment***

We have also identified as healing regulators several components of the machinery involved in the cells attachment to the extracellular matrix. *scab* encodes an integrin a-subunit that has been shown to be required for embryonic wound healing [165] and

dorsal closure [63]. We have observed that loss of *scab* affects both thorax fusion and imaginal discs healing, where it appears to act upstream of JNK activity. On the other hand, downregulation of Talin, an essential component of focal adhesions and a cytoskeletal linker that binds integrin and actin, also shows extreme thorax and wound closure defects. Talin appears to be required for almost all integrin functions in *Drosophila*. In its absence, embryonic muscles detach and round up, a hole in the dorsal epidermis of the embryo is produced, and the adult wing develops blisters, all of them landmarks of failures on integrin activities. Also, in vertebrates, Talin modulates wound healing and apoptosis [296]. Remarkably, scab and Talin display distinct healing phenotypes. In the absence of Talin, wound healing does not initiate and there is a remarkable absence of actin cytoskeleton remodelling. Interference in scab leads to milder defects and healing fails at a later stage, when the CE and PE epithelia could not complete closure. These results indicate that the linking of the actin cytoskeleton to pre-existent focal adhesions is essential at early time points during healing, while remodelling of ECM attachments constitutes a much later event in the process.

- ***Inflammatory responses***

A final class of genes functionally relevant for closure and healing is that highly related to the immune and inflammatory response including Tg, Spn55b and scarface.

Tg, is a protein-glutamine gamma-glutamyl transferase previously identified in microarray analysis as an important factor for haemolymph coagulation [297]. Its downregulation during thorax closure produces a phenotype in which several cells at edges round up and actin expression and filopodia are lightly affected in most anterior positions but strongly at the most posterior part of the imaginal fusing borders. Importantly, this seems to be a JNK-independent effect as *puc* expression is not altered. Remarkably, interference on its expression in a larva-healing model does not result in any phenotype [89]. Thus, *tg* seems to be a specific modulator of imaginal closure not be involved in directed cell migration, the primary mechanism of larval wound healing [198]. In vertebrates transglutaminases affect the stability of the extracellular matrix during healing [298, 299] and probably actin organization.

Conversely, Serpins in vertebrates regulate the activity of the serine proteases of the immune system, playing a role in many processes including migration and phagocytosis, limiting tissue damage and unwarranting cell death [300]. In humans, many serpins regulate the functions of proteases involved in the body's response to injury, including roles in coagulation, fibrinolysis, inflammation, wound healing, and tissue repair. Recently it has been described that the SerpinA1 has therapeutic potential as a wound-healing agent [301]. In *Drosophila*, the *serpin55B* was previously identified as a gene related to the immune response [302] and, importantly, is upregulated in the transcriptome profile of regenerating wing discs [273]. In fact, we found that, upon wounding, the downregulation of this gene results in healing defects. In the absence of *serpin55B*, healing progresses almost normally but the distal completion of closure and the final relaxation of the sealed tissue (by downregulation of actin accumulation) are never achieved (Phenotypic Class 7).

scarface codes for a secreted serine protease homologue catalytically inactive. This type of serine protease homologues control blood clotting during healing in mammals [303]. In *Drosophila*, scarface is a target gene of JNK signalling implementing a negative feedback in the pathway and it exhibits phenotypes resembling JNK hyperactivation [216]. Inhibition of scarface expression leads to late healing defects equivalent to those observed in *scab*. Interestingly, *scarface* mutations during dorsal closure alter the basal localization of laminin affecting cells attachments to the extracellular matrix as integrins do. It is then tempting to suggest that a specific functional network implicating JNK signalling, integrins (scab) and scarface regulating ECM attachments is instrumental for the final completion of wound healing in the imaginal discs.

In summary, we have uncovered a whole new set of genes functionally relevant for the control and implementation of wound healing in imaginal discs of *Drosophila* belonging to different ontology categories.

5. The spatial and temporal network of genetic interactions implementing wound healing

We have initially employed the JNK signalling pathway is an initial readout to select those cells engaged in wound repair in imaginal discs. The JNK signalling pathway is selectively activated at the leading edge cells during embryonic, larval, imaginal and adult wound healing and gives an entry point into the analysis of the system. Still, the JNK pathway, being essential for healing, is most probably not the only element channelling the healing response. In fact, we have found that interfering in *Cpα, Cpβ, sqh* and *spn55b* do not affect the expression of *puc* at the leading edge, but result in healing defects of distinct magnitude. Blocking actin polymerization (*Cpα, Cpβ*) or myosin activity (*sqh*) disturb late healing events, whilst preventing the inhibition of serine proteases (*spn55b*) suppress, upon healing, the final tissue relaxation. In all scenarios, JNK signalling activity is untouched. This strongly suggest that these events occur downstream of the JNK cascade or alternatively through independent functions.

Interference in three other genes (*CG12007, scarface and scab*), however, resulted in a strong reduction of *puc* expression in the healing area. Both *scarface* and *scab* expressions are regulated by JNK activity in different processes and both display very similar late phenotypes in imaginal discs healing. Here, in their absence the JNK activity is abolished suggesting the presence of a negative feedback loop. If this is consequence of the loss of cell matrix attachments potentially resulting from scarface or scab absence remains to be determined. The downregulation of JNK signalling consequence of interference in the CG12007 gene stands out from this potential loop. *CG12007* encodes for a Rab geranyl-geranyl-transferase that potentially affects cell shape and cell-cell adhesions. In the absence of *CG12007*, healing arrests at very early stages. The wounded tissue showed no signs of actin accumulation or filopodia formation at the leading edge, a phenotype very reminiscent of that resulting of the absence of JNK signalling [92]. This strongly suggest that CG12007 is involved in JNK signalling activation at very early healing stages. How this activation is achieved or how CG12007 could eventually participate in sensing wound signals, detecting the loss of tissue tension or responding to chemical messages remains to be studied.

