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Dissection of the Regulatory Elements of the Complex Expression Pattern of Puckered, a Dual-Specificity JNK Phosphatase

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ABSTRACT

For developmental processes we know most of the gene networks controlling specific cell responses. We still have to determine how these networks cooperate and how signals become integrated. The JNK pathway is one of the key elements modulating cellular responses during development. Yet, we still know little on how the core components of the pathway interact with additional regulators or how this network modulates cellular responses in the whole organism in homeostasis or during tissue morphogenesis. We have performed a promoter analysis searching for potential regulatory sequences of *puc* and identified different specific enhancers directing gene expression in different tissues and at different developmental times. Remarkably, some of these domains respond to the JNK activity, but not all. Altogether, these analyses show that *puc* expression regulation is very complex and that JNK activities participate in non-previously known processes during the development of *Drosophila*.

KEYWORDS

JNK, Drosophila, dual specificity phosphatase, gene expression

INTRODUCTION

Developmental biology focuses in the analysis of the events occurring during the development of the organism. It aims understanding the mechanisms directing how, from a single cell, pluricellular organisms, with their extraordinary variety of shape and forms, organs and tissues, are built. Nowadays, along complex morphogenetic processes.

Signaling pathways are key for morphogenesis as they transduce extracellular information regulating cell proliferation, determining developmental axis, specifying compartment domains, or modulating cell shape or apoptosis. Remarkably, the number of these pathways is finite. A few signaling modules are required once and again in various and divergent processes. To understand how the same transducing cascades could regulate so many different events is fundamental to gain knowledge in how their activity is controlled and how they control their target genes. The recent literature has led to new emerging ideas, considering these pathways more a subject of threshold events and regulatory feedback loops than of on/off signaling switches. It has thus become crucial to understand when, where and how these pathways are activated, for how long this activation lasts, which is their level of activity and how is this

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dynamically controlled in time and space.

JNK is an acronym for the cJun-N terminal kinase. The JNK belongs to the family of MAPKs (Mitogen Activated Protein Kinases) like ERK (Extracellular Signal Regulated Kinase) and p38 Kinase (1). Originally, the JNK signaling pathway was identified as a stress-response kinase cascade controlling cell survival and proliferation (2). It is highly conserved throughout evolution and homologues for JNK have been identified in mouse, humans, Zebrafish, *C. elegans* and *Drosophila* (3). The JNK pathway is essential for the amplification of cellular responses to growth factors and cytokines, controls cell shape, proliferation, adhesion and death; and regulates a plethora of different fundamental process like stress, innate immune response and apoptosis (4). During development, signaling mechanisms regulated by JNK have been shown to modulate epithelial fusion (5) and the cohesion of border cell clusters during migration (6) in *Drosophila*, as well as multiple morphogenetic processes and wound healing in vertebrates (7, 8). It has also a role in many different pathological conditions such as Atherosclerosis, Parkinson or Alzheimer disease (9, 10).

The JNK signaling responds to many different stimuli of physical and chemical nature; e.g., heat, oxidative and osmotic stress cytokines; growth factors (11, 12) or insulin (13). However, the information on the stimuli and receptors involved in activating JNK is far from being saturated, since they vary largely depending on the physiological context and the cell type. It would be extremely important to define the interaction between the core components of the pathway and additional regulators to understand how this network adequately modulates cellular responses in culture but also in the whole organism in homeostasis or during tissue morphogenesis.

In *Drosophila* the core of the cascade consists of the homologues of JNKK and JNK, which are encoded by the genes *hemipterous* (*hep*) (14) and *basket* (*bsk*) (15). Mutant analysis has revealed that the entire JNK cascade participates in the completion of dorsal closure (DC) in embryonic stages. Mutants for *hep*, *bsk* or *kayak* (*kay*) (Fos in *Drosophila*) display embryonic dorsal holes (16). When JNK signaling is defective, the leading-edge cells of the embryonic lateral epidermis slightly elongate but quickly afterwards revert to polygonal shapes. They fail to accumulate actin and myosin at the dorsal most edges and as a consequence they do not extend filopodia and the actin cable inherently built at the edge of the epithelia is not assembled (17). A restricted activation of the JNK cascade is required for the differentiation of the leading-edge cells during DC, where the JNK signaling pathway controls the expression of the gene *puckered* (*puc*). *puc* is an immediate early gene responding to JNK activity, that encodes

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a dual specificity MAPK phosphatase that acts to down-regulate DJNK/Bsk activity through dephosphorylation (18). The expression of Puckered in the leading-edge cells results in the formation a negative regulatory feedback loop of DJNK/Bsk activity (19). In *puc* mutants, the activity of JNK becomes upregulated, LE cells accumulate higher levels of actin and myosin and they undergo excessive progression resulting in puckering of the epidermis at the dorsal midline (17).

We previously performed a promoter analysis searching for potential regulatory sequences of *puc* and identified an enhancer specific for border cell expression during *Drosophila* oogenesis (6). Here we present a general overview of the results of this screening. Besides identifying the role of the JNK pathway in the collective-cell movements of border cells, we isolated multiple regulatory domains in *puc* directing gene expression in different tissues and at different developmental times. Remarkably, some of these domains respond to the JNK activity, but not all. Altogether, these analyses paint a complex scenario in which *puc* and the JNK activities seem to be required in many non-previously anticipated events during the development of *Drosophila*.

RESULTS

puc is expressed in multiple tissues: 1) a population of lateral cells (the leading edge) that delineates the boundary between the ectoderm and the amnioserosa during dorsal closure; 2) the amnioserosa itself (transiently); 3) the peripheral embryonic nervous system; 4) discrete neurons and neuronal precursors in the embryonic central nervous system (CNS); 5) epidermis and spiracles in the third instar larva; 6) specific cell populations in all thoracic imaginal discs (the proximal part of the wing, haltere and leg discs in the stalk where imaginal discs connect to the larval epidermis and in the peripodial epithelium). Later during prepupariation, *puc* expression is maintained in the peripodial membrane and marks the presumptive suture sites of imaginal discs with their neighbors; 7) all photoreceptor precursors (very weak); 8) sensory organs precursors in wing, leg and haltere imaginal discs (weak); 9) larval muscles (very strong expression); 10) different types of follicular cells and the border cells in egg chambers; 11) embryonic and larval hemocytes; 12) induced at the edge of wounds in epithelial cells in embryos, larval epidermis and imaginal discs; 13) epithelial regions surrounding necrotic areas. The expression of *puc* in these tissues has been in most cases identified as a result of studying different enhancer trap P elements inserted in or around the gene. The low level of expression

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of *puc* or the high instability of its transcripts has prevented direct observation of its expression and only has been reported at the epidermal leading edge in embryos during dorsal closure and in follicle cells (20) (18) (21) (22) (23).

In most locations, the expression of *puc* depends on the activity of the JNK signaling cascade and upstream regulators. However, in discrete events, *puc* expression is observed in the absence of JNK activity, in particular in necrotic cells in dorsal open embryos in *hep* (JNKK) and *bsk* (JNK) mutant conditions (E. Martin-Blanco, unpublished results). ln order to identify the regulatory elements involved in controlling the expression of *puc*, we developed different approaches. We analyzed the ability of different genomic fragments in and around the gene to drive the expression of a reporter. Further, we performed an evolutionary analysis *in silico* and identified highly conserved regions that we also tested as potential regulatory sequences (RS).

puc genomic (PG) domain analysis

puc cytologically maps on the right arm of the third chromosome (84E12-84E13) and its transcription unit comprises four exons. The P element insertions isolated so far are all inserted in different introns and exhibit different patterns of expression (**Figure 1**). This suggests that the intronic regions of *puc* can contain distinct enhancers regulating differential subsets of *puc* expression. The different P element insertions in homozygosis also show different phenotypes. Thus, the B48 allele inserted in the first intron reproduces a different pattern of expression in the follicle cell and the embryo during dorsal closure than the A251 insertion that is localized in the third intron (18).

We have subdivided the *puc* genomic region in 5 domains [Puckered Genomic Regions 1 to 5 (PG1 to PG5)]. These domains covered upstream sequences and the 5' untranslated domain (PG1); the first and second intron (PG2); the 5' half of the third intron (PG3); the 3' half of the third intron (PG4); and the 3' untranslated domain and downstream sequences (PG5). The candidate regulatory domains (PG1 to PG5) were cloned in the PPTGal4 vector and transgenic lines were created to study their regulatory effects *in vivo* (**Figure 1**).

