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Gene induction following wounding of wild-type versus macrophage-deficient *Drosophila* embryos

Brian Stramer[1,5+], *Mark Winfield*[2], *Tanya Shaw*[1], *Thomas H. Millard*[3], *Sarah Woolner*[1,4] & *Paul Martin*[1,3]

[1]Department of Physiology, [2]Department of Biological Sciences, and [3]Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol, UK, [4]Department of Zoology, University of Wisconsin-Madison, Madison, Wisconsin, USA, and [5]Royal Veterinary College, Veterinary Basic Sciences, London, UK

By using a microarray screen to compare gene responses after sterile laser wounding of wild-type and 'macrophageless' *serpent* mutant *Drosophila* embryos, we show the wound-induced programmes that are independent of a pathogenic response and distinguish which of the genes are macrophage dependent. The evolutionarily conserved nature of this response is highlighted by our finding that one such new inflammation-associated gene, growth arrest and DNA damage-inducible gene 45 (*GADD45*), is upregulated in both *Drosophila* and murine repair models. Comparison of unwounded wild-type and *serpent* mutant embryos also shows a portfolio of 'macrophage-specific' genes, which suggest analogous functions with vertebrate inflammatory cells. Besides identifying the various classes of wound- and macrophage-related genes, our data indicate that sterile injury *per se*, in the absence of pathogens, triggers induction of a 'pathogen response', which might prime the organism for what is likely to be an increased risk of infection.

Keywords: repair; inflammation; hemocyte; antimicrobial; GADD45

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INTRODUCTION

Similar to vertebrates, the survival of insects after tissue damage is critically dependent on a rapid repair response to seal the injured tissue layers and on an immune response that is necessary to prevent microbial invasion and subsequent sepsis. In vertebrates, a series of leukocytic lineages diapedese from nearby blood vessels and migrate to sites of tissue damage where they kill microbes, clear away cell and matrix debris, and release a plethora of signals that act on local cells at the wound margin. In the *Drosophila* embryo, haemocytes (*Drosophila* macrophages) show a similar rapid response to wounding. This intimate association between tissue damage and an immediate 'inflammatory' response has made it difficult to distinguish which of the vast array of genes upregulated at a wound site are triggered directly by wounding and which are an indirect consequence of the wound-associated inflammatory response.

Numerous studies have examined the genome-wide *Drosophila* response to septic injury and these have been instrumental in our understanding of the genetics of innate immunity, showing, for example, the crucial role of Toll receptor signalling in the immune response (De Gregorio *et al*, 2002). However, these studies do not differentiate between the response to tissue injury and the response to infection. Furthermore, without spatial resolution, array studies cannot determine whether increased gene expression is due to upregulation by local wound tissues, by activated haemocytes or by other immunosensitive tissues such as the fat body.

By using mutant *Drosophila* embryos that lack haemocytes and a laser wounding model that creates a sterile epithelial wound, we have recently disassociated inflammation from tissue repair and shown that in flies, as in mice that are genetically impaired in their inflammatory response (Martin *et al*, 2003), wound healing does not seem to be absolutely dependent on a cellular immune response (Stramer *et al*, 2005).

Here, we used microarray analysis to compare the transcriptional profiles of unwounded and wounded wild-type and haemocyte-null embryos. Besides identifying new haemocyte and wound-specific genes, this approach distinguishes which of the wound-induced genes are triggered by inflammatory signals and which are independent of haemocytes. Furthermore, this screen shows that in the absence of pathogenic infection, mechanical wounding is able to induce an antibacterial response that might prime the organism to fight what is perceived to be an increased likelihood of infection.

RESULTS AND DISCUSSION

To investigate haemocyte-specific genes and the macrophage-dependent and macrophage-independent gene induction after wounding of *Drosophila* embryos, we used a microarray approach to compare unwounded and wounded embryos that were either wild-type or *serpent* (*srp*) mutant and lacked the haemocyte

[1]Department of Physiology, [2]Department of Biological Sciences, and [3]Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK
[4]Department of Zoology, University of Wisconsin-Madison, 1117 West Johnson Street, Room 120, Madison, Wisconsin 53706, USA
[5]Royal Veterinary College, Veterinary Basic Sciences, Royal College Street, London NW1 0TU, UK
[+]Corresponding author. Tel: +44 (0) 207 121 1905; Fax: +44 (0) 117 928 8923; E-mail: bstramer@rvc.ac.uk