MMPs (matrix metalloproteases) belong to a large family of proteases, which have

been shown in different models to be expressed at high levels near wound edges. They help in degrading the basement membrane during healing. Matrix metalloproteinases (MMPs) are extracellular proteases normally involved in matrix removal and highly expressed at wound sites in vertebrates [170]. Knockout mice for MMPs display a mild delay in closing excisional wounds [171]. The *Drosophila* secreted MMP, MMP1, is required in larval epidermis repair, remodelling the basement membrane and actin cytoskeletal reorganization [90]. Interestingly, *mmp1* expression is upregulated by the JNK pathway upon wounding [90]. The secreted mmp1 is also strongly upregulated during imaginal discs healing but surprisingly we were unable to detect any healing defect when interfering in its expression. If this failure is due to the presence of redundant basal membrane degrading factor or is consequence of the inability of the employed RNAi line to interfere in its expression needs to be determined. Surprisingly, we found that MMP1 expression is unaffected in the absence of *CG12007* of *scarface*. Considering that upon interfering in *CG12007* or *scarface* expression, JNK signalling is abolished but MMP1 expression is sustained, points to a mechanism for the regulation of MMP1 expression independent of the activity of this pathway. Alternatively JNK signalling may be redundant for or could only partially influence on the expression of MMP1. Indeed, MMP1 expression in different systems has been shown to depend on distinct signals and mechanisms, ranging from its activation by TGFb or EGF to its dependence on Cdc42 or ERK [90, 99, 304-306]. Extrapolating, the interference in cell to matrix attachments hypothetically resulting from the absence of *scarface*, also appears to be insufficient to alter the late basal membrane remodelling that could potentially be mediated by MMP1.

On the other hand, MMP1 expression is altered in the absence of the capping protein subunits α or β. As described above, capping proteins cap the barbed ends of actin filaments with high affinity preventing the addition or release of actin subunits [203]. Downregulation of *Cpα* or *Cpβ* by UAS-RNAi resulted in incomplete healing. Heterotypic contacts were built but epithelial zippering of the CE was affected. Thus, the proper dynamics of the healing cells actin cytoskeleton seems to be critical for MMP1 expression, and hence proper basal membrane removal at intermediate stages of healing. We do not know how this is implemented.

Integrins are the major cell-matrix receptors and they have been implicated in

mammals in setting the attachments to the provisional wound matrix [307]. They were also found to be strongly upregulated in the epidermis in human chronic wounds and have become of great interests for clinical research [307]. Integrins have both structural and signalling roles, they act as links between the ECM and the actin cytoskeleton and also modulate signalling components, such as members of the Rho family of GTPases, focal adhesion kinase, Src kinase, and the ERK and JNK pathways [308]. *scab* codes for a *Drosophila* integrin α subunit and has been shown to be required for embryonic wound healing [165] and dorsal closure [63]. *scab* loss of function resulted in incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia fail to complete closure. *scab* also shows elevated levels of expression all along the leading edge during healing in imaginal discs. This expression is not affected in the absence of *scarface*. Thus, contrary to basal membrane turnover by MMP1, undermining actin polymerization (*Cpα* or *Cpβ*) at intermediate healing stages does not influence the actual matrix attachments mediated by *scab*. The absence of any effect of *scarface* on *scab* expression is not surprising as its role influencing matrix attachments is most probably conducted through modulating matrix components localization.

Conversely, interference in *CG12007, CG15611*, *verprolin* and *scab* itself (an internal control), resulted in strong Scab expression alterations and suppression in the healing area. Downregulation of *CG12007* resulted in a loss of uniformity in Scab expression, which becomes clumpy. This may endorse the potential role of *CG12007* modulating JNK activity, which itself could participate in directing early Scab expression. Alternatively, *CG12007* could just be affecting scab localization at matrix attachments by an unknown mechanism that could be related to endocytosis (*CG12007* codes for a Rab geranyl-geranyl-transferase). The downregulation of *CG15611* or *verprolin*, however, resulted in the complete loss Scab expression at the leading edge, both on the DP and PM epithelia. Verprolins regulate actin dynamics either by binding directly to actin or to proteins of the WASP family [184]. Interestingly, Verprolins have also been implicated in endocytosis [185]. Interference in *verprolin* yields an early healing phenotype in imaginal discs indistinguishable of that of *Act42a.* This hints at the inhibition of *scab* expression and the early abolition of cell-matrix attachments as a result of the full derangement of the actin cytoskeleton in these conditions. This possibility should be further explored by analysing in detail

matrix attachments at early healing time points in different mutant backgrounds. *CG15611* codes for a Rho guanyl-nucleotide exchange factor, which yields, upon interference, a relatively late healing phenotype. Despite, this late involvement on the healing process, it strongly suppresses scab expression in loss of function conditions. Indeed, its phenotype is indistinguishable from scab. Thus, late rebuilding of cell-matrix attachments may be dependent on the activity of this Rho GEF. As before, this possibility must be substantiated by future analyses. How *CG15611* activity and Scab expression could be linked and the actual make up of matrix-attachments in this condition must be further explored.

Talin is a FERM-domain containing protein that directly links integrin adhesion receptors to actin cytoskeleton. It is an important regulator of integrin function [309]. As expected, Talin in *Drosophila* is required for almost all integrin functions. *Talin* loss of function resulted in an early healing failure with the wounded tissue showing no signs of actin accumulation or filopodia formation. Talin expression is enhanced during imaginal disc healing at the leading edge. This increased expression is unaffected in *CG12007*, *Cpα* or *Cpβ* loss of function conditions indicating that neither early interference in JNK signalling (*CG12007*), nor blocking actin polymerization at healing intermediate time points (*Cpα* and *Cpβ*) seem to affect the early linkage of the actin cytoskeleton to integrins in focal adhesions that apparently is critical at this early stage.

