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piragua encodes a zinc finger protein required for development in *Drosophila*

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ABSTRACT

We isolated and characterized embryonic lethal mutations in *piragua* (*prg*). The *prg* locus encodes a protein with an amino terminus Zinc Finger-Associated-Domain (ZAD) and nine C_2H_2 zinc fingers (ZF). *prg* mRNA and protein expression during embryogenesis is dynamic with widespread maternal contribution, and subsequent expression in epithelial precursors. About a quarter of *prg* mutant embryos do not develop cuticle, and from those that do a small fraction have cuticular defects. Roughly half of *prg* mutants die during embryogenesis. *prg* mutants have an extended phenocritical period encompassing embryogenesis and first instar larval stage, since the other half of *prg* mutants die as first or second instar larvae. During dorsal closure, time-lapse high-resolution imaging shows defects arising out of sluggishness in closure, resolving at times in failures of closure. *prg* is expressed in imaginal discs, and is required for imaginal development. *prg* was identified in imaginal tissue in a cell super competition screen, together with other genes, like *flower*. We find that *flower* mutations are also embryonic lethal with a similar phenocritical period and strong embryonic mutant phenotypes (head involution defects, primarily). The two loci interact genetically in the embryo, as they increase embryonic mortality to close to 90% with the same embryonic phenotypes (dorsal closure and head involution defects, plus lack of cuticle). Mutant *prg* clones generated in developing dorsal thorax and eye imaginal tissue have strong developmental defects (lack of bristles and ommatidial malformations). *prg* is required in several developmental morphogenetic processes.

1. Introduction

Zinc-finger domains (ZF) are among the most prevalent and diverse domains in eukaryotic genomes (Laity et al., 2001). The diversity of proteins that harbor such domains, plus the different modifications suffered throughout evolution, creates opportunities and a conundrum: They are clearly very important and versatile, yet this very commonality and prevalence has somewhat obscured specific zinc-finger containing genes and their functions. In *Drosophila*, close to one hundred genes coding for ZF and Zinc finger-associated-domain (ZAD) are known, but few have been extensively studied.

Here we characterize one such gene: *piragua*, that illustrates their versatility: It is required multiple times, during gamete formation and embryogenesis (oogenesis, and late embryogenesis: Doral closure and head involution) and imaginal development (dorsal thorax and compound eye development). It also interacts with other genes, like the membrane receptor protein Flower.

1.1. Zinc finger proteins

The *Drosophila piragua* gene codes for a protein with nine instances of the abundant Cys_2His_2 zinc finger (C_2H_2-ZF) protein motif (Lander et al., 2001; Andreini et al., 2006). The C_2H_2-ZF motif bound by coordination to a zinc ion forms a digit-like shape in space that can interact with DNA, RNA, and proteins (Gamsjaeger et al., 2007; Matthews et al., 2000; Matthews and Sunde, 2002; Razin et al., 2012). C_2H_2-ZF encoding genes may harbor another zinc ion-bound-by-coordination motif, the ZAD or C_4DM (Chung et al., 2002). The ZAD domain forms a cloverleaf structure in space coordinated by a zinc ion and four cysteine residues.

Identified mutations in their ZAD or ZF domains gives rise to loss of function alleles (Chen et al., 2000). The Grauzone ZAD motif is involved in protein-protein interactions (Chang et al., 2010; Crozatier et al., 1992; Gaszner et al., 1999; Gibert et al., 2005). By extension, ZAD motifs in other proteins are thought to be protein-protein interaction modules. Recently, consensus binding DNA sequences for target genes were obtained for 21 ZAD-ZF-containing genes, using GST-chimeras, electrophoretic mobility shift assays, and bioinformatics, showing that these 21 genes are in general early embryonic regulators (Krystel and Ayyanathan, 2013).