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Figure 1

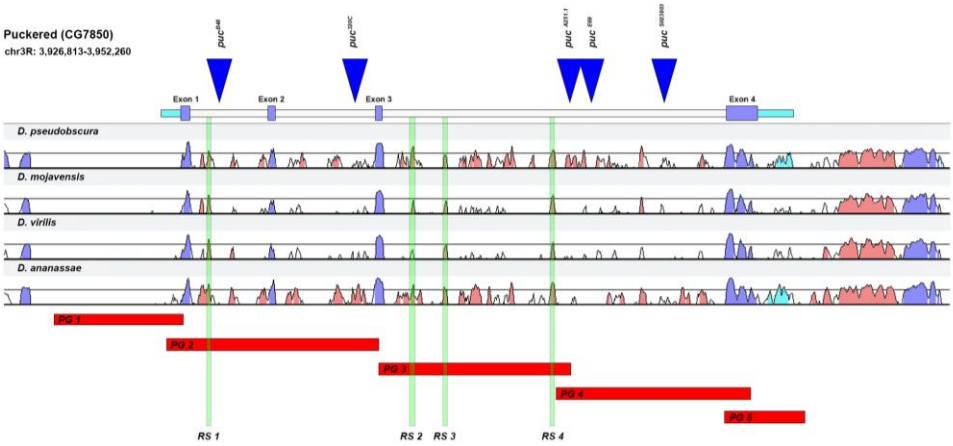

Figure 1: *puckered* genomic organization

The area expanding the gene *puckered* (*puc*) expands around 26 Kilobases. It includes 4 Exons (purple) that are conserved across all *Drosophilae*. Several P element insertions have been identified within the gene, and some of them, with known expression patterns (enhancer traps) or phenotypically characterized, have been mapped (blue triangles). We have subdivided the *puc* genomic region in 5 domains [red boxes - *puckered* Genomic Regions 1 to 5 (PG1 to PG5)]. These domains covered upstream sequences and the 5' untranslated domain (PG1); the first and second intron (PG2); the 5' half of the third intron (PG3); the 3' half of the third intron (PG4); and the 3' untranslated domain and downstream sequences (PG5). Four hyper conserved motifs (Regulatory Sequences (RS) - green vertical bars) in *puc* introns were identified in *D. mojavensis*, *D. virilis* and *D. ananassae*, the most divergent species from *D. melanogaster* sequenced so far. RS1 resides within the PG2 domain and RS2-4 map in the PG3 region.

Identification of *puc* regulatory regions *in silico*

To extend and refine our analysis of *puc* regulatory domains, we decided to make use of available bioinformatic tools to search for possible regulatory regions. The bioinformatic search was restricted to the intronic regions of *puc*. First, a comparative analysis to identify conserved regions was done using the VISTA Browser tool (UCSC). The comparison was performed with three species chosen based on the phylogenetic distance with *D. melanogaster (D. pseudobscura*, *D. virilis* and *D. mojavensis)* (**Figure 1**).

The four most conserved regions within the introns (RS1 in the domain covered by PG2, and RS2 to RS4 within the PG3) were scanned with the Position-Weight Matrices (PWM) of the TRANSFAC database in order to associate them to known transcription factors (Match tool).

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PM2 was the only conserved region that got a statistically accepted score. The matrices that theoretically bind this region are those involving c-Jun, Creb and CRE-BP1/c-Jun heterodimer. The rest of the conserved regions, did not get any matrix associated. Yet, these sequences may keep important functional roles considering their high level of conservation throughout the *Drosophila* genus and we decided to analyze them further. The candidate regulatory sequences (RS1 to RS4) were cloned as for the PG lines in the PPTGal4 vector and transgenic lines were built (**Figure 1**).

Analysis of lines carrying *puc* regions *in vivo*

To test the different Gal4 transgenic lines generated (PG1 – PG5 and RS1 – RS4), they were crossed with a multimerized UAS-GFP stock (24). Following the GFP expression during fly development allowed to determine if these lines reproduced partially or completely the expression pattern of *puc*. For each of the available Gal4 lines, expression was evaluated in embryonic tissues, in first instar larval brains and in third instar full larvae.

Upstream Sequences (PG1)

The region enclosing the 4 Kb 5' of the *puc* transcription start site plus its 5' untranslated domain do not seem to carry relevant transcriptional regulatory elements for embryonic development. None of the different insertions of the PG1 construct analyzed show detectable expression neither in embryonic tissues nor in first instar larva brains (beyond faint signal in the salivary glands and in the posterior spiracles) (**Figure 2A**). Yet, GFP expression could be detected in immunostained flat preparation of first instar larvae in neurons of the brain lobes and the thoracic ganglion (**Figure 2B**). This expression does not sustain at later stages and no signal was detected in third instar larval brains. The third instar larva shows strong expression on the salivary glands and on iterated group of cells corresponding to those involved in secreting the larval cuticle denticles (**Figure 2C**).

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Figure 2

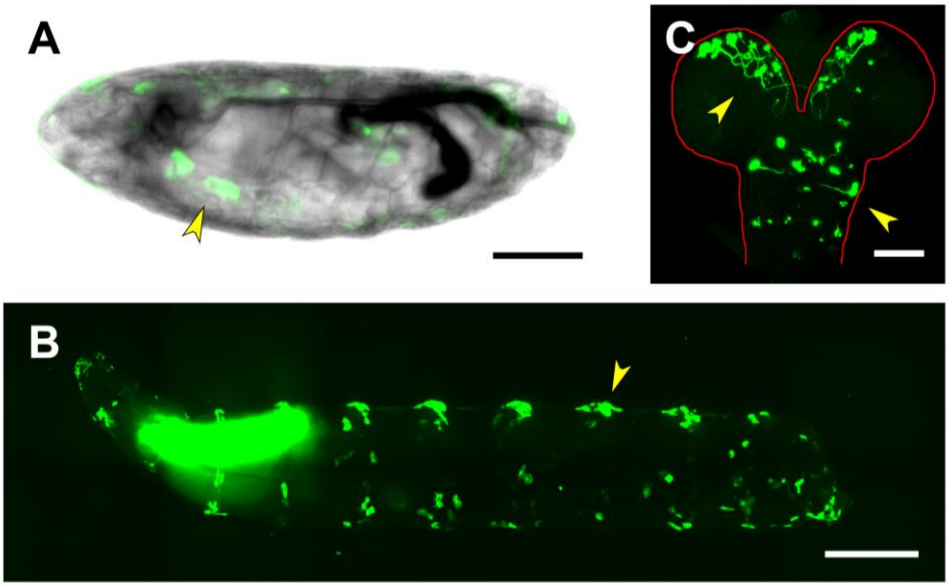

Figure 2: Expression directed by the Upstream Regulatory Sequences of *puc*

A) Expression of a PG1-directed GFP-reporter on a live late stage 17 embryo. Salivary glands (arrowhead) and conspicuous groups of epithelial cells are labelled. Scale bar 100 µm. **B)** Lateral view of a live third-instar larva, showing strong expression of the PG1-directed GFP-reporter in the salivary glands and in segmentally iterated groups of epithelial cells at the locations of the dorsal denticles (arrowhead). Expression is also observed in epithelial cells distributed in scattered ventral spots. Anterior is left. Scale bar 400 µm. **C)** Representative image of GFP-immunostained first-instar larva brains dissected from animals expressing the GFP-reporter under the control of the PG1 Gal4 line. Arrowheads point to PG1-driven expression in neurons of the brain lobes and the thoracic ganglion. The CNS perimeter is marked (red line) and anterior is up. Scale bar 20 µm.

First and Second Intron (PG2 + RS1)

The first and second introns of *puc* as well as its second exon are covered by the PG2 Gal4 line. This region appears to enclose different regulatory elements active in different tissues and periods. PG2-driven expression was first detected in the CNS of stage 15 embryos and patchy at the whole epidermis (**Figure 3A**). As development proceeds, expression was also observed all over on the tracheal system and at the posterior spiracles (**Figure 3B**). PG2 was additionally found to be active in a group of cells positioned in between the brain lobes, that could correspond to the foregut (**Figure 3C** and **3D**).