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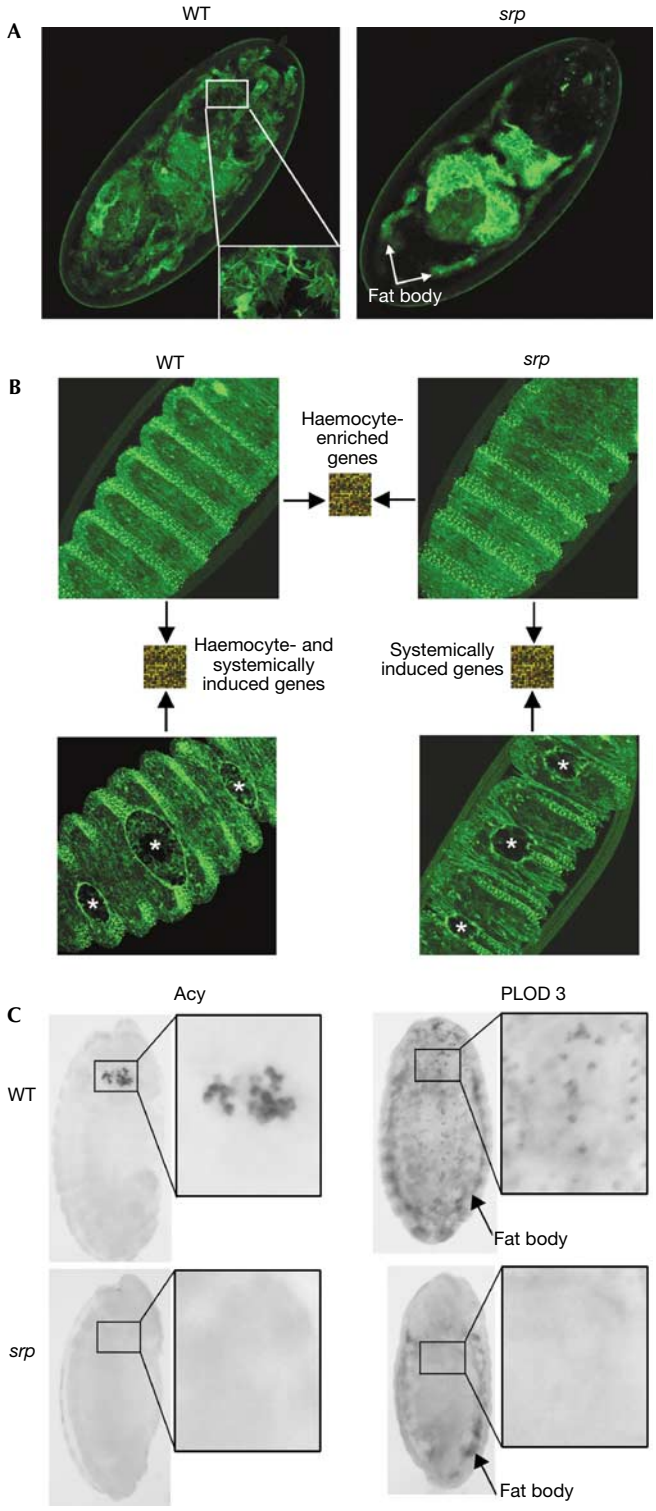

Fig 1 | Microarray schematic to elucidate haemocyte-specific, wound-induced and inflammation-dependent genes. (**A**) To illustrate the absence of macrophages without loss of other srp-dependent tissues, we used an srp-gal4 driver to drive GFP in wild-type (WT) and srp^{AS}/srp^{3} mutants. Wild-type embryos show a labelled fat body and large numbers of haemocytes (wild-type inset), whereas *srp* mutants have only a labelled fat body. (**B**) RNA from unwounded wild-type and *srp* mutant embryos was compared by using microarray analysis to uncover haemocyte-enriched genes. RNA from unwounded wild-type embryos and embryos with three wounds (indicated by asterisks) was compared to elucidate haemocyte-induced and systemically induced wound genes, whereas RNA from wounded and unwounded *srp* mutant embryos was compared to show only inflammation-independent genes. All embryos in this figure constitutively express GFP-moesin, which shows both the wounded epithelium and recruited haemocytes. Note the absence of *Drosophila* macrophages in *srp* wounds. (**C**) Procollagen-lysine dioxygenase 3 (PLOD3) was expressed in wild-type embryos by the fat body and plasmatocytes, whereas in *srp* mutants it was expressed only by the fat body. Aminoacylase (Acy) was expressed by the crystal cell haemocyte subpopulation in wild-type embryos and was absent in *srp* mutants. GFP, green fluorescent protein; srp, serpent.

