An Epidermal Barrier Wound Repair Pathway in *Drosophila* Is Mediated by *grainy head*

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We used wounded *Drosophila* embryos to define an evolutionarily conserved pathway for repairing the epidermal surface barrier. This pathway includes a wound response enhancer from the *Ddc* gene that requires *grainy head* (*grh*) function and binding sites for the Grh transcription factor. At the signaling level, tyrosine kinase and extracellular signal–regulated kinase (ERK) activities are induced in epidermal cells near wounds, and activated ERK is required for a robust wound response. The conservation of this Grh-dependent pathway suggests that the repair of insect cuticle and mammal skin is controlled by an ancient, shared control system for constructing and healing the animal body surface barrier.

Animals have evolved biological armor, an epidermally derived integument, to protect their bodies from physical injury and dehydration and have evolved control pathways to regenerate this barrier after wounding. A key component of this barrier in mammals is the stratum corneum of the skin, and a key component of the barrier in insects is the cuticle. In invertebrates, the immediate barrier response to wounding involves the formation of a temporary plug at wound sites, along with the activation of melanization and cross-linking enzymes that encapsulate invading microbes and help seal wound openings (*1*–*3*). In vertebrates, the immediate humoral response to vascular wounding results in the activation of proteases leading to the formation of a fibrin clot to help seal wound openings (*4*).

In both invertebrates and vertebrates, introducing infectious microbes through wounds results in the induction of the innate immune pathways (*5*). In two branches of these pathways, Toll-family transmembrane receptors or Imd-dependent signals trigger a signaling cascade that allows transcription factors from the NF-κB family to enter the nucleus, where they directly activate the transcription of genes that provide a first line of defense against pathogens (*5*–*7*).

Another response to epithelial wounds is mediated by wound healing pathways that re-epithelialize the breach (*8*). Genes that are required for regenerating the epithelial sheet after laser-induced or mechanically induced wounds in the *Drosophila* epidermis include those encoding Rho, Cdc42, and Jun N-terminal kinase (JNK) (*3*, *9*, *10*). Additional *Drosophila* genes have been implicated in the process of epithelial repair by means of their requirement for epidermal dorsal closure during late embryogenesis. These include genes encoding the *Drosophila* Jun and Fos transcription factors as well as Dpp, Ras, and Puckered (*11*, *12*). Homologs of most of these proteins, in addition to many others, are associated with the process of epithelial regeneration in vertebrates (*8*, *11*, *13*, *14*).

By comparison, the genetic pathways that respond to aseptic breaks in the barrier integument and provide for its regeneration are poorly understood. Although the integuments of both mammals and insects depend on a dense, highly cross-linked matrix of proteins and other macromolecules, heretofore there has been no reason to suspect common genetic control pathways in the repair of mammal skin and insect cuticle.

Ectopic sclerites in *Hox* or *spen* mutants correlate with loss of epidermal integrity. *Drosophila* embryos that lack all *Hox* gene function in a body region, or that are mutant for the *Hox*-interacting gene *spen*, develop ectopic sclerites (hard, melanized cuticular structures) in the trunk of first instar larvae (*15*–*18*). These sclerites were proposed to be ectopic head skeleton, but we observed that the sclerites in these mutant larvae often looked like the cuticular scar tissue that often surrounds the healed hole generated by a sterile needle in late-stage embryos (Fig. 1, B to D).

We therefore tested whether the sclerites observed in *spen* or *Scr Antp* double-mutant larvae were associated with breaks in the epidermal integument. To assay this, we injected rhodamine-labeled dextran into the perivitelline space of stage 17 wild-type, *spen* mutant, and *Scr Antp* mutant embryos. As a positive control, we injected rhodamine-dextran directly into the body cavity of a wild-type embryo (Fig. 1H). We observed that the fluorescent dye penetrated the body cavity of *spen* mutant (Fig. 1F) and *Scr Antp* double-mutant (Fig. 1G) embryos but not control wild-type embryos (Fig. 1E). We conclude that there are localized failures of epidermal integrity in late-stage embryos that lack the function of *spen* or both *Scr* and *Antp*.