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Figure 3

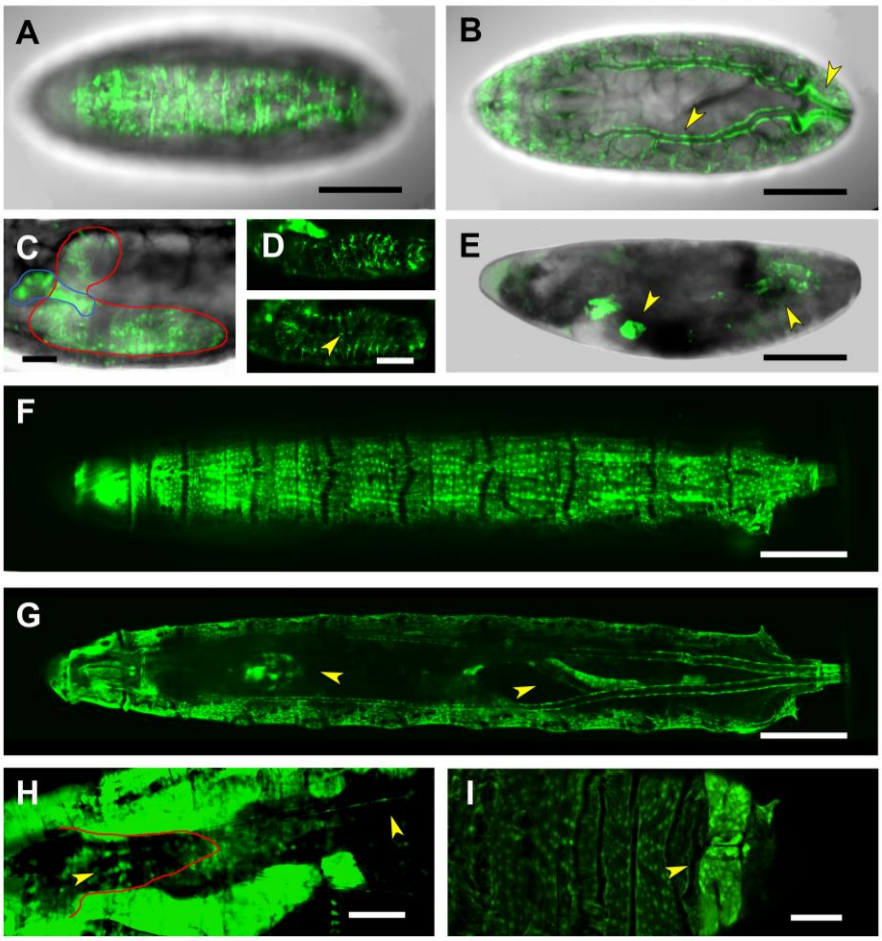

Figure 3: PG2-driven expression in embryonic and larval stages.

A and **B)** Lateral (**A**) and dorsal (**B**) views of a live, late-stage 17 embryo, expressing GFP under the control of the PG2 Gal4 line. Expression is detected throughout the epidermis (**A**), in the tracheal dorsal trunk wall, as well as in the posterior spiracles (arrowheads in **B**). Scale bar 100 µm. **C)** Image corresponding to a deeper focal plane of the embryo shown in (**A**). GFP-expression is observed in the foregut in between the two brain lobes (demarked by a blue line) as well as in the CNS (demarked by a red line). Scale bar 20 µm. **D)** Ventrolateral (upper panel) and mediolateral (lower panel) views of the ventral nerve cord portion of the CNS shown in (**C**). Activation of the PG2 fragment in the CNS is limited to the perineural / sub-perineural glia and the channel glia (arrowhead). Scale bar 20 µm. **E)** Lateral view of a stage 17, maternal-zygotic *hep* mutant embryo, with a characteristic dorsal open phenotype. Reporter activation by the PG2 fragment remains unaffected in the remnants of the salivary glands, the dorsal tracheal trunk and the posterior spiracles (arrowheads). No expression was observed in the epidermis. Scale bar 100 µm. **F** and **G)** Lateral (**F**) and dorsal (**G**) views of a live third instar larva, exhibiting strong GFP expression in the epidermis and the dorsal tracheal trunk (**F**), as well as in the brain and in the midgut (arrowheads in **G**). Scale bar 400 µm. In all cases anterior is left.

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The activity of the PG2 regulatory elements in the CNS was further analyzed by double immunostaining experiments performed in flat-dissected stage 16 embryos. The GFP reporter co-localized with Repo, a transcription factor involved in glial differentiation, but not with the pan-neuronal marker Elav, indicating that PG2 was active in the embryonic CNS glia (**Figure 4A** and **4B**). These glial cells were, by positional criteria, identified as perineural/sub-perineural [belonging to the subgroup that forms the Blood Brain Barrier (BBB)] and ensheathing glia (**Figure 4C**). The CNS expression observed in the late embryonic stages persisted in the first instar larvae. The number of glial cells activating the PG2 sequence increases considerably (**Figure S1**).

Figure 4

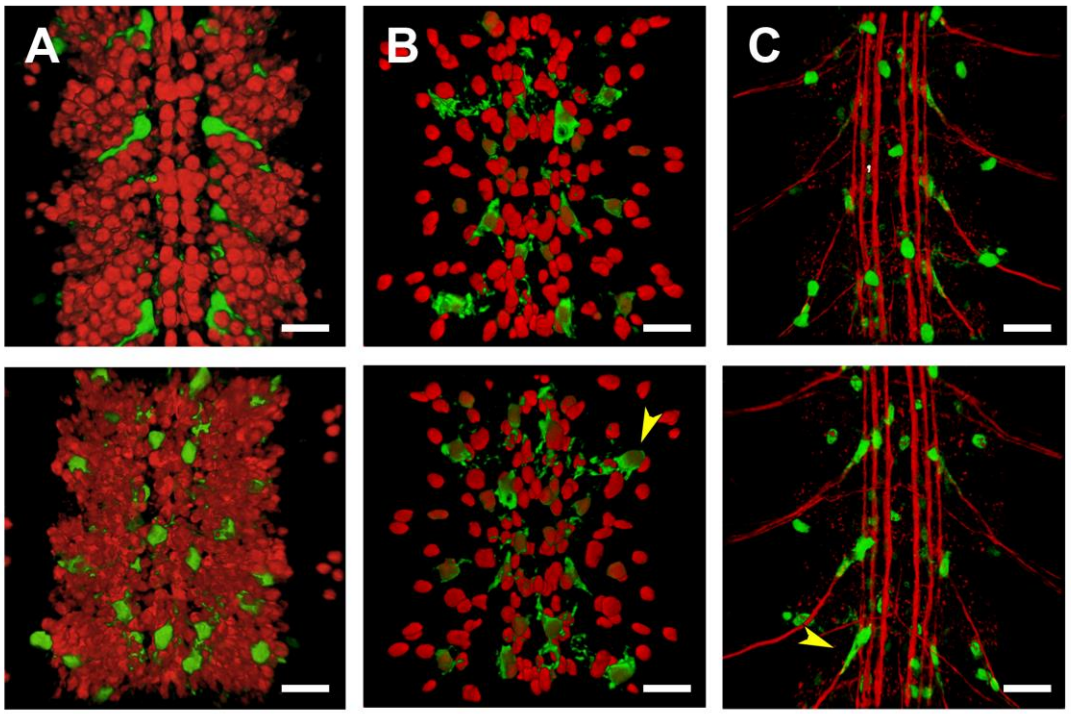

Figure 4: PG2 is expressed in glia

A to **C**) Flat preparation of embryos at stage 17 expressing GFP under the control of the PG2 Gal4 line. Dorsal (top) and ventral (bottom) views of three segments of the ventral nerve cord (VNC) are shown. **A**) Double staining with an anti-ELAV pan-neural antibody. **B**) Double staining with an anti-Repo antibody labelling most of the glial cells. Arrowhead points to cells co-expressing GFP an Repo (Glia). **C**) Double staining with an anti-Fasciclin 2 antibody expressed in the longitudinal, commissures, segmental and intersegmental nerves. Arrowhead points to an ensheating glial cell expressing PG2-directed GFP. Scale bar 10 µm. In all cases anterior is top.

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In the third instar larvae, the expression directed by the PG2 domain is sustained for most tissues, the epidermis, the tracheal system, the posterior spiracles and the foregut (**Figure 3E** and **3F**). In the nervous system it is refined to two rows of cells running along the VNC and to a limited number of glia ensheating the intersegmental nerves (**Figure 3H**). Further, it becomes prominent in the posterior midgut (**Figure 3G**) and the anal pad (**Figure 3I**).

In summary, the first and second introns of *puc* appear to be active regulatory domains probably containing multiple regulatory elements. In a first approach to identify these elements we took advantage of our genomic comparative analysis and explore the expression patterns generated in response to the RS1 motif, which as described above is one of the four hyper-conserved genomic regions found in *puc*. RS1 maps in its first intron within the PG2 regulatory domain (**Figure 1**). Expression driven by this line is detected in late embryonic stages, in the salivary glands; in some epidermal cells, in the posterior midgut/hindgut and in the posterior spiracles. It is not activated in the embryonic CNS. RS1 is thus reproducing in a very limited way the activation pattern of the PG2. Remarkably, it shows ex pression in a group of cells not found for PG2 around the maxillary primordia. (**Figure S2A**). In the third instar larva, RS1 also fails to recreate the expression observed for PG2. Its activity is restricted to scattered cells in the epidermis, a partial expression in the tracheal system and to the posterior spiracles. The perimaxillar group of cells expressing RS1 in embryos are still detected at this stage (**Figure S2C** and **S2D**).