lineage (Fig 1A). Homozygous mutant embryos of the strong srp^{3} allele die during embryogenesis as a result of a failure in germ band retraction, whereas srp^{AS}/srp^{3} embryos maintain srp expression in all the wild-type embryonic expression zones except for the head mesoderm from which the haemocyte primordia are derived (Rehorn *et al*, 1996). This results in an embryo that seems to be morphologically normal, with the majority of *srp*-dependent tissues, including the fat body, present, but which lacks haemocytes entirely (Fig 1A).

Many wild-type enriched genes are expressed by haemocytes

We began our microarray analysis by comparing the RNA profiles of unwounded wild-type versus *srp* mutant embryos (Fig 1B). Despite the fact that haemocytes make up only a small percentage of the total population of cells in a *Drosophila* embryo, our array comparison seemed to be an efficient filter for identifying haemocyte-specific genes. Approximately 20% of the transcripts we found to be expressed $\geqslant$1.5-fold higher in wild-type versus *srp* unwounded embryos have previously been shown to be enriched in haemocytes (supplementary Table 1 online). Furthermore, approximately 8% of the differentially expressed genes encode proteins with a likely role in haemocyte function or immunity in larvae and adult flies (supplementary Table 2 online). Others with no previous link to haemocytes or infection are shown here to be expressed by either plasmatocytes or crystal cells, two subpopulations of haemocytes (Fig 1C).

Of those genes not previously linked to haemocytes, several encode proteins with likely roles in the normal functioning of these cells during embryonic development. For example, haemocytes are known to be the principal synthesizers of extracellular matrix (ECM) and to lay down embryonic basement membranes (Olofsson & Page, 2005); therefore, it is not surprising that one of our haemocyte-enriched genes is an orthologue of human procollagen-lysine dioxygenase 3 (PLOD3), which is involved in post-translational modification of collagens for normal basement membrane maturation (Myllyla *et al*, 2007). For other genes suggested to be haemocyte enriched by our microarray analysis, mutant studies showed reduced haemocyte numbers during embryogenesis; for example, the cell-cycle regulator string (cdc25; Milchanowski *et al*, 2004). The wild-type versus *srp*