In contrast, Talin expression is modified in the absence of *scarface*, *scab*, *CG15611* or *sqh*. Interference in the expression of all these genes yields equivalent late healing phenotypes. *scarface*, *scab* and *CG15611* appear to be directly or indirectly implicated in the remodelling of cell-matrix attachments at late healing stages. A failure in this remodelling could prevent the novel recruitment of Talin and the establishment of new links to the actin cytoskeleton. The somehow more dramatic effect on Talin expression observed upon interference in *scarface* could be caused by alternative particular functions elicited by this secreted serine protease homologue. Myosin activity (*sqh*) disruption inhibits late healing events leading to increased accumulation of (disorganised) actin and abnormal cell shapes in the affected area provoking the incomplete sealing of the epithelium. As mentioned before the early spreading of the epithelia occurs in the absence of any myosin motor activity and only late events are affected. In this scenario, the derangement of Talin expression

observed at late healing stages in the absence of *sqh* might be related to this late motor function. Actomyosin contractility might be important for the generation of steady structural links to integrins necessary for healing progression.

Interactions between microtubules and the actin cytoskeleton underlie many fundamental cellular processes and are critical for wound healing in multiple models [115, 310-312]. Wounding of *Xenopus* leads to a dramatic reorganization of the microtubule cytoskeleton and inhibition of microtubule polymerization blocks repair [116]. In *Drosophila* embryo wounds, although not significant accumulation or rearrangements of microtubules have been found, the perturbation of the microtubule network has a negative effect on repair [119]. In *Drosophila*, microtubules exert fundamental structural roles but could also participate in the transport of other structural proteins such as actomyosin complexes [313] or signalling molecules such as Rho GTPase activating proteins (GAPs) and exchange factors (GEFs) fulfilling instructive activities [118] [255]. Wild type wounded imaginal discs show reorientation of microtubules towards the wound edges. This reorganization is more prominent in the CE epithelial side while a dense network of microtubules is seen on the PE side of the disc. Tubulin also shows co-localization with actin. A set of healing defective genes belonging to different phenotypic classes were analysed to evaluate the behaviour of the microtubules network as a result of their downregulation. *CG12007, scarface* and *scab* do not affect microtubules organization. The early stages of microtubules rearrangements appear, thus, to be independent of Rab geranyl-geranyl-transferase activity (CG12007), JNK signalling (*puc* expression) or the integrity of cell-matrix attachments (Scab expression). Their late reorientation seems also to be independent of the reorganization of matrix attachments associated to the final steps of epithelial fusion (*scarface* and *scab*).

Otherwise, interference in *Cpα, Cpβ*, *TCP1 ζ* and *TCP1 η* resulted in defects in microtubules organization at the wound edge. At intermediate steps of healing, the proper level of polymerization of actin microfilaments (mediated by *CPα* and *Cpβ*) is necessary for correct microtubule reorganization. In contrast, the TCP1 subunits appear to modulate the microtubule network behaviour at a much earlier time point. The TCP1 complex is an oligomeric particle that consists of eight distinct subunits, which are the products of individual genes (α, β, γ, δ, ε, ζ, η and θ) [125]. It is

required for the production of native actin and tubulin and an undefined set of other proteins. TCP1 is known to play a role during healing in mucosal wounds where mRNA levels of TCP1 ζ, δ, η and ε are significantly increased [137]. Both, TCP1 ζ and *TCP1* η were upregulated in direct comparisons in healing-engaged cells in wing imaginal discs. Healing assays upon interference in both cases yielded an open wound phenotype. Healing was blocked early and the wounded tissue showed no signs of actin accumulation or filopodia formation at the leading edge. In these conditions, the early organization of the microtubule network was disrupted. This is probably due to a direct role of the TCP1 complex assuring the correct folding of tubulin, most likely under a strong demand, as soon as the tissue initiates the healing process. However, an indirect effect on microtubules organization via the maintenance of an appropriate actin cytoskeleton (consequence of the actin folding capabilities of the TCP1 complex) cannot be discarded. See a detailed discussion on the TCP1 complex in the next section.

6. The TCP1 Complex

As just described, TCP1 is a cytosolic chaperone complex of about 900 kDa ubiquitously expressed in all tissues examined [314]. Each of its eight subunits is coded by individual highly diverged genes [315]. Apart from their role as a chaperone, TCP1 also assists the synthesis of some of its substrates [316]. Although many studies have been done on TCP1 much is still unknown about the mechanisms it employs to fold its substrates, which are its substrates, although it is well established that it participate in the folding of actin and tubulin monomers, and which could be its ultimate cellular role(s). Clinically, it has been linked to colorectal cancer progression and to prevention of polyglutamate-containing proteins (Poly Q) aggregation, the main cause of polyglutamate toxicity in neurodegenerative diseases like Huntington [317, 318].

Remarkably, two subunits of the complex TCP1 ζ and *TCP1* η are upregulated in *puc*-expressing healing-engaged cells in our transcriptomic analysis. Further, we have found that interfering in their expression lead to very similar strong thorax fusion defects and to an early arrest of healing in imaginal discs. This arrest is accompanied by a failure to organize actin microfilaments and generate filopodia at the wound

edges and by strong defects in the organization of the microtubular network. The TCP1 subunits healing phenotype is very similar to that observed upon interference in *Act42a* or the actin dynamics regulator *verprolin*. This suggests than one of the major roles the TCP1 complex could have during healing in discs could be to facilitate the folding of new actin monomers necessary to fuel the new demands of a severely compromised tissue that must actively and rapidly rebuild its cytoskeleton. The pre-wounding levels of TCP1 ζ and *TCP1* η may be insufficient to coop with this requirement and must be hyperexpressed accordingly. Providing sufficient level of folding capacity would complement the observed upregulation of actin and verprolin, which also seem to be insufficiently expressed in resting discs. The remainder of the subunits of the TCP1 complex, apparently, do not need to be upregulated and seem to be present at sufficient levels to coop with the exceptional requests of the healing process. This mechanism constitutes a new way to control actin dynamics parallel to those implemented by the conventional array of actin regulators.