Third Intron (PG3, PG4, RS2-4)

The examination of *puc*'s long third intron for residing cryptic enhancers was facilitated by splitting its sequence in two fragments and by the generation of the lines PG3 and PG4, with PG3 corresponding to the 5' portion of the intron. The PG3 domain covers the three hyper conserved motifs RS2, RS3 and RS4 (**Figure 1**).

Expression driven by the PG3 line is detectable only from late embryonic stages (stages 16-17), in the salivary glands, the pharynx/ring gland and weakly in the midgut. Yet, its most characteristic feature is to be expressed in all body wall muscles and the nervous system (**Figure 5A** to **5C**). Its expression in the embryonic CNS is distinct that than found for PG2. It is restricted to the Repo-negative midline glia (**Figure S3A and S3B**) shifting in the first instar larval brain to the surface glia. Here, the expression in surface glia becomes weak when

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compared to a massive expression observed in the tracheal branches penetrating the brain (**Figure S3C and S3D**).

Figure 5

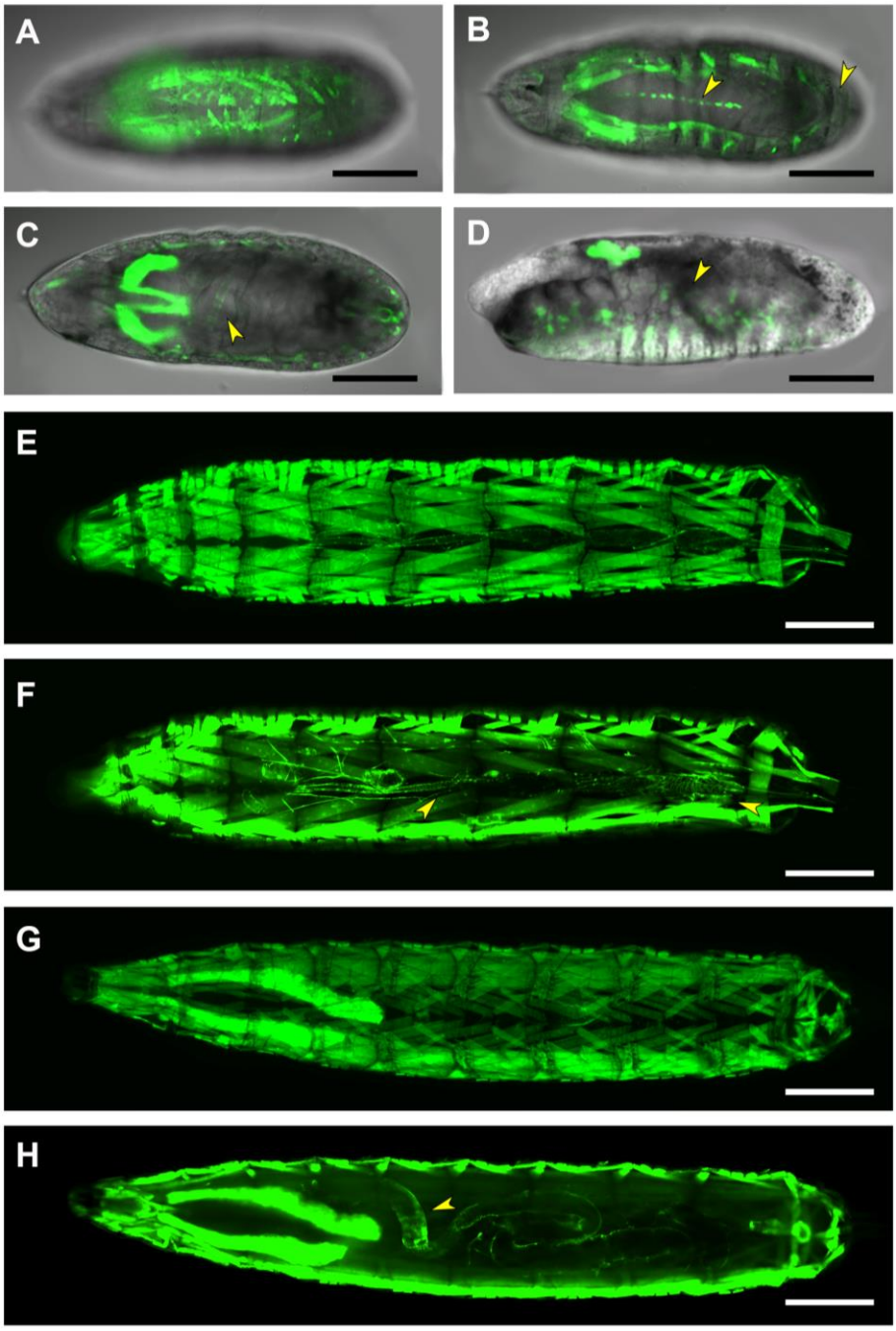

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Figure 5: Gene expression modulation by the PG3 domain

A-C) Ventral views, acquired at different focal depths (with dorsal directionality), of a live, stage 17 embryo, expressing GFP under the control of the PG3 Gal4 line. Expression is detected in all body wall muscles (**A**); in the CNS midline glia (arrowhead in **B**); and in the salivary glands, pharynx, intestinal tract and anal pad (arrowheads in **C**). Scale bar 100 µm. **D)** Lateral view of a dorsal-open, stage 17, *hep* null embryo. The activation of the PG3 regulatory sequence, in the salivary glands and in the remnants of the muscles (arrowheads) is sustained despite the loss-of-function of the JNK-activating kinase Hep. Scale bar 100 µm. **E** and **F)** Dorsal superficial and deep views of a live, third instar larva. PG3 exhibits strong activation in all body-wall muscles (**E)**. In a deep focal plane (**F**), PG3 activity is revealed in the heart tube, as well as in the alary muscles (arrowheads in **F**). Scale bar 400 µm. **G** and **H)** Ventral superficial and deep views of a living third instar larva. GFP-expression directed by PG3 is detected in the salivary glands (**G**) and in the intestinal tract (arrowhead in **H**). Scale bar 400 µm. In all cases anterior is left.

In the third instar larvae, the expression directed by the PG3 domain in muscles recapitulate that observed in the embryo (**Figure 5E**). Further, the heart and alary muscles (other mesodermal derivatives) are also stained (**Figure 5F**). The expression in the nervous system disappears and get strengthen in the midgut extending to the hindgut, and the salivary glands (**Figure 5G** and **5H**).

Three out of the four identified hyper-conserved *puc* sequences (RS2-4) map in the PG3 intronic fragment. The complex embryonic expression pattern observed for the PG3 domain is only in part reproduced by the RS2 to RS4 conserved sequences. The RS2 motif, residing at the 5' end of the PG3 sequence, is activated at the end of embryogenesis (late stage 17) in the salivary glands and in a subset of the lateral body wall muscles, in the posterior spiracles and in the CNS (**Figure 6A** to **6C**). CNS expression is found in both neurons and glia (**Figure S4A** to **S4D**) and persists in the first instar larva stage glia (**Figure S4E** and **S4F**). In third instar larva, the RS2-directed GFP expression persists in a fraction of body-wall muscles, in the heart tube, in the alary muscles and at the periphery of the anal pad (**Figure 6E** to **6G**). No expression is detected in the nervous system. The expression of RS2 in the heart and auxiliary structures is restricted to mesodermal derivatives all along the anterior posterior axis of the heart tube (**Figure 6H** and **6I**).