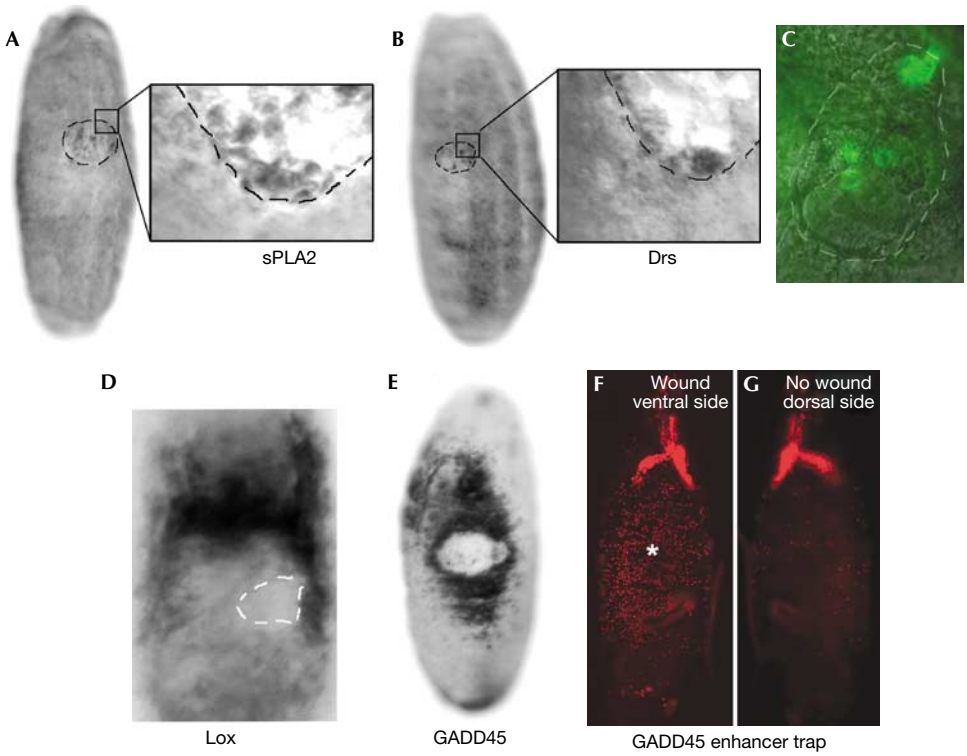

Fig 2 | *In situ* hybridization showing the expression domains of wound-induced genes 90 min after injury. Wound-activated haemocytes express (**A**) phospholipase A2 (sPLA2) and (**B**) the antimicrobial peptide drosomycin (Drs). (**C**) Drs induction by haemocytes is confirmed by wounding of the Drs-GFP reporter line. (**D**) Lox induction is seen in the wound epithelium and the underlying fat body. (**E**) *In situ* hybridization shows that GADD45 is induced in a broad band of epithelium spreading back from the wound edge. (**F**,**G**) A GADD45 enhancer trap line confirms GADD45 expression extending from the wound site (**F**), but this inductive wave fails to spread to the unwounded side of the embryo (**G**). The asterisk indicates a closed wound and the dotted lines indicate the wound site. GADD45, growth arrest and DNA damage-inducible gene 45; GFP, green fluorescent protein.

comparison also identified genes that indicate likely functional parallels between *Drosophila* immune cells and vertebrate leukocytes. For example, several glutathione-*S*-transferases (supplementary Table 3 online) were shown to be enriched in *Drosophila* macrophages, reflecting the high glutathione levels of haemocytes (Tirouvanziam *et al*, 2004), and this parallels the importance of glutathione for vertebrate immune function (Droge & Breitkreutz, 2000).

Identifying wound-induced genes

Next we used microarray analysis to compare the RNA profiles of wounded versus unwounded wild-type and *srp* mutant embryos. To increase the number of wound-activated cells per embryo, and thus reduce the dilution of wound-induced genes, we generated three large laser wounds in each embryo. Despite their large size, each of these wounds healed as efficiently as smaller wounds (Stramer *et al*, 2005). Even with three wounds, there are inevitable effects of dilution and, as a result, this microarray comparison yielded few significantly upregulated or downregulated genes. However, this strategy does enrich for wound-induced genes, and we have gone on to screen and elucidate the tissue-specific induction of several candidates identified in this way by using *in situ* hybridization. These studies showed several genes that were expressed by haemocytes at the wound site. Examples of such wound-activated haemocyte genes are secreted phospholipase A2 (*sPLA2*; CG14507) and the antimicrobial peptide drosomycin (*Drs*; Fig 2A–C). Induction of genes such as phospholipase A2 suggests the existence of evolutionarily conserved inflammatory signals, as the phospholipase A2 gene family is pivotal in the production of eicosanoids during the mammalian repair response and, in this way, regulates several aspects of leukocyte behaviour (Ninnemann, 1988). Interestingly, there is also evidence that eicosanoids might be involved in insect haemocyte immune responses (Stanley-Samuelson *et al*, 1991).

Our screen also showed several genes induced in the wounded epithelium or systemically in other distant tissues. A lysyl oxidase-like gene (CG11335) was induced by epithelial cells as well as other tissues during the repair process (Fig 2D). Lysyl oxidases are involved in crosslinking elastin and collagen molecules and this might be important in repairing ECM damage (Kagan & Li, 2003). Also upregulated by the wound epidermal cells was the *Drosophila* orthologue of the growth arrest and DNA damage-inducible gene 45 (*GADD45*; CG11086; Fig 2E–G), which is a known stress response gene with an ability to regulate MAP-kinase signalling (Takekawa & Saito, 1998) and thus affect, in several crucial ways, the repair process. It is intriguing that, in mammalian cells, GADD45 has been shown to 'unsilence' genes by excisional repair-dependent DNA hypomethylation (Barreto *et al*, 2007) and, although it is generally believed that the *Drosophila* genome is not regulated by methylation, it might be the case that GADD45 has