It has been shown that the different subunits of the TCP1 complex carry some sort of specificity regarding the target polypeptide to be recruited for folding [319]. Remarkably, interference in the expression of any (most) of the subunits of the complex lead to thorax closure defects indicating that affecting the integrity of the complex by any mean readily affect its function: i.e. all the subunits must be expressed at sufficient levels (and cannot be compensated by others) to be able to modulate the TCP1 folding activity. This supports a scenario in which the systemic activity of the complex is necessary to fulfil its function during fusion and healing and claims against a potential specificity in terms of the target polypeptide to fold. This last argument further points to actin (and may be tubulin) as the main substrate(s) to be targeted by the TCP1 complex during imaginal discs fusion or healing.

The TCP1 complex in *Drosophila* thus appears to share its major substrates with other organisms [320]. We have substantiated this assertion further by noticing, employing Drosophila S2R+ cells, that in culture interfering with dsRNAs in the expression of at least two subunits of the complex, X and Y, results in strong alterations of their morphology and their actin and tubulin organization and dynamics. Moreover, cells deficient for TCP1 subunits show a drastic increase of non-native G-actin and are unable to recover their natural levels of polymerized F-actin upon a pulse of Latrunculin A.

Last, we found that the upregulation of certain subunits of the TCP1 complex, eta in particular, is shared in the early response of murine and human epidermis to experimental wounds. Importantly, the knockdown of its human homologue by siRNAi impairs wound repair in scratch assays. Thus, the critical function we uncovered for the TCP1 complex facilitating wound healing in *Drosophila* appears to be conserved between invertebrates and vertebrates.

7. A comparison to vertebrates healing

A variety of different microarray analyses of murine and humans have identified characteristic profiles of gene expression at the wound site [321, 322]. However, very few functional analysis addressing the role of genes potentially involved in healing regulation have been performed. Comparative studies between vertebrates and invertebrates should be instrumental for the identification of conserved genes relevant for wound healing across species.

We have performed comparative bioinformatic analysis of our datasets to data obtained from different transcriptomic profiles obtained to the early phase of healing in humanized skin [323] and mouse. Those genes that change their level of expression during healing in both humans and mouse were used to search for *Drosophila* homologues using different tools [168]. A final short list of 93 genes, that seems to be conserved through phylogeny (41 upregulated and 52 downregulated), appear modified in all the systems and in the same manner (Supplementary Table 7). In this final signature we found, amongst others, genes involved in cytoskeleton regulation (like ACTC1, CCT6A, ARPC1B or TWF1), extracellular matrix remodeling proteins (like MMP10, MMP3 or TIMP) [90, 279], transcription factors (like MYBBP1A, SUB1 or ELK3) and different members of the JAK/STAT signaling pathway (like STAT3 or IL4R) [281, 324]. The 41 human homologues of these 41 upregulated genes were analysed in scratch assays in human cells. These analyses identified 26 genes in whose absence healing is impaired.

On top of previously described tests performed on the imaginal fusion model [168], we functionally analysed a subset of these transcriptionally conserved genes on healing in imaginal discs. From those surveyed genes (10), we found that interference in l(1)1Bi (No Discs), reptin (Class 1), Act42A (Class 1), TCP1 ζ (Class 1), sqh

(Class 5), scab (Class 5), aats-Trp (Class 7) and spn6 (Class 7) and lead to different types of defects. Timp (TIMP1 in humans) and Nopp140 (NOLC in humans) do not yield any phenotype. All these genes with the exception of l(1)1Bi have been thoroughly described in previous sections

- l(1)1Bi codes for the *Drosophila* homologue of the transcriptional regulators MYB binding protein (P160) 1a (MYBBP1A). MYBBP1A coordinates ribosomal DNA transcription and rRNA preprocessing and is required for P53 activation [325]. *MYBBP1A* is essential for early mouse development prior to blastocyst formation and in its absence the expression of key cell cycle regulators is altered and apoptosis is induced [295]. We were unable to assess the ability of imaginal discs to heal upon interference in l(1)1Bi in an En-Gal4 background as it results in a failure in the development of imaginal discs. Still, in thorax closure assays with Pnr-Gal4 its absence leads to strong clefts at the dorsal midline suggesting a role in cell migration.
- Reptin is a DNA helicase that is a component of the PRC1 Polycomb complex. The human reptin homologue RUVBL2 is also involved in chromatin remodelling, functionally interacting with c-myc and the wnt signalling pathways [326]. Interestingly, Reptin is methylated in hypoxic conditions. Methylated Reptin binds to the promoters of a subset of hypoxia-responsive genes and negatively regulates transcription modulating cellular responses to hypoxia [327]. As described before interference in reptin results in an early arrest of the healing process in imaginal discs. How chromatin silencing, proliferation arrest or response to hypoxia mediated by *reptin* could impinge in wound healing is an open question.
- Act42A (ACTC1 - cardiac alpha actin 1 in humans) is essential for the earliest steps of the healing process in imaginal discs. Loss of ACTC1 affects heart development as a consequence of the disorganization of actomyosin filaments and has been implicated in congenital heart disease [328]. Its role in healing, despite its strong upregulation in mice and human transcriptomic analysis, is unknown, although it may play an equivalent role to that taken by Act42A in flies, to facilitate the rapid remodelling of the actin cytoskeleton.
- TCP1ζ (TCP1 subunit 6A in humans), as Act42A, acts at early stages of healing

facilitating actin (and probably tubulin) folding. Importantly, the TCP1 complex in mice has been found to be a potent regulator of wound healing [137, 329].