Sclerotization genes are rapidly activated near aseptic wound sites in late embryos. Next, we tested whether two genes required for normal cuticular sclerotization were activated in the wound regions that developed scars. The genes were *Ddc*, which encodes dopa decarboxylase, and *pale* (*ple*), which encodes tyrosine hydroxylase. These proteins contribute to the formation of the larval and adult cuticular skeleton in epithelial cells through the production of catecholamines that are converted to quinones by phenol oxidases (*19*). The reactive quinones then cross-link protein polymers and chitin polymers to generate the largely impermeable integument of insects (*20*). First instar larvae that are doubly mutant for *Ddc* and *ple* have almost no melanization and sclerotization of the head skeleton (Fig. 2, A and B). A key

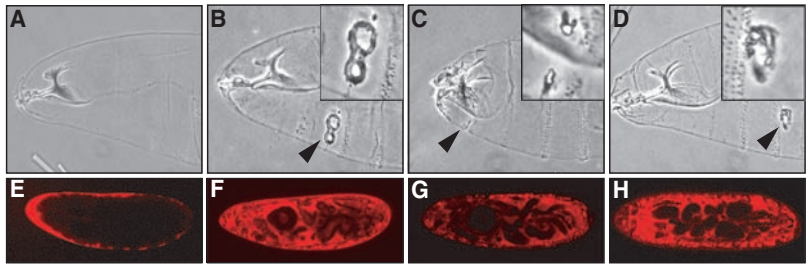

Fig. 1. *spen* mutants and *Scr Antp* double mutants have defective epidermal integrity. Cuticle preparations showing the position of normal and ectopic sclerites in (**A**) a wild-type embryo, (**B**) a *spen* mutant embryo, (**C**) a *Scr Antp* double-mutant embryo, and (**D**) an aseptically injured wild-type embryo. Insets are enlargements of sclerites indicated by the arrowheads. At 17 to 19 hours after egg lay, rhodamine-dextran was injected into the perivitelline space of (**E**) wild-type embryos, (**F**) *spen* mutant embryos, or (**G**) *Scr Antp* double-mutant embryos; or (**H**) directly through the epidermal layer of wild-type embryos. Red stain in the body cavity indicates the presence of breaks in the epidermis.

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regulatory step in the localized deposition of hard, dark cuticle is exerted at the transcriptional level of these genes, given that the hard skeleton–producing cells of the late embryo and early larva accumulate abundant *Ddc* and *ple* transcripts (Fig. 2, C and D).

In aseptically wounded late embryos, transcripts from both *Ddc* and *ple* accumulate to high levels in the epidermal cells near the wound site (Fig. 2, E and F). Transcripts from these genes can be detected within 30 min after injury (*21*), suggesting that these genes are direct targets of a wound-induced signal transduction pathway. Transcription of *Ddc* and *ple* is also abundant in the defective epidermal regions that develop sclerotic scar tissue in *spen* mutants and *Scr Antp* double mutants (Fig. 2, G to J). Control in situ hybridizations were done to eliminate the possibility that the increased *Ddc* and *ple* signals at wound sites were an accessibility artifact in late embryos (*21*).