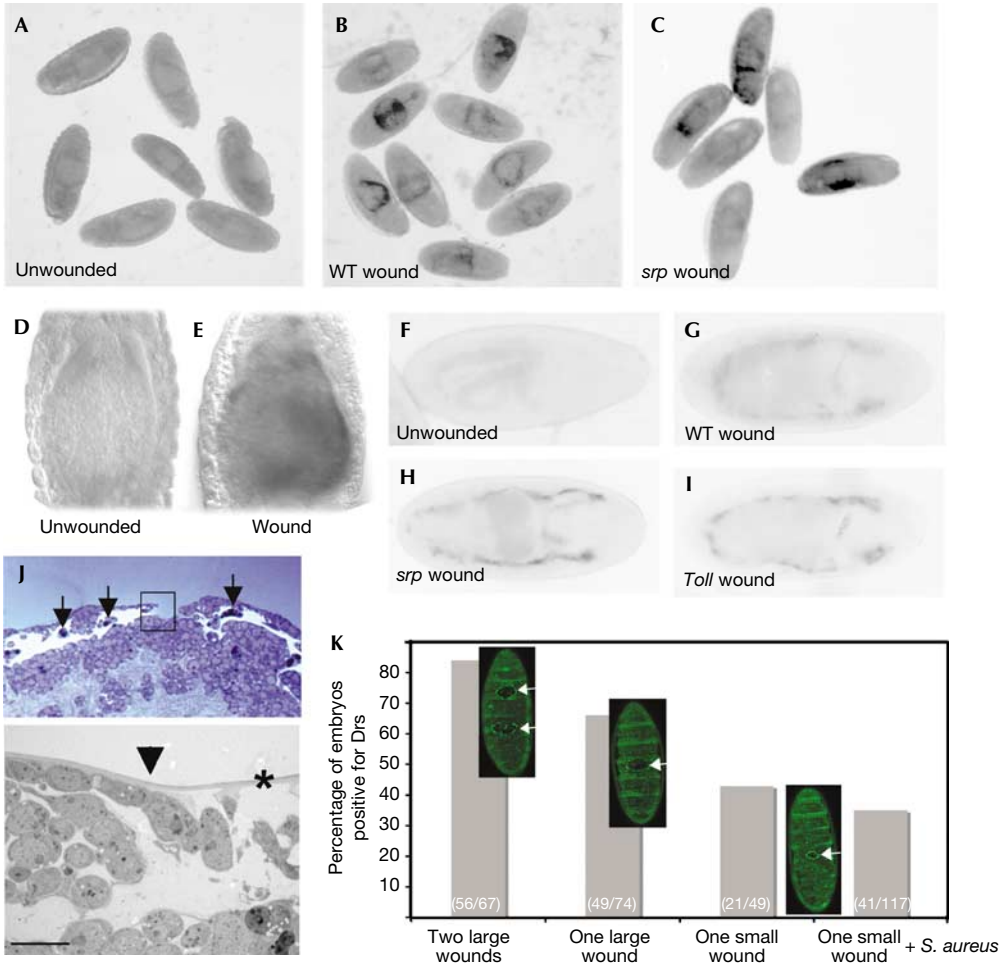

Fig 3 | Systemic induction of drosomycin after sterile wounding. *In situ* hybridization of (**A**) unwounded, (**B**) wounded wild-type (WT) and (**C**) *srp* mutant embryos shows that Drs is upregulated in the yolk after injury independent of haemocytes. (**D**,**E**) High-magnification view to compare Drs expression in the yolk of an unwounded and wounded embryo. (**F–I**) A Drs-GFP reporter line (colour inverted) shows that from stage 17, the fat body also upregulates Drs after laser wounding of (**G**) wild-type, (**H**) *srp* and (**I**) *Toll* mutants. (**J**) A methylene blue-stained resin section through a 90 min wound with recruited haemocytes indicated by arrows (top panel). The inset corresponds to an electron microscopic view (lower panel) illustrating how the vitelline membrane (indicated by the asterisk) remains intact beyond the epithelial wound edge (arrowhead). Scale bar, 5 μm. (**K**) Wound severity correlates with fat body expression of Drs. Two large wounds induced the Drs reporter in 84% of embryos, one large wound induced 66%, one small wound 43% and one small wound + *Staphylococcus aureus* 35%. GFP-moesin embryos were wounded to highlight wound sizes. Drs, drosomycin; GFP, green fluorescent protein; srp, serpent.

some role in the epigenetic regulation of wound target genes in migrating epidermal cells.