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Figure 6

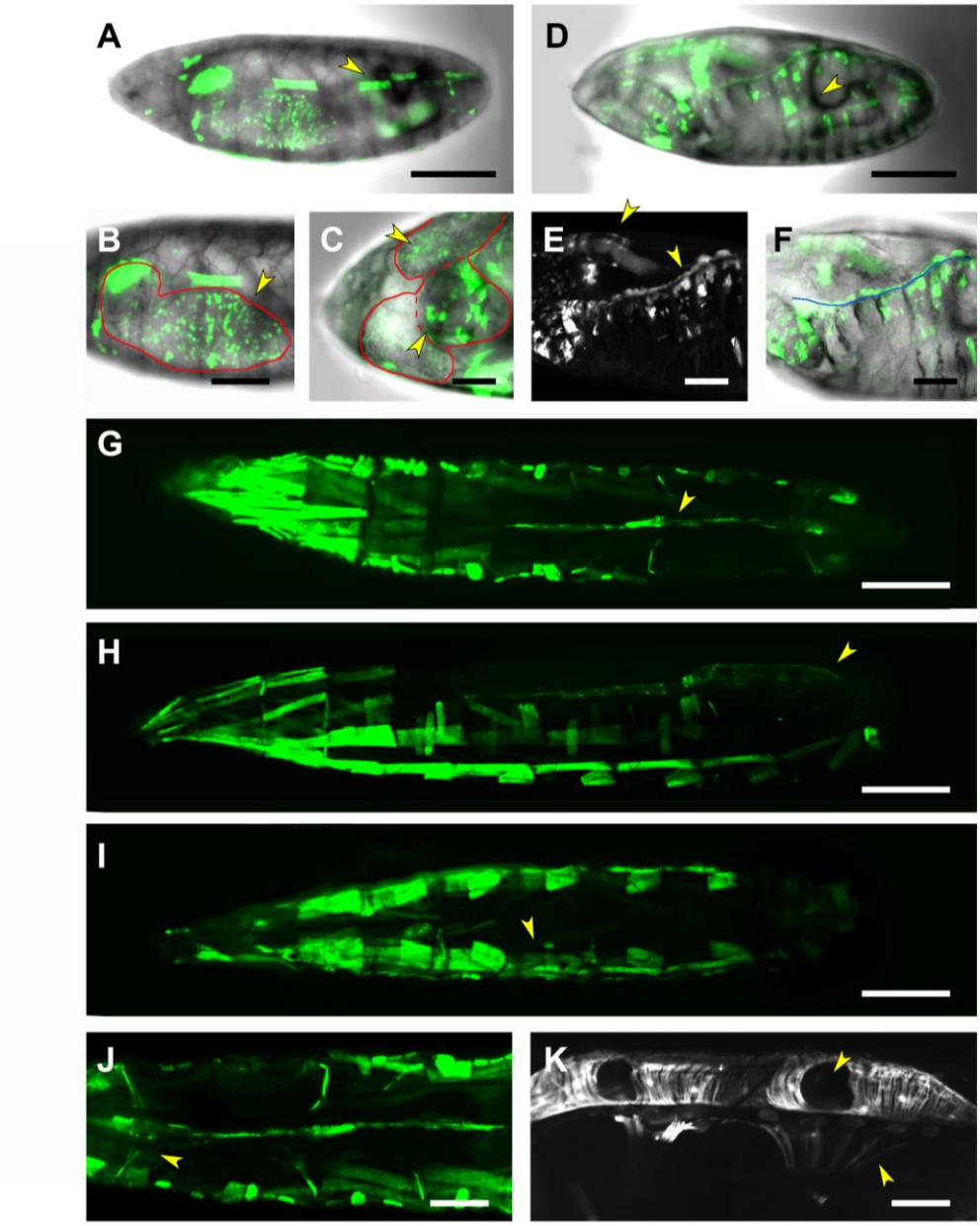

Figure 6: Complex gene expression modulation by the RS2 motif

A) Lateral view of a live, stage 17 embryo expressing GFP under the control of the RS2 motif. Expression is detected in a subset of body wall muscles (arrowhead), the CNS and, faintly, in the posterior midgut. Scale bar 100 µm. **B** and **C)** high magnification views (**B**) lateral and (**C**) ventral of the embryonic CNS (delimited by a red outline) of (**A**). Arrowheads point to neurons in the optic lobes and perineural and subperineral glia on the Ventral Nerve Cord. Scale bar 20 µm. **D)** Lateral view of a dorsal-open, stage 17, *hep* null embryo. The activation by the RS2 motif in the remnants of the muscles, the CNS and the midgut is sustained (arrowheads). Scale bar 20 µm. **E** and **F)** high magnification views showing the GFP signal (**E**) or the signal in a brightfield background (**F**) of the epithelial surface of (**D**). Arrowheads point to the ectopic expression of the marker in the anterior midgut

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and the epidermal cells (blue line) at the edge of the open hole consequence of the dorsal closure failure in *hep* mutants. Scale bar 20 µm. **G** to **I**) Dorsal (**G**), lateral (**H**) and ventral (**I**) views of a live third instar larva showing RS2-directed GFP expression in a subset of body-wall muscles, in the heart tube and in the alary muscles (arrowheads). Scale bar 400 µm. **J)** High magnification image of the heart tube and the attached alary muscles (arrowhead) expressing RS2-directed GFP. Scale bar 100 µm. **K)** 3D-reconstruction of the heart tube and the attached alary muscles (asterisks). Arrowheads point to the hemolymph exit pores along the cardiac tube. Scale bar 100 µm. In all instances, anterior is left.

RS3 directed expression is quite distinct of that resulting from the PG2 domain. RS3, in embryos at late-stage 17th, besides the salivary glands, targets expression to the posterior spiracles, the trachea and to the epidermal cells (**Figure 7A** to **7C**). No CNS or mesodermal expression was observed. In third instar larva, the RS3-directed GFP expression in the epidermis and the posterior spiracles is maintained. The tracheal expression becomes more complex and it is not limited to the main trunks. The most distal tracheal cells do now display at high levels RS3-directed expression (**Figure 7E** to **7G**).

Figure 7

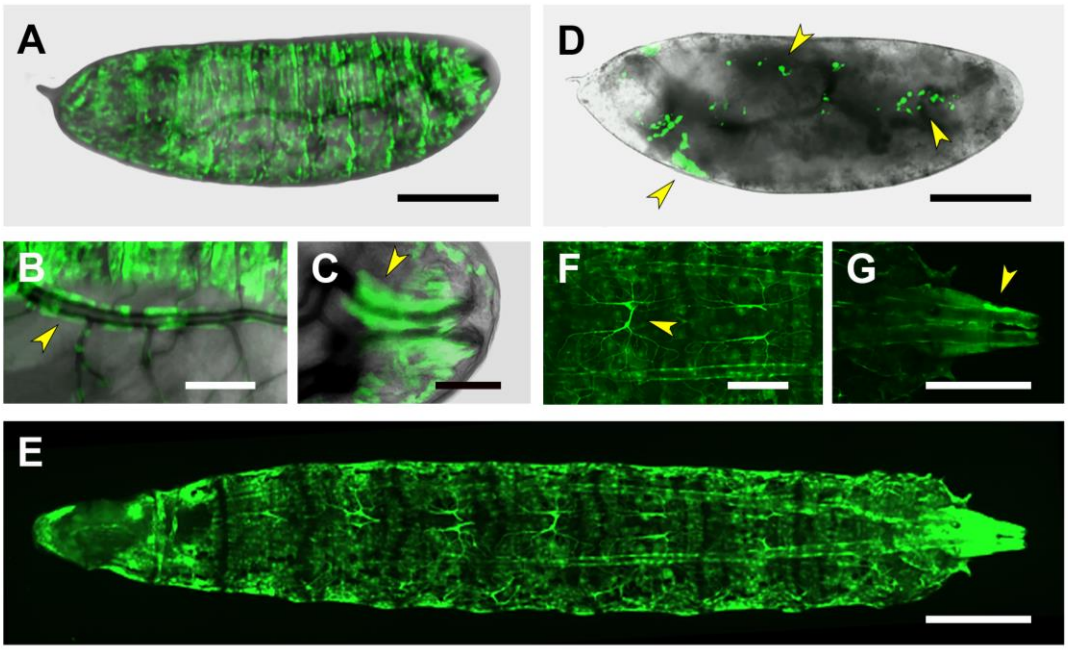

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Figure 7: RS3-driven expression in embryonic and larval stages

A) Dorsal view of a live, stage 17 embryo, expressing GFP under the control of the RS3 Gal4 line. Strong activation of this motif is observed throughout the epidermis. Scale bar 100 µm. **B** and **C)** Details from the embryo shown in (**A**). RS3 activation is observed in the trachea wall (arrowhead in **B**) and in the posterior spiracles (arrowhead in **C**). Scale bar 20 µm. **D)** Lateral view of a dorsal-open, stage 17, *hep* null embryo. In *hep* null embryos, activation of the RS3 motif is abolished from the epidermis but not from the trachea. Arrowheads point to remnants of the trachea that sustain RS3-induced GFP expression. Scale bar 100 µm. **E)** Dorsal view of a live third-instar larva, showing strong expression of the RS3-directed GFP-reporter in the epidermis, the trachea and the posterior spiracles. Scale bar 400 µm. **F** and **G)** High magnification images showing details of RS3 expression in third instar larval tracheal system. Arrows point to the tracheal terminal arborizations at the dorsal midline (**F**) and at the posterior spiracles (**G**). Scale bars 20 (**F**) and 50 µm (**G**) respectively. Anterior is left.

RS4 expression in embryonic stages is limited to the salivary glands and the posterior spiracles, not showing any of the characteristic patterns observed for PG3 (**Figure S5A** and **S5B**). In third instar larva, RS4 becomes active at the maxillary region, the tracheal trunks, scattered epidermal cells, the anal pad, and weakly at the posterior midgut and the CNS (**Figure S5C** and **S5D**).