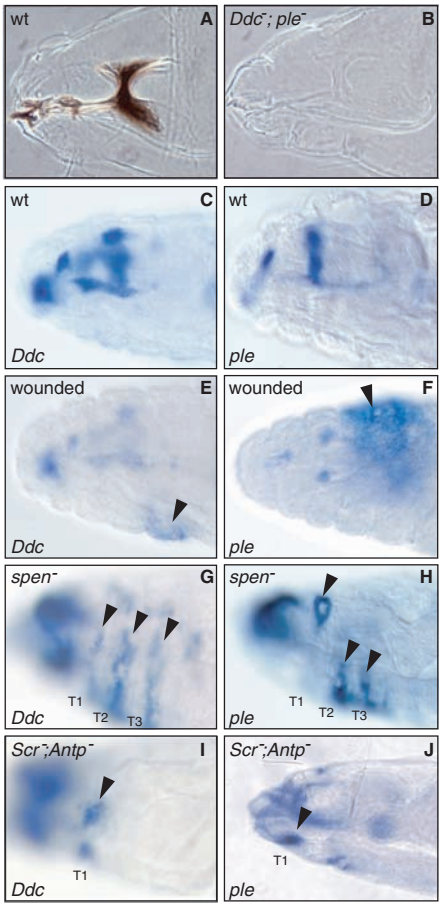

Fig. 2. *Ddc* and *ple* are transcriptionally activated where sclerotized structures develop. Cuticle preparations are shown for (**A**) wild-type (wt) first instar larva and (**B**) *Ddc; ple* double-mutant larva. The rest of the panels show localization of *Ddc* and *ple* RNA with in situ hybridization (*21*) in wild-type embryos (**C** and **D**), aseptically wounded wild-type embryos (**E** and **F**), *spen* mutant embryos (**G** and **H**), and *Scr Antp* double-mutant embryos (**I** and **J**). Arrowheads show ectopic *Ddc* or *ple* transcripts surrounding puncture wounds or epidermal breaks in stage 17 embryos. T1, T2, and T3 mark the first, second, and third thoracic segments, respectively.

A wound response regulatory element upstream of the *Ddc* transcriptional start. To dissect transcriptional regulatory inputs involved in the activation of epidermal wound response genes, we first analyzed the regulatory regions of *Ddc* (*22*, *23*). We began by testing the expression pattern provided by a 7.5-kb segment of DNA that included a hemagglutinin-tagged *Ddc* protein coding sequence (Fig. 3A). This 7.5-kb region provides the normal *Ddc* expression pattern during embryogenesis (Fig. 3B shows the head skeletal pattern) and is also activated near wound sites in late-embryonic epidermal cells (Fig. 3, C and D).

Deletion analyses of lacZ and/or green fluorescent protein (GFP) reporter constructs fused to the *hsp70* basal promoter show that sequences between –1.4 kb to the *Ddc* transcription start are sufficient for a wound response, but sequences from –381 base pairs (bp) to the transcription start are not (Fig. 3, A, E, and F). The –1.4-kb *Ddc*-GFP reporter is activated over many cell diameters near wound sites (Fig. 3G), and the extent of activation increases with larger wounds and longer incubations after injury. The graded nature of the response (Fig. 3G) suggests that a signal is produced at the injury boundaries that activates the wound response enhancer in a dose-dependent fashion in nearby epidermal cells. We also tested the –1.4-kb *Ddc*-GFP reporter (Fig. 3A) in *Scr Antp* double-mutant embryos and found that the wound response reporter is activated in regions where cuticular scars develop (Fig. 3H). Similar results were obtained in *spen* mutants.

Response of the –1.4-kb *Ddc* wound response enhancer in mutants for the NF-κB and AP-1 pathways. To determine whether the aseptic wound response pathway (as defined by the *Ddc* wound response enhancer) and the infectious wound response pathways overlapped, we tested for activation of the –1.4-kb *Ddc*-GFP wound response reporter after aseptic wounding of zygotic mutants of the innate immunity signaling pathway genes *Toll*, *tube*, *imd*, and *18-wheeler* as well as in zygotic mutants of the innate immunity transcription factor genes *rel*, *Dif*, and *Dl* (*Dl* and *Dif* were tested as a double-mutant combination). In all of these mutant backgrounds, the –1.4-kb *Ddc*-GFP reporter was activated near wounds, as it was in wild-type embryos. We also tested *Toll* maternal/zygotic