Drs, which is expressed by haemocytes at the wound site, was also systemically induced in the yolk after wounding (Fig 3A–E). An immune role for the embryonic yolk nuclei is consistent with the finding that cecropin, another antibacterial gene, is similarly expressed in this tissue during embryonic infection (Tingvall *et al*, 2001). We found that in older embryos (stage 17), the fat body, which is the main immune responsive tissue in the adult fly, also gained the ability to upregulate Drs after laser wounding (Fig 3F,G) in a haemocyte-independent manner (Fig 3C,H). However, diptericin, an antimicrobial peptide that is regulated by a different immune response pathway (De Gregorio *et al*, 2002), was not induced in the fat body after wounding in a diptericin-green fluorescent protein (GFP) reporter line (data not shown).

Although we believe that our laser wounds were sterile and did not seem to breach the vitelline membrane (Fig 3J), we cannot completely exclude the possibility that some pathogens were present on or within the vitelline membrane, thus inducing Drs after wounding. However, Drs induction after wounding was, at the very least, independent of any present Gram-positive or fungal infection, as *Toll* mutant embryos also expressed Drs following wounding (Fig 3I). We also found an increase in susceptibility to induce Drs within the fat body of embryos with increasing wound severity and number, but preincubation of embryos with a Gram-positive bacteria before wounding failed to increase the Drs response (Fig 3K), showing that the vitelline membrane was still a competent barrier to infection after laser ablation. These data support the idea that tissue damage *per se* might be a crucial component in triggering a pathogen response. Braun *et al* (1998)

showed that although infection without injury was able to induce an immune response, the level of induction was significantly greater when infection was accompanied by injury. A more recent study has shown that survival rates are enhanced by previous wounding, indicating that tissue damage might prime the host's defence to future infections (Apidianakis *et al*, 2005).

'Inflammation'-dependent and -independent wound genes

Microarray comparison of wounded *srp* and wild-type embryos also allowed us to screen which of the wound-induced genes was dependent on an inflammatory response. For example, our array analysis suggested that Lox was equivalently upregulated after wounding of both wild-type and *srp* embryos, and our *in situ* hybridization data confirmed similar expression levels in both genotypes after wounding (Fig 4A). However, it seemed that *Drosophila GADD45* was much more robustly induced by wounding of wild-type embryos, and *in situ* hybridization showed that this gene was strongly inflammation dependent (Fig 4B). Our data suggest that *Drosophila* macrophages might secrete signals that are necessary for full *GADD45* induction in the wounded epithelium. There is precedent for a paracrine signalling role for haemocytes during an immune response; after septic injury, an unpaired (Upd)-like cytokine is secreted by haemocytes and is necessary for Jak/Stat signalling in the fat body (Agaisse *et al*, 2003). Although *GADD45* is not a known Jak/Stat target, it is responsive to Toll signalling (De Gregorio *et al*, 2002). However, *Toll* mutants showed a similar epithelial wound induction of *GADD45* (Fig 4C,D), and expression of an activated form of the Toll ligand, spatzle (spz), in the epithelium of *Drosophila* embryos failed to induce *GADD45* expression (Fig 4E,F), suggesting that *GADD45* induction following wounding is Toll independent.

Our data suggest that *GADD45* is an 'inflammation-associated' wound response gene in insects. To determine whether this response is conserved in mammals, we re-analysed microarray data from an analogous experiment in mice comparing the gene profiles of wounds in the presence and absence of an inflammatory response (Cooper *et al*, 2005). These data showed that a murine homologue of *Drosophila GADD45* (Affymetrix *MGU74A*, 102292_at) was upregulated rapidly after wounding and that this response was much reduced in PU.1 null mice in which inflammatory cells were missing (Fig 4G). The expression of GADD45 protein after injury was examined by western blotting and showed rapid and transient induction by 1 day after wounding (Fig 4H). Furthermore, immunostaining showed that, as in *Drosophila*, murine *GADD45* was induced in the wound epithelium (Fig 4I). This finding provided further evidence for an evolutionarily conserved repair response in flies and vertebrates, and highlights how useful *Drosophila* might be in elucidating new mechanisms regulating various aspects of vertebrate tissue repair.