The complex pattern targeted by the lines mapping at the 5' half of *puc*'s third intron was not as such for the PG4 line, which covers the 3' half of this intronic sequence. In the embryo, PG4 is activated at stage 15 and drives expression in the salivary glands and the epidermis (in a patchy pattern) (**Figure 8A** and **8B**). Further, strong expression was also detected in a group of four to five cells, possibly components of the ring gland, medially positioned along the embryo's dorso-ventral axis in between the brain lobes, and in the posterior spiracles (**Figure 8C** and **8D**). No expression was observed in the embryonic CNS. However, in the first instar larva the PG4 sequence is activated in specific, interneurons of both the brain lobes and the ventral nerve cord, as well as in a group of posterior neurons with axonal projections outside the CNS. A subset of astrocytes and ensheathing glial cells also appear to express the reporter (**Figure S6A to S6C**). In third instar larvae, no obvious expression was hold in the CNS but it was maintained in the epidermis and the posterior spiracles. The ring gland is heavily stained as they are the posterior midgut and specific cells at the trachea segmental junctions (**Figure 8F** and **8G**).

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Figure 8

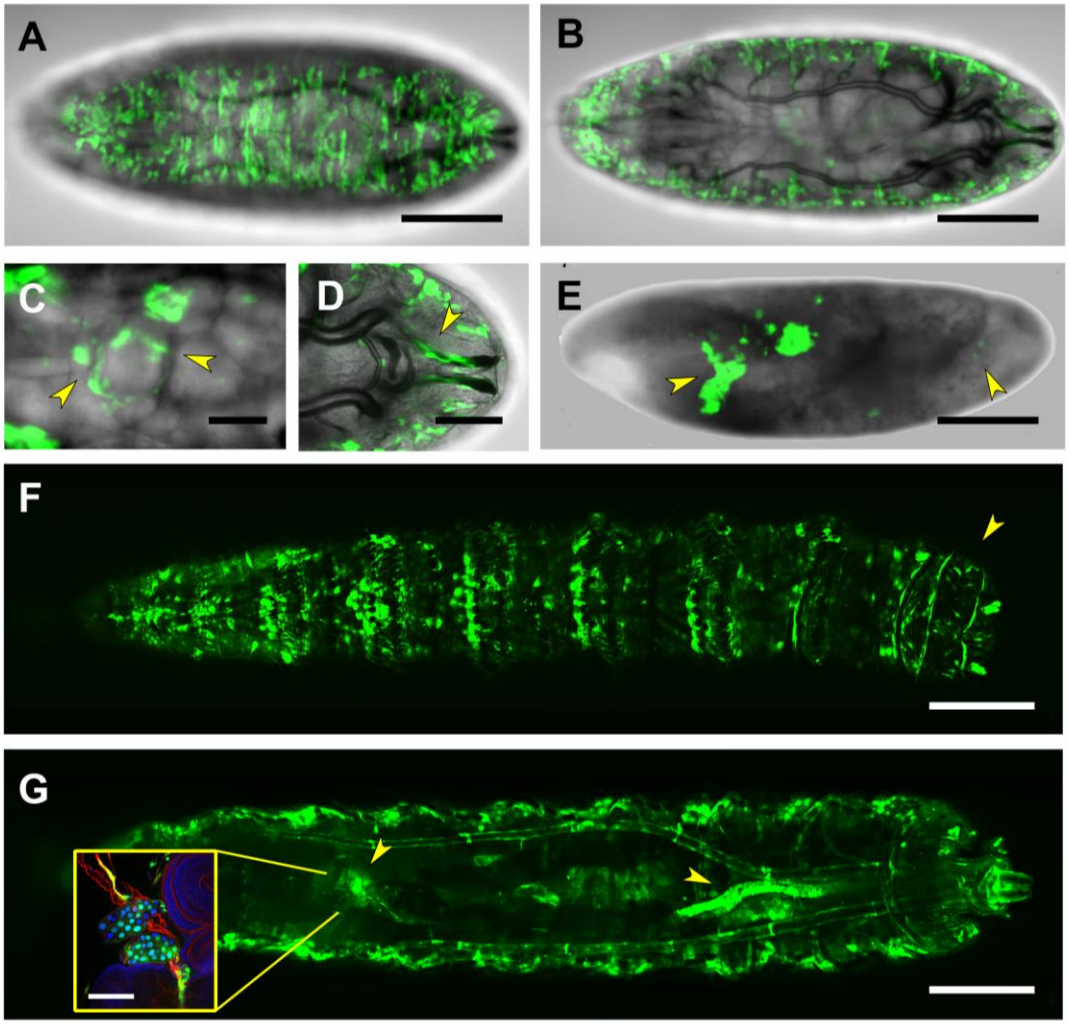

Figure 8: Gene expression modulation by the PG4 domain

A and **B)** Dorsal views at different focal planes of a live stage 17 embryo showing strong epidermal GFP expression directed by the PG4 Gal4 line. Expression by this line is also driven in a group of 4-5 cells positioned medially along the embryo's dorso-ventral axis (arrowhead in **B**). Scale bar 100 µm. **C** and **D)** High magnification images from (**A**). PG4 is activated in the ring gland and anterior midgut (arrowheads in **C**); as well as in the posterior spiracles (arrowhead in **D**). Scale bar 20 µm. **E)** Lateral view of a dorsal-open stage 17 *hep* null embryo, showing PG4 activation in the salivary glands and on a medially positioned group of cells and the remnants of the posterior spiracle (arrowheads). Scale bar 100 µm. **F)** Ventral view of a live, third instar larva, exhibiting activation by PG4 sequence in the anal pad (arrowhead) and the epidermis. Scale bar 400 µm. **G**) Dorsal view of a third instar larva showing distinct PG4-directed expression predominantly in the midgut and the ring gland (arrowheads) but also in scattered positions along the dorsal trachea trunk and posterior spiracles. Scale bar 400 µm. Inset shows a high magnification of the ring gland expressing GFP as a marker for PG4 activity. DAPI is blue and Phalloidin is red. Scale bar 20 µm. Anterior is left, in all instances.

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Downstream Sequences (PG5)

puc 3' non-coding sequences examined (PG5 Gal4 line) includes its last exon, 3'UTR and sequences downstream. The PG5 sequence is activated during the last embryonic stage (stage 17) in the salivary glands and in a few cells at the anterior tip of the embryo. Expression is also detected in the posterior spiracles, in a group of cells, positioned dorsally and slightly posteriorly with respect to the brain lobes and, in cases, in cells occupying symmetrical positions at the branching points of the tracheal dorsal trunk and the epidermis (**Figure 9A** and **9B**). No expression was detected in the CNS. In third instar larvae, PG5 is active in the salivary glands, in restricted group of cells positioned below the dorsal denticles and in the posterior spiracle (**Figure 9C**).

Figure 9

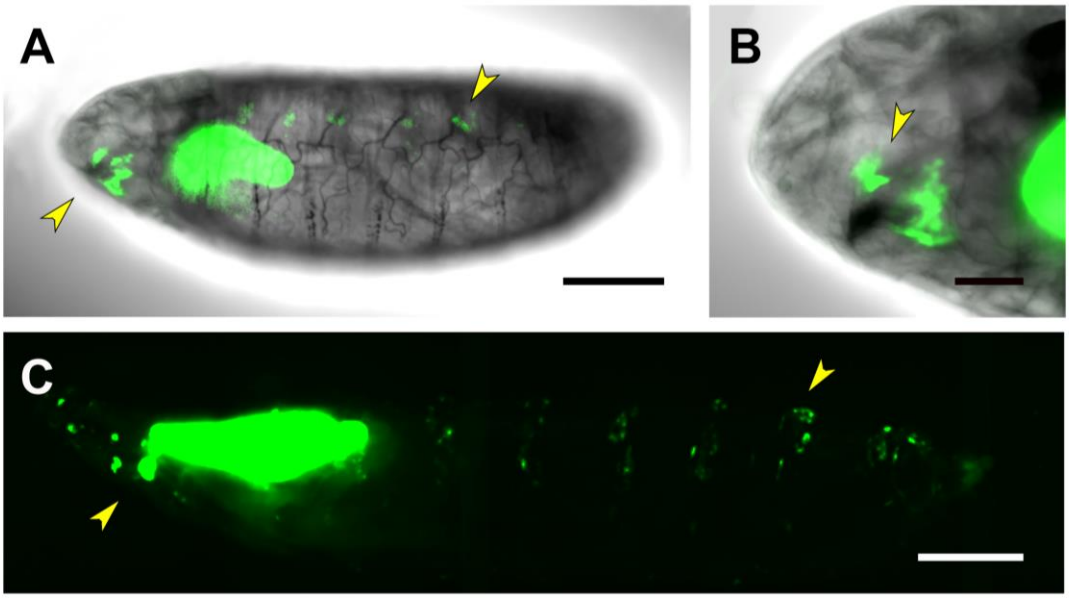

Figure 9: Gene expression modulation by the PG5 domain

A) Lateral view of a live stage 17 embryo expressing GFP under the control of the PG5 Gal4 line. Expression is detected in the salivary glands, in cells surrounding the maxilla and in iterated group of epidermal cells that will give rise to the larval denticles (arrowheads). Scale bar 100 µm. **B)** High magnification of the image in A focus on those PG5 positive cells around the embryonic maxilla. Scale bar 20 µm. **C)** Lateral view of a live third instar larva showing the persistence of the embryonic PG5 activation pattern. Arrowheads point to the cells surrounding the maxilla and the denticle bearing cells. Scale bar 400 µm. Anterior is left.