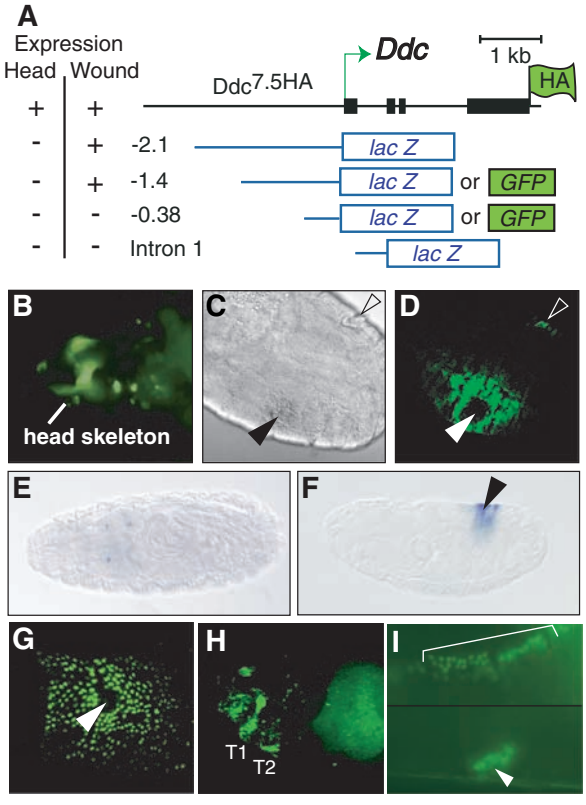

Fig. 3. The region between –1.4 and –0.38 kb of the *Ddc* transcription start site functions as a wound response enhancer. (**A**) Diagram of the *Ddc* locus and the reporter constructs used to identify embryonic transcriptional activation functions. A summary of the expression patterns seen with each *Ddc* regulatory construct is shown to the left of each construct: +, expression; –, no expression. (**B**) Immunofluorescent detection of the hemagglutinin (HA) tag in an embryo carrying the $Ddc^{7.5HA}$ transgene showing normal *Ddc* head skeleton expression (*21*). (**C**) Nomarski image of an aseptically wounded stage 17 $Ddc^{7.5HA}$ embryo. Black and open arrowheads indicate entry and exit wounds, respectively. (**D**) Anti-HA stain of the embryo in (C), showing wound response activation of the $Ddc^{7.5HA}$ transgene. (**E**) Unwounded embryo carrying the –1.4-kb *Ddc-lacZ* reporter, hybridized to detect *lacZ* transcripts (*21*). (**F**) Aseptically wounded embryo carrying the –1.4-kb *Ddc-lacZ* reporter, hybridized to detect *lacZ* transcripts. Black arrowhead, entry wound. (**G**) Closeup of an aseptically wounded embryo carrying the –1.4-kb *Ddc*-GFP reporter (*21*), showing GFP expression in a gradient surrounding the entry wound (white arrowhead). (**H**) *Scr Antp* mutant embryo carrying the –1.4-kb *Ddc*-GFP reporter, showing GFP expression in the first and second thoracic segments (T1 and T2). (**I**) JNK mutant *bsk* with –1.4-kb *Ddc*-GFP reporter; bracket denotes arrested dorsal leading edge of epidermis. Jun mutant *Jra* and Fos mutant *kay* with the same wound reporter show identical staining pattern as *bsk* mutants (*24*).

mutants and *tube* maternal/zygotic mutants, and we observed activation of the wound response reporter at the breaks in the epidermal integument that occur in these mutants. Additional evidence that the *Toll* and *imd* pathways are not required to activate the *Ddc* wound response enhancer is reported in the supporting online material (*21*).

Previous studies have indicated that the JNK pathway is required for the process of wound healing in embryos and adults (*9*–*12*). We found that the –1.4-kb *Ddc*-GFP wound response reporter is still activated at aseptic wound sites of zygotic mutants in either *Drosophila* JNK (Fig. 3I), Jun, or Fos (*24*) and is also activated in cells at and near the dorsal epidermal leading edge "wound boundaries" (brackets in Fig. 3I) that form when dorsal closure fails in these mutant backgrounds. The wound response reporter is not activated in dorsal epidermal leading edge cells in wild-type embryos. We conclude that the zygotic functions of JNK, Jun, and Fos are dispensable for the activation of the –1.4-kb *Ddc* wound response reporter, even in the cells where the zygotic functions of these genes are required for the migration and sealing of epithelial cell sheets during dorsal closure.

***grainy head* is required for activation of the *Ddc* wound response enhancer.** The *Drosophila grainy head* (*grh*) gene encodes two major transcription factor isoforms (Grh-N and Grh-O). Grh-N is expressed in regions of the central nervous system, whereas Grh-O is expressed in barrier epithelia such as the embryonic epidermis, the foregut, the hindgut, and the tracheal system (*25*, *26*). Zygotic mutants in *grh* die at the embryonic/larval transition with weak epidermal cuticle, malformed head skeletons that are composed of discontinuous grainy sclerites, and abnormal tracheal trunks (*26*–*29*). Clones of *grh* mutant cells in the adult epidermis have defects in pigmentation, cell polarity, and epidermal hair differentiation (*30*).