METHODS

Fly stocks. Mutant embryos for microarray analysis and for *in situ* hybridization were generated by using a heteroallelic combination of alleles srp^{3} and srp^{AS} (Rehorn *et al*, 1996); wild-type embryos were rucuca strain (stock no. 576, Bloomington Stock Center). To visualize Drs (Drs-GFP) and diptericin expression, GFP reporter lines were wounded (Ferrandon *et al*, 1998; Tzou *et al*, 2000). To visualize epithelial wounds and haemocyte recruitment, a fly line expressing moesin fused to GFP was used (Kiehart *et al*, 2000). To examine Drs and GADD45 expression in *srp* and *Toll* mutants, lines containing Drs-GFP; srp^{AS}/TTG (GFP balancer), Tl^{r4}/TTG and Drs-GFP; Tl^{r4}/TTG were created and mutant embryos were selected by their absence of fluorescence. For the GADD45 enhancer trap (stock no. 103594, Kyoto Stock Center, Kyoto, Japan), UAS-red stinger (stock no. 8546, Bloomington Stock Center, Bloomington, IN, USA) was recombined with this line before wounding. To express the spz ligand in the epithelium, an active form of spz (Ligoxygakis *et al*, 2002) was driven in the epithelium with the Engal4 driver.

Laser wounding and microarray analysis. Embryos were collected at stage 15 and wounded by using laser ablation (Wood *et al*, 2002). Embryos were collected after 90 min for *in situ* hybridization and microarray analysis, and total RNA was extracted from collections of 400 embryos for each time point by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the RNeasy Cleanup Kit (Qiagen, Crawley, UK). Complementary DNA was labelled by using amino-allyl reverse transcription labelling. The samples were hybridized to an in-house cDNA array (Fred Hutchinson Cancer Center, Seattle, WA, USA) containing 12,144 probes derived from the following 3 collections: Dgcr1, Dgcr2 and the Northwest Drosophila Microarray Consortium.

For the unwounded wild type versus *srp* comparison, the experiment was conducted twice, yielding two independent replicate measurements. In addition, wounded versus unwounded wild-type embryos and wounded versus unwounded *srp* comparisons were made, and used to screen for inflammation-dependent and inflammation-independent transcripts. Analysis was performed by using the GeneSpring GX programme. Briefly, per spot and per chip intensity-dependent (Lowess) normalization was performed. Transcripts with unreliable scores were removed from the analysis on the basis of the cross-gene error model by using replicate scores. Genes were screened on the basis of *t*-test *P*-values and fold change. All raw microarray data are available in an MIAME-compliant format under accession number GSE10225 of the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/).

To examine Drs and diptericin expression by using GFP reporter lines, stage 17 embryos were wounded and examined 4 h later for fat body expression. To examine GADD45 induction with the enhancer trap line, stage 15 embryos were wounded and imaged after 12 h. For the *Staphylococcus aureus* experiment, embryos were preincubated in 1×10^{7} CFU of *S. aureus* (reference strain ATCC 25923) before wounding.

***In situ* hybridization and electron microscopy.** Immunohistochemical whole-mount *in situ* hybridization was carried out using standard methods (Lehmann & Tautz, 1994) with digoxigenin-substituted RNA probes generated by transcription of expressed-sequence tag clones from the Berkeley Drosophila Genome Project: LP04931 (Lox), RH27007 (CG14507), RE38345 (GADD45), LP03851 (Drs), LD37702 (PLOD3), RE63537 (aminoacylase). Embryos for transmission electron microscopy were frozen under high pressure, and then fixed and stained during the freeze substitution step with osmium tetroxide and uranyl acetate before embedding in resin. Thick sections were stained with methylene blue.

Murine histology, western blotting and immunostaining. Male, 8-week-old ICR mice were used according to UK Home Office regulations. Mice were anaesthetized by halothane inhalation and excisional wounds were made on the shaved back on either side