- *spaghetti squash (sqh)* encodes for a myosin light chain regulatory subunit (MYLPF in humans). In vertebrates epithelial wounds heal by purse-string contraction of an actomyosin ring that is regulated by the myosin light chain kinase (MLCK) and rho kinase (ROCK) [330]. Immediately upon wounding, actin and myosin II regulatory light chain accumulated at two locations: (1) in a ring adjacent to the tight junctions that circumscribed the wound, which serve as attachment points and (2) in fibers at the base of the cell in membranes extending over the wound site [114]. Later on, polarization and cell migration proceed. ROCK activity is required for assembly of the myosin ring, and myosin II activity is required for contraction but not for basal membrane extension [114]. Interestigly, during the early acute-phase of myosin II activation, pharmacological approaches reveal p38-MAP kinase (an stress responding kinase highly related to JNK) as having critical roles in myosin II phosphorylation driving cytoskeletal assembly [331]. In flies, *sqh* participates in the wound response of embryos, larvae and imaginal discs regulating the formation of actomyosin rings [85, 119]. Downregulation of *sqh* produce severe wound healing phenotypes in imaginal discs leading to increased accumulation of (disorganised) actin and abnormal cell shapes in the affected area provoking the incomplete sealing of the epithelium. As detailed before, the early steps of healing are unaffected in this condition. Detailed genetic analysis of the potential Myosin II regulators (MLCK and ROCK) in the imaginal discs healing model are missing and the precise spatiotemporal regime of myosin motor activity and function remains to be clarified.
- *scab* codes for an integrin heterodimeric transmembrane receptor α-subunit (ITGA5 in humans) [212]. Through their cytoplasmic domains, integrins interact with cytoskeletal elements and cause changes in cell behaviour in response to ligand binding. Integrins regulate multiple developmental and physiological processes. ITGA5 Zebrafish mutants develop cataracts, characterized by defects in fiber adhesion, elongation, and packing [332]. Cell motion analysis also showed that cell migration in posterior cranial placodes in ITGA5 morphants becomes highly erratic and unfocused, impairing

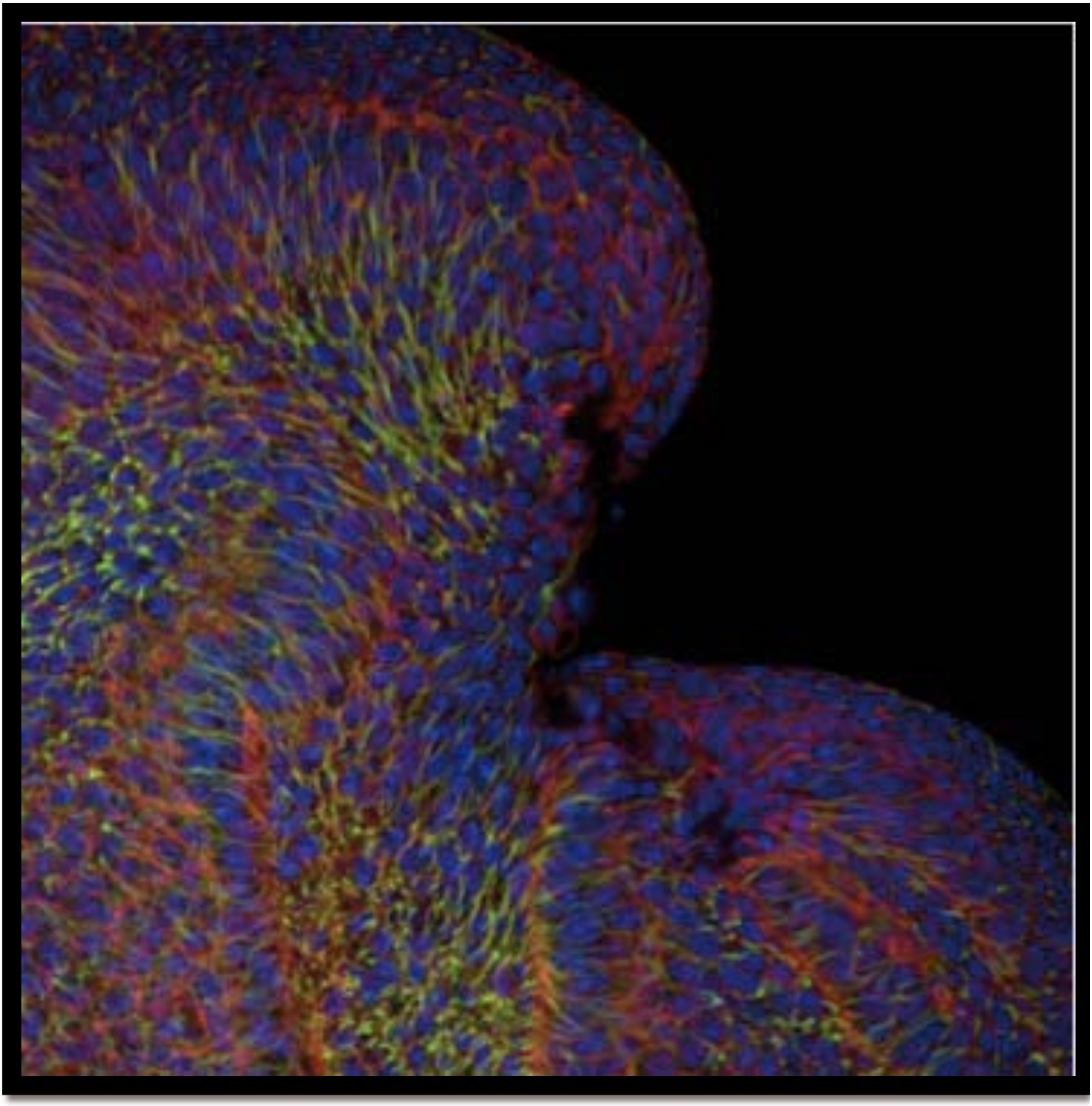
convergence [333]. ITGA5 also serves as a key mediator of Src and ErbB2-survival signalling by blocking anoikis acting as a potential metastasis suppressor [334]. Last, although integrins are best known for mediating cell adhesion and migration, integrins in wound epidermis also control cell survival, proliferation, matrix remodelling, and paracrine crosstalk to other cellular compartments of the wound [335]. ITGA5, amongst other integrin subunits, is expressed *de novo* in the epidermis in healing wounds in mammals binding to ligands in the provisional wound matrix [336]. The *Drosophila* gene scab is transcribed in tissues undergoing morphogenetic movements [211] and in its absence, dorsal closure fails [213]. Downregulation of scab in healing assays yielded incomplete healing which halts after 12 hours of culture. Both, CE and PE epithelia fail to complete closure. As previously discussed, these defects could be adscribed to a failure to generate adequate cell-matrix attachments at late stages during the healing process. Indeed, scarface loss of function conditions result in similar phenotypes supporting this possibility. However, the early partial disruption of Scab expression in *CG12007* interference conditions we observed points to potential additional roles. If these involve inside-out or outside-in signalling or paracrine crosstalks remains to be analysed.