The similarity of the *grh* mutant phenotype to *Ddc* and *ple* mutants prompted us to test whether *grh* function is required for activation of –1.4-kb *Ddc* wound response element, which has two evolutionarily conserved Grh binding sites (Fig. 4A). In aseptically wounded *grh* mutant embryos, the –1.4-kb *Ddc* wound response reporter is at most weakly activated in a few cells immediately adjacent to the wound border (Fig. 4B). This is consistent with the abnormal wound healing in *grh* mutant larval cuticle (Fig. 4, E and F). When compared with wild-type wounds, the *grh* mutant wound sites are deficient in normal cuticle regeneration, as well as in displacement of the melanized plug that forms immediately after wounding (*3*). A similar phenotype is seen in wounded *Ddc* mutants, although the remaining plug is less melanized (Fig. 4G).

Grh and CREB/AP-1–like consensus binding sites are required for the function of *Ddc* wound response enhancers. The −1.4-kb *Ddc* wound response enhancer has consensus transcription factor binding sites for Grh, NF-κB/Rel proteins, adenosine 3′,5′-monophosphate response element–binding protein (CREB)–A proteins, and AP-1–like/basic-leucine zipper proteins (Fig. 4A), which we believed were candidate sites required for the function of the wound response enhancer. Transgenic embryos for the –1.4-kb *Ddc*-GFP reporter with point mutations in the six consensus sites for NF-κB family proteins (fig. S1) showed normal wound-induced activation (*24*), whereas a similar reporter with point mutations in the single CREB-A and the three AP-1–like consensus sites showed a marked reduction in wound-induced activation compared with wild-type reporter controls (Fig. 4C).

Further deletion analyses define a minimal *Ddc* epidermal wound response element from –472 bp to the start of transcription, in which sequences from –472 to –381 bp are required for wound response function (Fig. 4, A and D). The –0.47-kb *Ddc* wound response element from *D. melanogaster* has only five blocks of marked sequence conservation (a perfect match of 6 bp or greater; fig. S1) with a *D. virilis Ddc* promoter proximal fragment from base pairs –392 to +13, which provides a wound response when attached to reporter genes in *D. melanogaster* embryos (Fig. 4, A and H). The blocks of sequence conservation are each 12 to 13 bp and they include one Grh site, one AP-1 consensus site, one ETS consensus site, a GGGGGATT motif (which overlaps with one of the NF-κB consensus sites), and the TATA box region (fig. S1). In *D. melanogaster*, the conserved GGGGGATT motif, AP-1–like site, and ETS site are all within the interval of –472 to –381 bp that is required for wound response function. The conserved Grh site, which is closer to the promoter, is required for the –472-bp element function, given that its mutation abolished wound response reporter activation (Fig. 4, A and I).

To identify potential wound response enhancers at the *D. melanogaster ple* gene, we scanned the sequences from 10 kb upstream to 10 kb downstream of the *ple* transcription start for clusters of evolutionarily conserved Grh, AP-1, ETS, and GGGGGATT sites and selected two regions. One is a 3.0-kb DNA fragment beginning 2.9 kb upstream of the *ple* transcription start, which contains two Grh sites, two AP-1 sites, four ETS sites, and three GGGGGATT sites (fig. S2). The second is a 995-bp fragment just upstream of the *ple* transcription start, which conserves one Grh, one AP-1, and three ETS sites but no GGGGGATT motif (fig. S3). We tested both fragments in red fluorescent protein (DsRed) reporter constructs (*31*) and