Despite commonality of ZAD-ZF genes and efforts to characterize their function (Jauch et al., 2003; Krystel and Ayyanathan, 2013),

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relatively little is known about them. Some might have redundant functions (Chung et al., 2007). ZAD-ZF genes expression patterns in *Drosophila* embryos tend to be widespread. This might suggest, likewise, widespread functions in developmental processes (Knight and Shimeld, 2001)

1.2. piragua in embryonic tissues: dorsal closure and head involution

At mid-embryogenesis, the *Drosophila* embryo undergoes dorsal closure, a process where its dorsal aspect is covered by the future tegument. Lateral epithelia (LaE) stretch dorsally over the amnioserosa (AS); both LaE and AS are ectodermally derived tissues. (Rios-Barrera and Riesgo-Escovar, 2013). The AS cells relax and contract in an oscillatory pattern before and during DC. (Fernandez et al., 2007; Muliyil et al., 2011; Reed et al., 2004; Solon et al., 2009). During DC contralateral postmitotic LaE cell sheets extend in a dorsalward fashion and close the embryo dorsally (Kiehart et al., 2000; Layton et al., 2009; Peralta et al., 2007; Peralta et al., 2008).

Three basic categories of genes are required for DC: Jun-N-terminal kinase (JNK) pathway genes, other signaling genes like *dpp*, *Rho*, and *Dcdc42* and thirdly, cytoskeletal proteins like tubulin, integrins, and a non-muscle myosin. (Bloor and Kiehart, 2002; Dutta et al., 2002; Harden, 2002; Homsy et al., 2006; Jankovics and Brunner, 2006; Riesgo-Escovar and Hafen, 1997a; Riesgo-Escovar and Hafen, 1997b; Riesgo-Escovar et al., 1996; Rios-Barrera and Riesgo-Escovar, 2013; Young et al., 1993; Zeitlinger et al., 1997). Besides these, another ZF protein has been implicated in DC: *cabut*, with three C_2H_2-ZF (Belacortu et al., 2011; Munoz-Descalzo et al., 2007; Munoz-Descalzo et al., 2005).

Head involution starts at about the same time as dorsal closure, and is the rearrangement of tissues at the anterior end of the embryo. This basically involves internalization of the six head segments. There are close parallels between dorsal closure and head involution, including shared genetic components (VanHook and Letsou, 2008). Here we show *piragua* is required for both dorsal closure and head involution.

1.3. piragua in cell-cell competition

Cell-cell competition was discovered many years ago in imaginal disc tissue (Morata and Ripoll, 1975). Since then, several genes have been found to be critical: Minute mutations (mutations in genes encoding ribosome proteins), and *dmyc*, that triggers ribosome biosynthesis (Moreno and Basler, 2004). It is thought that 'fitter' cells incorporate higher amounts of survival factors and signaling molecules, translated in a 'survival code' that is secreted extracellularly, read by competing cells, leading to the culling of 'loser' or slower growing cells (Rhiner and Moreno, 2009). In a screen designed to isolate genes expressed early in 'loser' cells, *prg* levels were found to increase, and, via RNAi experiments, to be required for apoptosis of imaginal 'loser' cells. Among six such genes, *prg* was the sole transcription factor (all remaining five genes were membrane proteins) (Rhiner et al., 2010; Rhiner and Moreno, 2009).

In the same cell-competition screen where *prg* was isolated, another gene, *flower* (*fwe*), was isolated that had been cloned before (Yao et al., 2009a). *fwe* is considered the best-characterized cell-competition gene (Casas-Tinto et al., 2011; Merino et al., 2013; Moreno and Rhiner, 2014; Rhiner and Moreno, 2009). *fwe* loss of function homozygous mutant cells in clones in developing imaginal tissue fail to suffer apoptosis. Here we show that *prg* and *fwe* interact genetically.