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Regulatory control of *puc* expression by the JNK signaling cascade

As mentioned above, *puc* expression (detected from enhancer trap lines) have been thoroughly employed as a readout of JNK signaling activity. Yet, *puc* has, in places, been found to be expressed in the absence of JNK. We tested a subset of the new enhancers generated in our screening for dependence on JNK activity and evaluate the expression of these new lines in a *hep* (JNKK) minus maternal and zygotic background. A stock carrying a hep^{1} mutation and a multimerized UAS-GFP inducible marker was built. Homozygous, maternally rescued, hep^{1} females were crossed to males of the Gal4 lines PG2 to PG4 and PM1 to PM3. Hemizygous hep^{1} male embryos not maternally rescued were identified in the progeny by their characteristic dorsal open phenotype. GFP expression was monitored in these living embryos. Remarkably, much of the expression directed by the new enhancers is not JNK dependent.

For PG2, covering the first and second intron of *puc*, the hep^{1} embryos display GFP expression in the remnants of the salivary glands and the trunks of the tracheal system. The epidermal expression seems to be reduced but some signal still remains (**Figure 3E**). No conclusion could be reached regarding the nervous system. The RS1 motif included in the PG2 domain, however, responds to JNK activity but in an unexpected way. In the absence of JNK activity, RS1-directed expression shows up strongly on the epidermis, while in the wild type condition is limited to scattered epidermal and tracheal cells and to a posterior domain associated to the spiracles. This distinct pattern appears to not be affected in hep^{1} embryos.

The expression patterns on salivary glands, body wall muscles and the midgut directed by the 5' fraction of *puc* third intron (PG3) are unaffected in hep^{1} embryos (**Figure 5D**). It was very difficult to assess any affectation of the PG3-directed nervous system or heart expression as these were, originally, quite low.

The patterns of expression of RS2 and RS3 enclosed within the PG2 domain respond differentially to the JNK activity (we did not evaluate RS4 as its expression pattern is quite unspecific and remarkably low).

RS2-directed expression recapitulates in part the PG3 muscle pattern but not its tracheal expression. It also shows a quite distinct expression in the CNS mostly in glial cells. In the absence of JNK activity the muscle and CNS expression (as it happens for PG3) are maintained (**Figure 6D**). Interestingly, RS2 becomes ectopically expressed in the midgut and in a row of cells at the edge of the opening dorsal hole (**Figure 6E** and **6F**). These last cells have been previously observed to activate $puc^{A251.1}$ and puc^{E69} enhancer trap expression both in hep^{1} and

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bsk^{2} allelic conditions (unpublished observations and J. Riesgo-Escovar, personal communication).

The RS3-directed expression pattern is quite dissimilar of the PG3 one showing widespread epidermal and tracheal expression. Specially, the tracheal cells of the terminal branches and the posterior spiracles. In *hep*^{1} mutants, remnants of GFP expression in the salivary glands and scattered cells that seem to be of tracheal origin are found. The epidermal and terminal tracheal cells expression seems to be abolished in the loss of JNK activity condition (**Figure 7D**).

DISCUSSION

JNKs represent a signaling hub in many physiological responses and have pivotal functions in cell proliferation, differentiation, development and death (4). JNKs can be selectively inactivated by DSPs and transcriptional induction of DSP expression is well documented as a negative-feedback mechanism (25). In *Drosophila*, this negative feedback loop modulates the level of JNK activity in different developmental processes such as epithelial sheet expansion (dorsal closure and imaginal disc fusion) (14) (5) and morphogenetic death (26).

In our analysis of the mechanisms regulating the expression of *puckered (puc)*, the gene encoding the *Drosophila* Jun N-terminal kinase (JNK) Dual Specificity Phosphatase (DSP) (18), we uncovered regulatory sequences (PG2) directing its expression to egg chamber border cells. This expression can also be observed in the *puc* mutant line *puc*^{B48} (18) and in diverse protein trap lines (27). PG2 expands across the first and second introns of *puc*, where the *puc*^{B48} insertion is located and its activity depends on JNK activity (22). All generated PG2 lines show the same expression pattern.

Analyzing in more detail the genomic region spanning *puc* and its surrounding domains, we have found that both, 5' and 3' of its transcription unit, regulatory motifs are seldom. The PG1 and PG5 Gal4 lines, despite expression in salivary glands and posterior spiracles, which appear to be unspecific readouts present for all constructs studied, just revealed activity in a subset of neurons and scattered and unpattern epidermal cells. On the other hand, *puc* first and second introns (PG2 line), beside directing expression in border and follicle cells as described above, can activate gene expression in different tissues and at different stages as described. Remarkably, their activity in the nervous system responds to a very dynamic control and affect distinct neural subpopulations, evolving through embryonic and larval stages. *puc* third intro

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is highly enriched with regulatory domains, affecting , in particular, to mesodermal derivatives (PG3), the ring gland (PG4) and different components of the CNS (both). Other tissues targeted by the regulatory motifs present in *puc* third intron are the epidermis, the tracheal system, the anal pad, the midgut and the maxillary primordia (**Table 1**).

Table 1

	EMBRYO	CNS EMBRYO/1st INSTAR LARVAE	3rd INSTAR LARVAE
PG1	Salivary Glands		Salivary Glands
	Posterior Spiracles		Posterior Spiracles
		Brain lobes Neurons	Denticles precursors
		Thoracic ganglion Neurons	
PG2	Posterior Spiracles		Posterior Spiracles
	CNS	CNS Glia	CNS
	Epidermis (patchy)	· Perineural/Subperineural	· VNC subset
	Tracheal System	· Ensheating	· Glia intersegmental nerve
	Foregut		Epidermis
			Tracheal System
			Foregut
			Posterior midgut
			Anal pad
RS1	Salivary Glands		
	Posterior Spiracles		Posterior Spiracles
	Hindgut		Epidermis (patchy)
	Maxillary primordia		Tracheal System (patchy)
	Epidermis		Maxillary primordia
PG3	Salivary Glands		Salivary Glands
	CNS	CNS Glia	Midgut / Hindgut
	Midgut (weak)	· Repo-negative midline	Body wall muscles
	Body wall muscles	· Surface	Heart
	Pharynx/ring gland	VNC tracheal arborization	Alary muscles
RS2	Salivary Glands		Salivary Glands
	Posterior Spiracles		
	CNS	CNS	Body wall muscles (subset)
	Body wall muscles (subset)	· Neurons	Heart
	Healing Epithelia	· Glia	Alary muscles
	Midgut		Anal pad periphery
RS3	Salivary Glands		Salivary Glands
	Posterior Spiracles		Posterior Spiracles
	Epidermis		Epidermis
	Tracheal System		Tracheal System
			· Dorsal trunks
			· Distal arborization
RS4	Salivary Glands		Salivary Glands
	Posterior Spiracles		Posterior Spiracles
			Tracheal System
			Anal pad
			Maxillary primordia
PG4	Salivary Glands		Salivary Glands
	Posterior Spiracles		Posterior Spiracles
	Epidermis (patchy)	Brain lobes / VNC Interneurons	Epidermis (patchy)
	Ring gland	Nerves axonal projections	Ring gland
		Astrocytes	Posterior midgut
		Ensheathing glia	Tracheal junctions
PG5	Salivary Glands		Salivary Glands
	Posterior Spiracles		Posterior Spiracles
	Epidermis (scattered)		Epidermis (scattered)
	Maxillary primordia?		

Table 1: Summary of expression patterns

The patterns of expression directed by each Gal4 line (PGs and RSs) in embryos and third instar larvae, and on the embryonic and first instar larvae CNS when relevant, are summarized. Grey boxes relate to the non-specific expression detected in salivary glands and posterior spiracles (although we could not discard, they may represent bona fide targets). Green boxes highlight expression patterns not observed in the wild type condition that are switched on in the absence of JNK activity (*hep*^{1} maternal and zygotic condition). Red boxes point to patterns of expression that are eliminated in *hep*^{1} mutants.