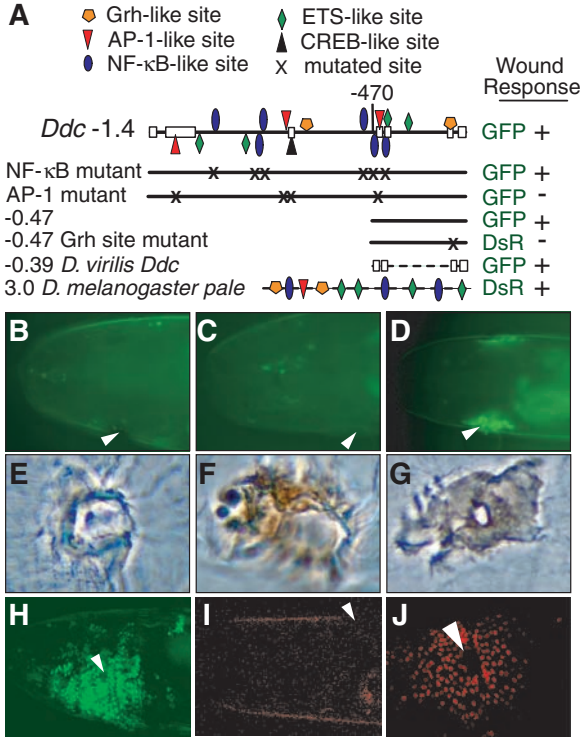

Fig. 4. Analysis of *Ddc* and *ple* wound response enhancers. (**A**) Schematic of *Ddc* and *ple* reporter constructs. Sequence blocks conserved between *D. melanogaster*, *D. pseudoobscura*, and *D. virilis* (figs. S1 and S2) are indicated by white blocks on construct –1.4, as are consensus Grh (orange pentagons), CREB (black triangle), AP-1–like (red triangles), NF-κB (blue ovals), and ETS sites (green diamonds). Mutated consensus sites are indicated by X's on the pertinent constructs, and a summary of the reporter response to aseptic wounding is shown in the column to the right of the construct. (**B** to **D**) GFP reporter expression patterns after aseptic wounding (arrowheads indicate wound sites) of stage 17 embryos with the following genotypes: (B) *grh* mutant with the –1.4-kb *Ddc*-GFP construct; (C) wild-type embryo with the –1.4 kb $^{mutAP\text{-}1\text{–}like}$*Ddc*-GFP construct, which has one CREB and three AP-1 consensus sites mutated (*21*); and (D) wild-type embryo with the –0.47-kb *Ddc*-GFP construct, the smallest sequence sufficient for a wound response. (**E** to **G**) Cuticle surrounding wound sites in representative early first instar larvae after wounding with a sterile micropipette at embryonic stage 17 (*21*) (E) wild-type embryo, (F) *grh* mutant, (G) *Ddc* mutant (*21*). (**H**) Wild-type embryo with the *D. virilis* –392-bp *Ddc*-GFP wound response enhancer (fig. S1). (**I**) Wild-type embryo with the –0.47-kb *Ddc*-GFP construct in which the Grh binding site is mutated (fig. S1). (**J**) Wild-type embryo with the *ple* 3.0-kb wound response enhancer (fig. S2).

found that the 3.0-kb *ple* fragment robustly activates reporter expression around aseptic wounds (Fig. 4, A and J), whereas the 995-bp fragment shows a very weak and slow response (*24*).

Activation of ERK-MAP kinase is required for robust wound enhancer function. The involvement of mitogen-activated protein (MAP) kinases in epithelial injury response (*32*) prompted us to test for receptor tyrosine kinase or MAP kinase activation in cells that induce wound response enhancers. Using antibodies directed against phosphotyrosine (p-Tyr), we found an increase in p-Tyr staining in the cells near aseptic wounds (Fig. 5B), as well as in the wounded thorax of *spen* mutants (Fig. 5C), when compared with controls (Fig. 5A). This increase in p-Tyr correlates well with cells that activate the –1.4-kb *Ddc*-GFP wound response construct (green), although at the times we could test for both (2 hours postwounding), some cells showed activation of the wound response enhancer without a detectable increase in p-Tyr.

To assay MAP kinase pathway activation near embryonic wound sites, we used antibodies that detect the active, dually phosphorylated form of ERK (dpERK). As shown in Fig. 5, D to F, there is an increase in activated ERK surrounding embryonic wound sites. ERK activation is detected within 30 min after aseptic injury, with a variable extent of activation. In *grh* mutant embryos, increased dpERK staining was still detected at wound sites (Fig. 5G), suggesting that dpERK is upstream of, or acting in parallel to, Grh in the barrier wound response pathway. Control antibody stains (*21*) were done to eliminate the possibility that the increased pTyr and dpERK staining at wound sites was an accessibility artifact.

Mutant alleles that eliminate ERK function in embryos but still develop into first instar larvae are not currently available. To test whether activated ERK is required for the wound response, we injected PD98059, a MAP kinase kinase (MEK) inhibitor that reduces the phosphorylation and activation of the ERK-MAP kinase (*21*), into the perivitelline space of late-stage embryos carrying the –1.4-kb *Ddc*-GFP wound response reporter. After a 45-min incubation, the embryos were aseptically wounded, and 4 hours later they were assayed for activation of the wound response reporter (*21*). Compared with controls (Fig. 5H), the activation of the *Ddc* wound response reporter was less frequent and at lower levels (*21*) in the PD98059-treated embryos (Fig. 5I).