- Aats-Trp codes for the tryptophanyl-tRNA synthetase (WARS in humans) with a direct role in translational regulation. Interestingly, WARS appears to carry over acquired additional functions, including cytokine activities in inflammatory and angiogenic signalling [337]. Recently, spliced or N-truncated forms of WARS have been found to hold angiostatic activity in vivo in chick embryos, and in the neonatal and adult mouse. Furthermore, the human Aats-Trp homologue is induced by cytokines and mediates the responses to the mechanical force of shear stress, shear stress-induced gene expression and cell alignment in endothelial cells [338]. These effects are linked to its ability to complex to VE-cadherin and VEGFR2, which promote cell survival signals. Interference in Aats-Trp expression during thorax closure leads to pupal lethality and during healing it blocks the late cytoskeleton relaxation restoring the shape of the tissue upon closure. Would this phenotype be related to a potential inhibitory role equivalent to the anti-angiogenic activities of truncated forms of its vertebrate homologues? Indeed, we detected that Aats-Trp shows

alternative splicing and ESTs lacking the coding domains for the N-terminal end of the protein have been annotated. However, considering that Aats-Trp appears to affect dendrite pruning in the embryonic *Drosophila* CNS, a phenomena structurally related to angiogenic growth indirectly suggest that this mechanism could be plausible. Functional tests will be necessary to clarify this possibility.

- The gene Serpin 55B codes for a serine-type endopeptidase inhibitor (SERPINB1 in humans). SERPINB1 is an inhibitor of proteases, including elastase (NE) and cathepsin G (cat G) found at high levels in the cytoplasm of neutrophils. Recent studies suggest that SERPINB1 could provide protection in the airways by regulating excess protease activity associated with inflammatory lung disorders [339]. Further, overexpression of SERPINB1 effectively suppresses the invasiveness and motility of malignant cancer cells [340], although its ectopic expression promotes cell motility [341]. Remarkably, serpins have been implicated in affecting tissue fibrosis as a consequence of the accumulation of excessive levels of collagen in the ECM. Collagen accumulation depends on two factors: an increased rate of synthesis and/or a decreased rate of degradation by cellular proteolytic activities mediated by urokinase/tissue type plasminogen activator (uPA/tPA) and plasmin. The activities of uPA/tPA/plasmin and plasmin-dependent MMPs rely mostly on the activity of the plasminogen activator inhibitor-1 (PAI-1), a canonical serpin. Under normal physiologic conditions, PAI-1 maintains the tissue homeostasis. During healing, elevated levels of PAI-1 inhibit uPA/tPA/plasmin and, thus, help expedite wound healing. Under pathologic conditions, high levels of PAI-1 contribute to excessive accumulation of collagen in the wound area, and thus preserves scarring. Lack of PAI-1 protects different organs from fibrosis in response to injury-related profibrotic signals [342]. In this scenario, interference in the expression of Spn55B, which is upregulated in healing-engaged cells, causes a failure of imaginal discs fusion and a thorax cleft and to late defects in the healing of imaginal discs. The distal completion of closure and tissue relaxation of the sealed area (downregulation of actin accumulation) was not achieved. It will be interesting to explore if this late failure could be related to deregulation of collagen levels and the proper turnover of the ECM.

Considering the low genetic redundancy of *Drosophila* and the high degree of conservation of fundamental cellular functions and signalling pathways when matching to vertebrates, data obtained from comparative analyses using *Drosophila* must facilitate targeting those genes of functional interest in wound healing studies. There is promise that our newly identified wound-healing genes will guide further work for researchers interested in the more complex processes of mammalian tissue repair. The limited survey of those genes showing equivalent transcriptional responses during healing across phyla may help to select potential candidates deserving deeper analysis in vertebrate models. These candidates could become clinically relevant in the development of better therapies.



CONCLUSIONS

1. *In vitro* healing requires the activation of JNK signaling leading to the formation of transient contacts between the epithelia.

2. A genome wide transcriptional profiling has been performed to identify new genes transcriptionally modulated in imaginal disc wound healing.

3. Functional analysis by RNAi and overexpression has allowed identifying new genes required for imaginal disc fusion and healing.

4. Phenotypic grouping of healing defective genes uncover the different stages leading to the proper healing of imaginal discs.