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Although our analysis does not cover every aspect of the newly identified motifs in the *puc* genomic domain, it provides several important clues on the activity and function of the regulatory control mediated by *puc* on JNK signaling: 1) the known expression of *puc* in mesodermal derivatives (body wall muscles and heart), follicle and border cells, and the tracheal system can now be ascribed to identified genomic domains; 2) *puc* expression is modulated by a more complex array of motifs than expected and it targets multiple unforeseen tissues where the role of the JNK pathway has not been previously explored, such as the ring gland, distal tracheal cells, alary muscles or the anal pad; 3) *puc* expression, in many places, appears to be independent of JNK activity, or at least it is not directly targeted by Hep (JNKK). [This has been previously documented in the wing imaginal discs for the control of JNK activity by small GTPases. Here, *hep* is required downstream of Dcdc42 to activate *puc*. Yet, the Rac activity on *puc* expression is not affected by the absence of *hep* (21). This suggest that a JNK-related activity is present in discs acting downstream of Rac or that alternative regulatory mechanisms can affect *puc* expression.]; and 4) eliminating JNK activity (maternal and zygotic *hep* loss of function) can lead to the activation of *puc* expression. We have detected overactivation of *puc* expression in the embryonic epidermis mediated by the RS1 domain in *hep* mutants. Further, in the absence of *hep*, RS2 becomes active in the midgut and in the cells at the edge of the dorsal open embryonic epidermis. RS2 in wild type animals does not label leading edge epidermal cells (although they are characteristic JNK positive - *puc* expressing cells (puc^{E69} and $puc^{A251.1}$ LacZ reporters). These cells are prompt to die and strongly initiate RS2-dependent expression once JNK signaling is switch off. If this activation could respond to a caspase-dependent but JNK-independent cell death pathway, as the one described in the *Drosophila* eye imaginal discs mediated by Eiger [tumor necrosis factor (TNF)] (28) or to another cascade, remains to be elucidated. RS2 directed expression in the absence of JNK activity is key as *puc* expression has been employed in a regular basis as a readout of JNK-dependent cell death-inducing activity. It might not be correct to assume that the JNK pathway is active every time that *puc* expression is turned on.

The c-Jun N-terminal kinase (JNK) pathway does not just play an important role in regulating a wide range of cellular activities but it is also fundamental for tumor growth (29). In this scenario the JNK pathway has been linked to both Ras-induced tumorigenesis and, in association to the Hippo pathway, on tissue growth control (30, 31). The links between these signaling elements are still unclear. In particular, in *Drosophila*, has been found that JNK signaling can activate or suppress Yorkie activation depending on context (32). The

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identification of these new regulatory elements controlling the expression of *puc* provides a great opportunity to investigate the implication of other regulatory cascades on the negative feedback control of JNK activity in different scenarios.

METHODS

Cloning of *puc* genomic sequences

In order to amplify the upstream, intronic and downstream potential regulatory sequences of *puc* we designed six pair of primers that were employed to amplify fragments of genomic DNA. As the expected fragments are around 4 to 6 Kb long, we used a long template PCR system from Roche. These PCR fragments were first cloned in a PGEM T easy vector. Secondly, we subcloned these fragment in a Gal4 expression vector, the pPTGal (33). pPTGal is a vector that contains two terminals 5 'L TR and 3 'L TR transposase targets that permit its integration in the fly genome. This vector carries also a *white* + gene marker which will lead to a red eye color in a white - background. Transgenic flies were generated by injections in *y- w-* embryos with a helper plasmid that contains a transposase gene. The hatched flies were crossed individually with *y- w-* flies. Generally, one female or male potential transgenics with 3 other *y- w-* flies. To map these insertions in the second or third chromosome, the flies were crossed with a double balancer stock (*w; If/CyO; MKRS/TM6B*).

Potential regulatory sequences (RS)

Regulatory Sequence 1 (94 bp)

AAAAAATGTAAAGCAAAGCAGGTTTCTCAAGCGGCCTGGCAACGCTGAAAAACC
CGCTTTGAAACGACCTTTCGAGTACGAATTCATCGGCACA

Regulatory Sequence 2 (127 bp)

GAGACGCAAAAAAGGGGGCACGAGGTGGGAACGAGGAGAGAACTTTAGCCGTG
GAATATAATGCTGACGTCAATCGCATTTTTCCATTTCCATTTTCTATGTCCAAAGC
TGTTCATCAAGTATTTTT

Regulatory Sequence 3 (58 bp)

TTGTTGTTGCTGTTGCGTGTTGGCGTTGGCAGACAGCATGGCGGCAGCGGCGTCG

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TCG

Regulatory Sequence 4 (91 bp)

GGTGACATTTTATCGACCAAGCGAAAAAAAATGTTGGCCCCCGTGCGCATAATTT
CAATATTCGTCAGCCGGCTATTTTTAAAATGCAAAA

RSs were cloned (after amplification employing appropriate primers) in the Bgl2 site of the pPTGal Vector. Multiple transgenic lines driving the expression of the transcription factor Gal4 under the control of these potential regulatory sequences were generated, mapped and balanced.

Drosophila Strains and Genetics

The following stocks were used:

pJFRC81-10XUAS-IVS-Syn21-GFP-p10 (attP2) (Dr. Todd Laverty, Janelia Research Campus)

hep^{1}*;; 10XUAS-IVS-Syn21-GFP-p10* (this work)

w; If/CyO; MKRS/TM6B

All crosses were performed at room temperature.

Drosophila embryo fixation and dissections

Embryos were collected at 25ºC and aged at 18ºC for 17-19h, then dechorionated in 100% bleach for 90s. They were washed thoroughly with water and processed. Whole mount embryos were fixed and de-vitellinized according to (34).

Drosophila embryo dissections for generating flat preparations were performed according to (35). Briefly, crosses were maintained in embryo collection baskets at room temperature and synchronized by repetitive changes of juice-agar plates, with a time interval of 2 hours. All embryos laid within this period of time were aged for approximately 13 hours at 25ºC, or until reaching mid-stage 16 (3-part gut stage). At this point, the chorion was removed with bleach for 1 min and the embryos were rinsed extensively with water. For dissection, dechorionated embryos were transferred with forceps on the surface of a small piece of double-sided tape, adhered on one of the sides of a poly-L-Lysine coated coverslip. After orienting the embryos dorsal side up and posterior end towards the center of the coverslip, the coverslip was flooded

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with saline (0.075 M Phosphate Buffer, pH 7.2). Embryos were manually de-vitellinized using a pulled glass needle and dragged to the center of the coverslip, where they were attached to the coated glass with their ventral side down. A dorsal incision was performed using the glass needle directed from the anterior to the posterior end of the embryo. The gut was removed by mouth suction and a blowing stream of saline was used to flatten the lateral epidermis. Tissue fixation was done with 3.7 % formaldehyde in saline for 10 minutes at room temperature. After this point standard immunostaining procedures were followed.

Immunohistochemistry

Immunostaining of whole mount or flat-dissected stage 16-17 Drosophila embryos was performed using the following primary antibodies: mouse anti-Fas2 (1:100, clone 1D4, DHSB), rabbit anti-GFP tag polyclonal (1:600, Thermo Fisher Scientific), mouse anti-Repo (1:100, clone 8D12 DHSB) and rat anti-Elav (1:1000, clone 7E8A10 DHSB).

The secondary antibodies used for detection were: Goat anti-Rabbit IgG (H+L), Alexa Fluor 488 conjugate (A-11008) and Goat anti-Mouse IgG (H+L) Alexa Fluor 555 conjugate (A-21422). All secondary antibodies were used in a dilution of 1:600 and were from Invitrogen.

Image Acquisition

Immunostained embryos were oriented on Poly-L-Lysine coated slides, and mounted in Vectashield anti-fading medium (Vector Laboratories, USA) using a 22mm x 6mm glass coverslip (thickness 1) as a spacer between the slide and the overlaying coverslip. Flat-prepped immunostained embryos were mounted in the same way.

Image acquisition was performed on a Zeiss LSM 700 inverted confocal microscope, using a 25X oil immersion lens (1.3 NA) for whole mount embryos and a 40X oil immersion lens (1.3 NA) for flat-dissected embryos respectively. Z-stacks spanning the whole embryo thickness were acquired with a step size of 2 µm.

Larvae live imaging

Third instar larvae were anesthetized in a chamber saturated with ether fumes for 8 min at room temperature. Next, they were stretched and aligned with forceps on a piece of double-sided

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tape, adhered on a glass slide and covered with a 3:1 mix of Halocarbon Oil 27/700 with a Refraction Index of 1.47. Two 22mm x 6mm glass coverslip (thickness 1) were used as spacer between the slide and the coverslip (thickness 1.5). Images were acquired on a Superesolution DRAGONFLY 505 (Andor) installed on an inverted confocal microscope (Nikon Eclipse Ti2) using a 10 X multiple immersion lens (0.8 NA). 150 slices with a step size of 2 µm were collected.

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AUTHOR CONTRIBUTION

KK performed all biological tests and EMB designed the study, analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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