Discussion. We provide evidence for a wound response pathway that senses aseptic breaks in the barrier epidermis of *Drosophila* embryos. A minimal 472-bp *Ddc* wound response enhancer requires a Grh protein binding site and the function of the *grh* gene, and all of the naturally evolved *Drosophila Ddc* or *ple* wound response enhancers we tested contain evolutionarily conserved consensus binding sites for Grh, ETS, and AP-1 transcription factors, as well as GGGGGATT sequence motifs (Fig. 5J and figs. S1 and S2) (*21*). Robust induction of *Ddc* wound response reporters also requires activated ERK kinase, which may transduce the wound signal to Grh, given that Grh protein can be phosphorylated by ERK in vitro (*33*). The signaling pathway components that lead to activation of the Grh-mediated wound response need further investigation, because the molecules that sense breaks in the epithelial integument and transmit this information to the *Ddc* wound enhancer in adjacent cells are still unknown.

A major biological role of the Grh-mediated activation of *Ddc* and *ple* near *Drosophila* wounds is apparently to provide the abundant cross-linking molecules that repair and replace the cuticular barrier. This integument repair process is apparently often too vigorous at the borders of cuticular wound sites, resulting in scars (Fig. 1). Larvae that do not rapidly repair the integument as a result of the absence of phenol oxidase in the cuticle and epidermis (*3*, *34*) die rapidly from blood loss (*3*).

The expression patterns and functions of structural orthologs of the *grh* gene in other species are consistent with the evolutionary conservation of this barrier wound response pathway. The *C. elegans* genome encodes an ortholog of the *Drosophila* Grh protein called Ce-GRH-1 (*35*). Depletion of Ce-GRH-1 function with RNA interference results in larvae with fragile epidermal cuticle, similar to the larval cuticle phenotype of *Drosophila grh* mutants. There are three mouse orthologs of *grh*, which are expressed abundantly in developing epithelial cells (*36*–*39*). Interestingly, in a study complementary to ours, a mutation in one of the mouse *grh* orthologs, *Grainy head–like 3* (*Grhl-3*), results in embryos with defective epidermal wound repair (*40*). Grhl-3 function is also required in developing embryos for the epidermal expression of *Transglutaminase 1*, which is required for epidermal protein barrier cross-linking, similar to *Ddc* and *ple* in flies. Thus, although *grh* orthologs are required for a diverse set of functions in animal epithelial cells, one evolutionarily conserved function in mice and flies is a *grh* requirement for transcriptional activation of epidermal barrier cross-linking genes, and the regeneration of an epidermally derived barrier after wounding. The ability to study a conserved barrier wound response pathway with *Drosophila* genetics may provide new insight into wound healing pathways in mammals.

Our evidence indicates that activation of the *Drosophila Ddc* wound response enhancer does not require the zygotic functions of either the signaling proteins and transcription factors that mediate the response to septic injury, or the JNK pathway that mediates re-epithelialization of wounds. However, our current assays of embryonic wound response enhancers are only