1.4. piragua in imaginal tissues: dorsal thoracic closure and eye development

Published RNAi experiments driving two *prg* RNAi constructs in the dorsomedial portion of embryos, larvae, and adults using the *pnr-Gal4* (pnr^{MD237}) line lead to adult flies that showed lack of thoracic bristles and pigmentation defects in the thorax and pupal death (Mummery-Widmer et al., 2009). Observation of the published mutant phenotypes also shows thoracic clefts. Here we observe lack of bristles and thoracic clefts in *piragua* mutant clones. Compound eye development is a classic and very well described structure (Bate and Martinez Arias, 1993). Here we report a surprising mutant phenotype: Lack of bristles, gross external cone malformations, and extensive tissue disarray in *prg* mutant clones.

2. Results

2.1. prg locus and mutants

We have isolated mutations by imprecise P-element excision in a locus we named *piragua* (*prg*). *piragua* means small boat in Spanish. A small fraction of mutant embryos sport a hole in the dorsal aspect of the cuticle, resembling such a vessel. *prg* is located (locus CG9233 in FlyBase (2003)) on the left arm of the second chromosome at 29D1 (2L: 8,464,488 to 8,466,694) (Fig. 1A). The *prg* locus theoretically codes for two transcripts of 2149 and 1984 bp long, respectively, differing only in the length of the 3′ trailer sequences (these transcripts are also known as *fu2*). There are two reported fully sequenced cDNAs for *prg* (BT025217 and RE69756), of 1997 bp each. *prg* encodes a hypothetical protein of 558 aminoacids (aa) encoding an amino terminus ZAD domain and nine classical C_2H_2 zinc fingers, with no close homologs in non-dipteran species (Fig. 1B).

Prg is evolutionarily conserved in Drosophilids and Dipterans (Fig. S1). The first six Prg ZF are contiguous and separated from the other three ZFs by 40 aa. The Prg ZAD domain is in the first 90 aa of the putative protein, containing a very conserved arginine between the first two cysteines (Figs. 1B and S2).

We generated three mutant *prg* alleles (prg^1, prg^2, and prg^3) by excision of the P-element transposon in the $P\{GT1\}fu2^{BG02741}$ strain whose insertion point is in the *prg* transcription unit (Fig. 1A, *prg* schematic). This P-element is inserted in the 5′ leader sequence of the transcript (at 2L: 8,464,503, 15 nucleotides downstream from the proposed transcription star site) (Fig. 1A). This stock is viable and fertile and has no mutant phenotypes. All three mutant alleles derived from $P\{GT1\}fu2^{BG02741}$ are homozygous lethal. The three alleles fail to complement each other.

Sequencing of the locus failed to reveal molecular abnormalities in prg^1 and prg^2, arguing that the defects were not present in the coding region. Semi-quantitative RT-PCR failed to reveal significant reductions of *prg* mRNA in prg^1 and prg^2 mutants. In contrast, prg^3 mutants have a significant reduction of *prg* mRNA, strongly arguing that locus CG9233 codes for *prg* (Fig. 1C).

In addition, prg^3 has point mutations not present in flies with the same genetic background (prg^1 and prg^2) and the wild type control. These point mutations in prg^3 are present in the promoter region upstream of the start point of transcription, plus two missense point mutations in the ORF, that change an aspartic acid (D) to an alanine (A), and a glutamic acid (E) to an aspartic acid (D) at positions 57 and 76 respectively, both within the ZAD domain. The E to D change at position 76 most likely does not affect Prg function, as a D is found (instead of an E as in *D. melanogaster*) in all *Drosophila* species surveyed with a recognizable Prg homologue (Fig. S2). In contrast, the 57 D position is conserved in six *Drosophila* species with an identifiable Prg homologue. Three others have a conservative change to E. A polar aminoacid is, thus, very conserved in position 57 in 10 orthologs of *Drosophila* (the *Drosophila virilis* ortholog does not have a ZAD domain). Other *D. melanogaster* ZAD containing genes also have an E/D residue in the equivalent position. This residue is found in the context of an evolutionarily conserved VQYER motif, probably important for ZAD function. Despite having a tyrosine residue in the middle, this motif is not predicted for phosphorylation. A non-conservative change at 57 D to alanine would likely alter function, and may account for the prg^3 phenotype, together with the significant reduction in expression of the mRNA (Figs.