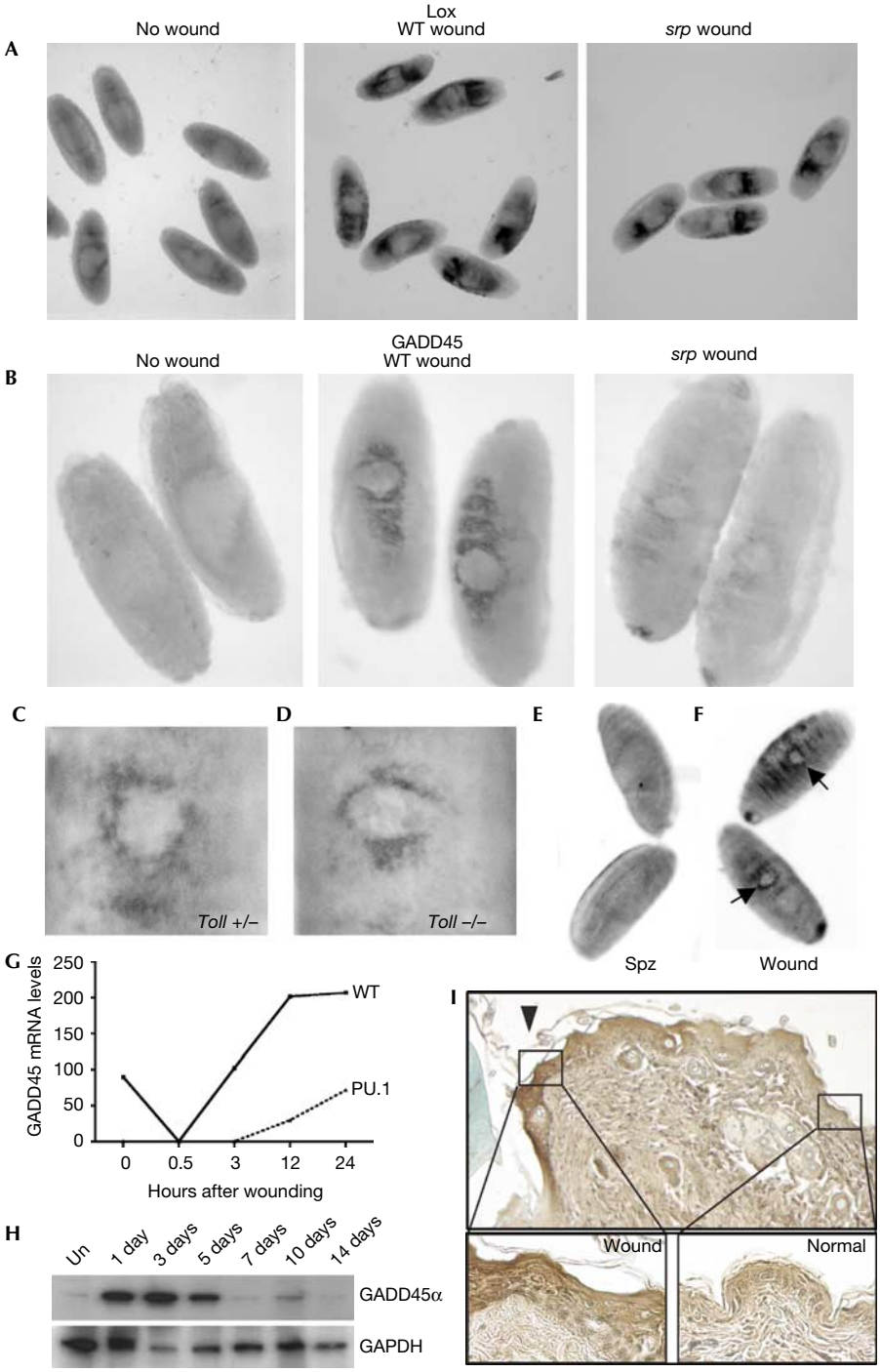

Fig 4 | Inflammation-dependent and inflammation-independent induced genes. (**A**) *In situ* hybridization studies show that Lox is induced by wounding, independent of a haemocyte response. (**B**) By contrast, GADD45 is robustly induced at wounds only in wild-type (WT) but not in *srp* mutant embryos. (**C**) *Toll* heterozygotes and (**D**) *Toll* mutants were wounded to show similar levels of GADD45 induction. (**E**) Ectopic expression of an activated form of spz in the epithelium failed to induce GADD45 expression, (**F**) unless wounded (arrows). (**G**) Temporal GADD45α messenger RNA expression following wounding of wild-type (solid line) and PU.1 mutant (dashed line) murine skin. (**H**) Western blot analysis shows transient GADD45α protein levels following excisional wounding of murine skin. (**I**) Immunostaining of GADD45α in 3-day murine wounds shows induction at the epithelial wound margin (arrowhead). The insets show a high-magnification view of increased GADD45α expression in wounded compared with normal epithelium. GADD45, growth arrest and DNA damage-inducible gene 45; spz, spatzle; srp, serpent; Un, unwounded.

of the dorsal midline with a 4-mm biopsy punch. Tissue was fixed in 10% formalin for embedding in paraffin. Wax sections (6 μm) were immunostained by using a GADD45α antibody, as described previously (Yamasawa *et al*, 2002). Protein samples were separated on Tris-glycine gels (Invitrogen) and blotted according to standard protocols by using GADD45α (Santa Cruz, Santa Cruz, CA, USA) and GAPDH (Abcam, Cambridge, UK) antibodies.
Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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