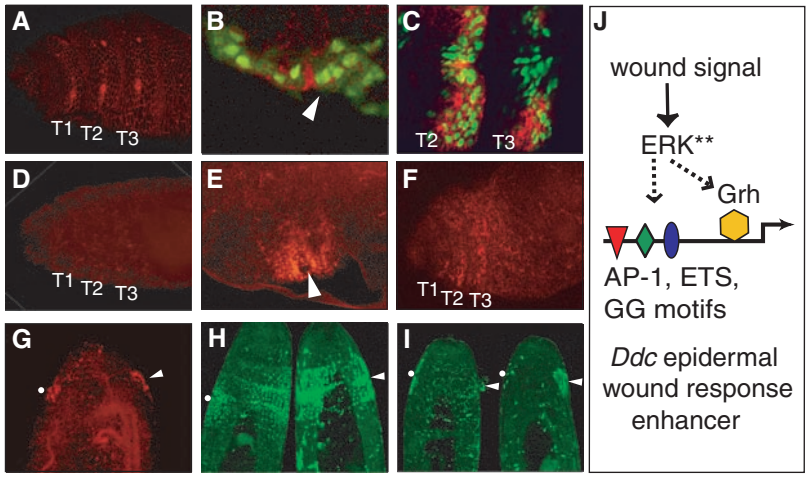

Fig. 5. Phosphotyrosine and diphospho-ERK (dpERK) concentrations are increased surrounding wound sites in wild-type or *spen* mutant embryos. Anti–p-Tyr staining (red) in (**A**) unwounded wild-type embryos carrying the –1.4-kb *Ddc*-GFP construct, (**B**) aseptically wounded embryos with the –1.4-kb *Ddc*-GFP construct (green, wound response enhancer expression), and (**C**) *spen* mutant embryos with the –1.4-kb *Ddc*-GFP construct. Anti-dpERK staining in (**D**) unwounded wild-type embryo, (**E**) aseptically wounded wild-type embryo, and (**F**) *spen* mutant embryo. (**G**) *grh* mutant embryo, wounded and stained for dpERK. (**H**) Two wounded embryos with the –0.47-kb *Ddc*-GFP construct that were treated only with 1% dimethyl sulfoxide. (**I**) Two wounded embryos with the –0.47-kb *Ddc*-GFP construct, treated with 100 μM PD98059 (MEK inhibitor) in 1% dimethyl sulfoxide (*21*). T1, T2, and T3, thoracic segments; white arrowheads, aseptic entry wounds; white dots, exit wounds. (**J**) Model of the epidermal barrier wound response pathway in *Drosophila* epidermal cells. A wound signal (unknown) activates ERK, which may signal through modification of Grh or another required factor (dotted lines). Evidence from mutagenesis and evolutionary comparison indicates that sequence motifs TGANTCA (AP-1), CMGGAW (ETS), and GGGGGATT (GG) are also important for barrier wound response enhancers.

qualitative, and it is still possible that these pathways have a quantitative influence on the epidermal barrier wound response.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S3
References and Notes

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Complement Factor H Polymorphism in Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) is a major cause of blindness in the elderly. We report a genome-wide screen of 96 cases and 50 controls for polymorphisms associated with AMD. Among 116,204 single-nucleotide polymorphisms genotyped, an intronic and common variant in the complement factor H gene (*CFH*) is strongly associated with AMD (nominal *P* value $<10^{-7}$). In individuals homozygous for the risk allele, the likelihood of AMD is increased by a factor of 7.4 (95% confidence interval 2.9 to 19). Resequencing revealed a polymorphism in linkage disequilibrium with the risk allele representing a tyrosine-histidine change at amino acid 402. This polymorphism is in a region of CFH that binds heparin and C-reactive protein. The *CFH* gene is located on chromosome 1 in a region repeatedly linked to AMD in family-based studies.

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world. Its incidence is increasing as the elderly population expands (*1*). AMD is characterized by progressive destruction of the retina's central region (macula), causing central field visual loss (*2*). A key feature of AMD is the formation of extracellular deposits called drusen concentrated in and around the macula behind the retina between the retinal pigment epithelium (RPE) and the choroid. To date, no therapy for this disease has proven to be broadly effective. Several risk factors have been linked to AMD, including age, smoking, and family history (*3*). Candidate-gene studies have not found any genetic differences that can account for a large proportion of the overall prevalence (*2*). Family-based whole-genome linkage scans have identified chromosomal regions that show evidence of linkage to AMD (*4*–*8*), but the linkage areas have not been resolved to any causative mutations.

Like many other chronic diseases, AMD is caused by a combination of genetic and environmental risk factors. Linkage studies are not as powerful as association studies for the identification of genes contributing to the risk for common, complex diseases (*9*). However, linkage studies have the advantage of searching the whole genome in an unbiased manner without presupposing the involvement of particular genes. Searching the whole genome in an association study requires typing 100,000 or more single-nucleotide polymorphisms (SNPs) (*10*). Because of these technical demands, only one whole-genome association study, on susceptibility to myocardial infarction, has been published to date (*11*).

Study design. We report a whole-genome case-control association study for genes involved in AMD. To maximize the chance of success, we chose clearly defined phenotypes for cases and controls. Case individuals exhibited at least some large drusen in a quantitative photographic assessment combined with evidence of sight-threatening AMD (geographic atrophy or neovascular AMD). Control individuals had either no or only a few small drusen. We analyzed our data using a statistically conservative approach to correct for the large number of SNPs tested, thereby guaranteeing that the probability of a false positive is no greater than our reported *P* values.

We used a subset of individuals who participated in the Age-Related Eye Disease Study (AREDS) (*12*). From the AREDS

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