5. Actin-related genes and signaling molecules form a major class of healing regulators.

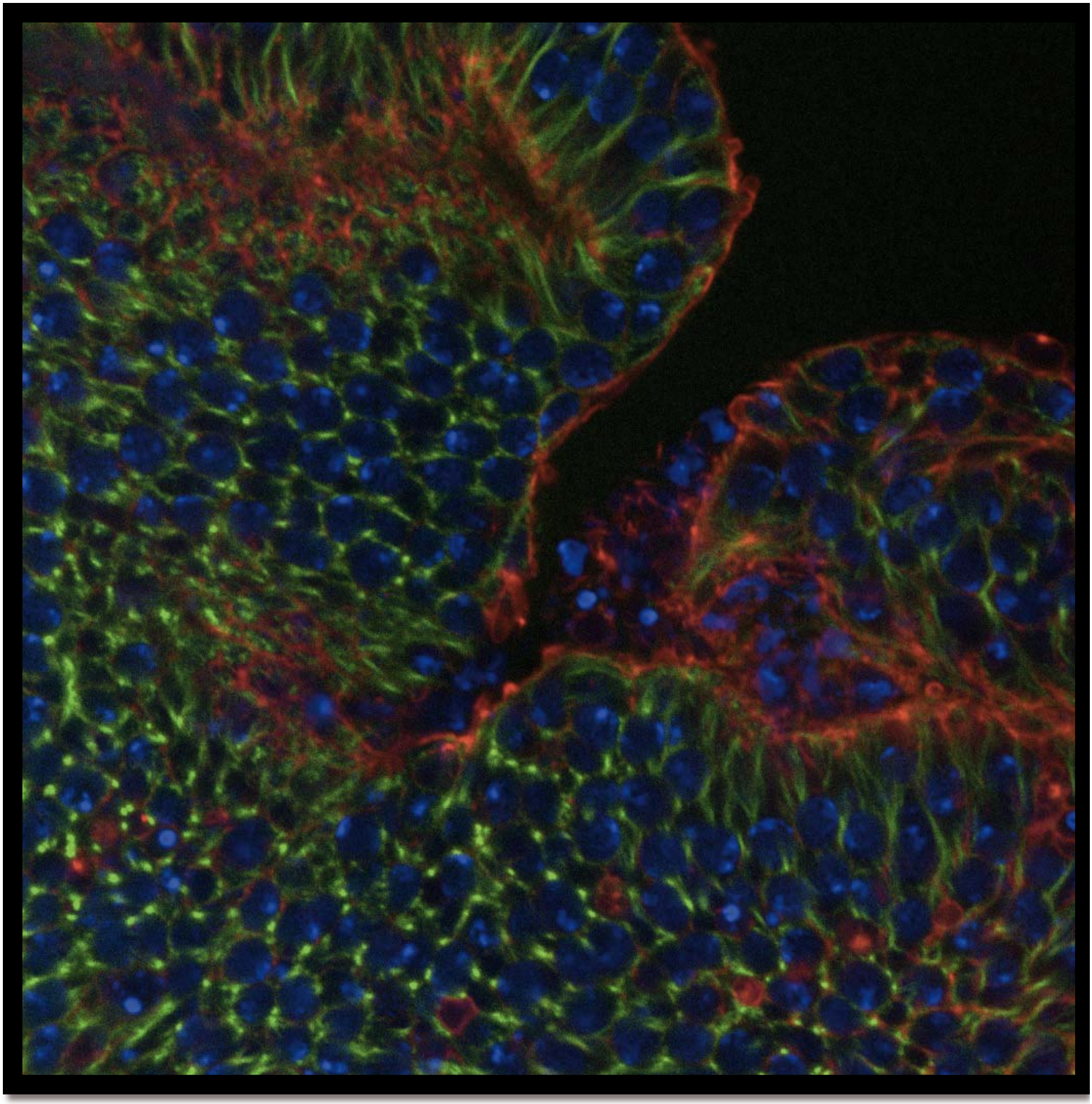
6. The functional characterization of a subset of genes transcriptionally activated during healing analyzing the expression of key markers has let to uncover their spatial and temporal coordination.

7. The TCP1 chaperone complex function is essential for wound healing.

8. Interference in the expression of TCP1 subunits affects imaginal discs fusion, healing, and actin and tubulin dynamics

9. Comparative studies between vertebrate and *Drosophila* healing transcriptomes identifies genes conserved through phylogeny.

10. Functional analysis of conserved genes between Drosophila and vertebrates identifies conserved functions and points to potential candidates for translational studies.



RESUMEN EN ESPAÑOL

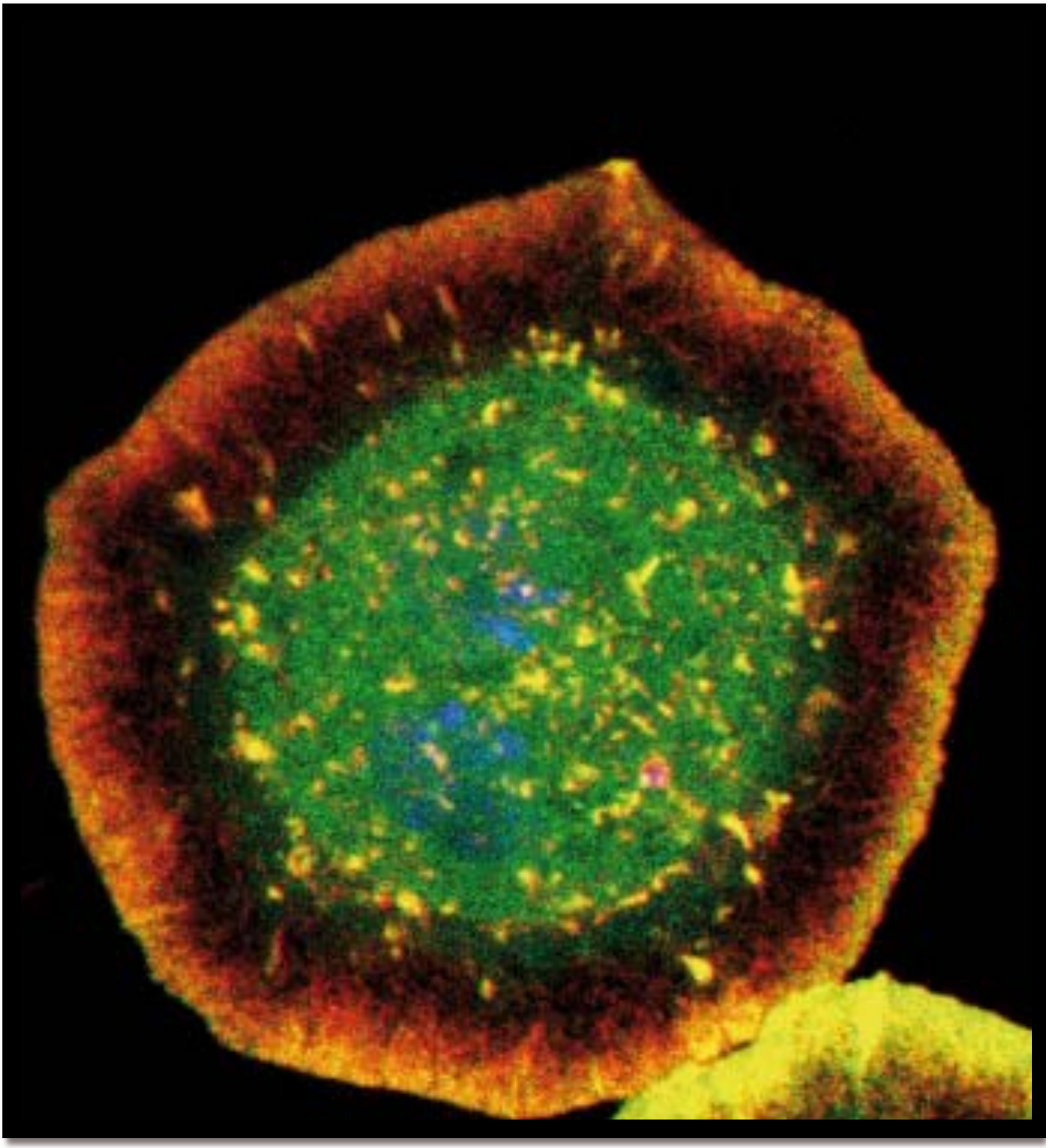
La ciencia básica ha de engarzarse con las necesidades sociales. Así, nuestro laboratorio esta involucrado en identificar genes específicamente modulados durante el proceso de cicatrización mediante estudios transcriptómicos en colaboración con diversos grupos. En esta línea, en esta Tesis se han perseguido una serie de objetivos concretos: 1) Realización de un análisis funcional de nuevos genes implicados en cicatrización (discos imaginales). 2) Comparación de los genes identificados en cicatrización en *Drosophila* con genes identificados en modelos de cicatrización en vertebrados. Más en detalle:

- Estudio y descripción de la cicatrización en discos imaginales de *Drosophila in vitro*.
- Identificación de genes cuya expresión cambia en las células activas en el proceso de cicatrización.
- Caracterización fenotípica de genes que afectan al proceso de cicatrización (definición de categorías fenotípicas)
- Caracterización detallada de un subset de genes involucrados en la regulación del proceso de cicatrización
- Identificación de reguladores de cicatrización conservados en la filogenia.
- Análisis del papel del citoesqueleto de actina en el proceso de cicatrización (el papel del complejo TCP1 (una chaperonina esencial)
- Exploración del papel del complejo TCP1 en la regulación del citoesqueleto de actina y tubulina

A lo largo de este trabajo hemos alcanzado las siguientes conclusiones:

11. La cicatrización *in vitro* requiere la actividad de la cascada de señalización de la JNK que controla la formación de contactos transitorios entre los epitelios
12. Han sido identificados una serie de nuevos genes modulados transcripcionalmente durante el proceso de cicatrización.
13. El análisis por interferencia o sobreexpresión de estos genes ha permitido identificar aquellos funcionalmente relevantes en la fusión de los discos imaginales y en la reparación del tejido imaginal.
14. El agrupamiento fenotípico de estos genes ha permitido definir a nivel celular las etapas requeridas para la reparación precisa de los discos imaginales.

15. Las clases principales de genes involucrados en el proceso de cicatrización son las de los reguladores del citoesqueleto de actina y transductores de señales.
16. La caracterización funcional de un subset de genes involucrados en cicatrización analizando marcadores específicos ha permitido definir la red de interacciones entre ellos y su coordinación espaciotemporal.
17. El complejo TCP1 es esencial para la cicatrización de los discos imaginales.
18. La interferencia en la expresión de distintas subunidades del complejo TCP1 demuestra su papel en la fusión de discos imaginales, cicatrización y control del citoesqueleto (actina y tubulina) en *Drosophila*.
19. Estudios comparativos entre *Drosophila* y vertebrados a nivel transcriptómico ha permitido identificar una serie de genes conservados entre distintos phila y regulados a nivel transcripcional durante la cicatrización.
20. Análisis funcionales de estos genes demuestran un papel conservado a lo largo de la filogenia apuntando a candidatos potenciales para estudios translacionales.



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ANNEX

Ontology analysis of Healing genes

Initially, to obtain a global genomic description of the process we compared the expression level for each *Drosophila* gene in cells involved in healing (*puc*-GFP) versus neighboring cells not involved in healing (non *puc*-GFP) in wounded discs by one to one comparisons. Further, dual comparisons let to define two relevant subpopulations: genes modified in *puc*-GFP cells in wounded discs and unwounded disc but with differential expression in between both (*dw1_dnw1_dd1*) and genes only modified in *puc*-GFP cells of wounded discs (*dw1_dnw0_dd1*). Modified genes that belong to the *dw1_dnw0_dd1* subsets will be specific to wound healing but not for discs eversion and fusion. Those genes included in the *dw1_dnw1_dd1* subsets must be important for both. A hypergeometric test (GOStats) was performed to assess GO terms overrepresentation relative to aleatory assignation in our selected categories (*dw1_dnw0_dd1* and *dw1_dnw1_dd1*). GO terms and relationships for *Drosophila* were downloaded (gene_ontology_edit.obo, release 1.2 and gene_association.fb, release 1.94) from the GO web site. Different gene sets annotations were done at level 3 of the 'molecular function' ontology tree. We climbed up in the ontology to obtain the corresponding parent term at level 3 for those genes that were annotated at deeper levels in the hierarchical tree. A statistical analysis was performed to test the probability of observing a given GO term significantly enriched in genes belonging to such clusters using a hypergeometric distribution. Functional annotations lead to classify the genes with differential expression pattern during healing according to their GO terms. Indeed, specific GO terms are differentially overrepresented or underrepresented in the individual subpopulations (*dw1_dnw0_dd1* or *dw1_dnw1_dd1*), while others are relevant in both groups.

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