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The Sac1 Lipid Phosphatase Regulates Cell Shape Change and the JNK Cascade during Dorsal Closure in *Drosophila*

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Summary

The Sac1 lipid phosphatase dephosphorylates several phosphatidylinositol (PtdIns) phosphates and, in yeast, regulates a diverse range of cellular processes including organization of the actin cytoskeleton and secretion [1]. We have identified mutations in the gene encoding *Drosophila* Sac1. *sac1* mutants die as embryos with defects in dorsal closure (DC). DC involves the migration of the epidermis to close a hole in the dorsal surface of the embryo occupied by the amnioserosa. It requires cell shape change in both the epidermis and amnioserosa and activation of a Jun N-terminal kinase (JNK) MAPK cascade in the leading edge cells of the epidermis [2]. Loss of Sac1 leads to the improper activation of two key events in DC: cell shape change in the amnioserosa and JNK signaling. *sac1* interacts genetically with other participants in these two events, and our data suggest that loss of Sac1 leads to upregulation of one or more signals controlling DC. This study is the first report of a role for Sac1 in the development of a multicellular organism.

Results and Discussion

The phosphoinositides (PIs) are important regulators of a wide range of cellular processes including cytoskeletal organization, vesicular trafficking, apoptosis, proliferation, and differentiation (reviewed in [3]). The synthesis and turnover of PIs are regulated at discrete membrane sites by lipid kinases, lipid phosphatases, and phospholipases. A variety of PIs are produced by the reversible phosphorylation of the inositol ring of PtdIns at one or several positions (D3, D4, and D5 hydroxyls). Sac1p is a *S. cerevisiae* phosphoinositide phosphatase localized to ER and Golgi membranes that can dephosphorylate PtdIns3P, PtdIns4P and PtdIns(3,5)P_2 (reviewed in [1]). The *SAC1* gene was originally isolated by virtue of a mutant allele suppressing the phenotypic effects of a temperature sensitive actin mutation, *act1-1*^{ts} [4]. *sac1* mutants exhibit an 8- to 10-fold increase in PtdIns4P levels and up to an 80% reduction in PtdIns(4,5)P_2 levels [5, 6].

Alleles of the predicted gene *CG9128*, which encodes a *Drosophila* Sac1 homolog [7, 8], were picked up during F2 lethal screens directed at obtaining mutations in the 61D-F cytogenetic region [9]. A lethal P element insertion (*BG02228*) located 30 bp downstream of the predicted starting point of transcription of *CG9128* belongs to the same complementation group [9]. *CG9128* is predicted to encode a 592 amino acid protein with a Sac phosphoinositide phosphatase domain, which is composed of approximately 400 amino acids organized into seven conserved motifs [1] (Figure 1).

We chose three lethal alleles of *sac1* for characterization. Two of these, *sac1*^{2107} and *sac1*^{L2F}, are EMS-induced while the third, *sac1*^{BG02228}, is the P element insertion line *BG02228* described above. *sac1*^{2107} shows a G to R substitution at position 251 in conserved motif 4 of the Sac domain (Figure 1). *sac1*^{L2F} has a premature stop codon at position 438 and is predicted to encode a truncated protein lacking conserved motifs 6 and 7 of the Sac domain and two transmembrane domains [9] (Figure 1). Data on Sac1p suggest that the activity of this truncated protein would be severely disrupted. Sac1p has two hydrophobic stretches similar to those in *Drosophila* Sac1 that allow Sac1p to traverse the ER membrane twice, with the bulk of the protein, including both termini, facing into the cytosol [10]. Removal of the transmembrane region impedes the ability of Sac1p to carry out efficient PtdIns4P turnover [6].

Individuals homozygous for any of the three lethal *sac1* alleles die as embryos. To confirm that the various phenotypes we describe below are due to loss of Sac1, we performed transgenic rescue experiments in *sac1* mutant backgrounds with a 2.8 kb genomic construct containing the *sac1* transcription unit and upstream sequences. A single copy of this construct was able to rescue the embryonic phenotypes associated with *sac1*^{2107} and *sac1*^{L2F}. We confirmed that the lethality associated with *BG02228* was due to the P element insertion by mobilizing the element and picking up viable excisions.

Embryos homozygous for these *sac1* alleles show dorsal anterior holes in cuticle preparations (Figures 2D–2F). The same phenotype is seen in embryos bearing a heteroallelic combination of these alleles or when these alleles are placed in *trans* to either of two deficiencies disrupting *sac1*, *Df(3L)B67*, and *Df(3L)Fpa2* [9] (Figures 2G and 2H, and data not shown). Embryos bearing the *sac1*^{L2F} allele as a homozygote, hemizygote, or in a heteroallelic combination also show "puckering" of the dorsal cuticle and an absence of dorsal hairs along the midline

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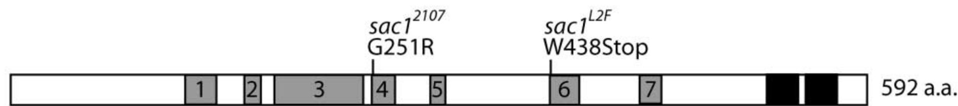

Figure 1. Diagram Showing the Organization of the Sac1 Protein and the Positions of the Mutations in the *sac1*^{2107} and *sac1*^{L2F} Alleles
The seven conserved motifs in the Sac domain are shown as numbered gray boxes; black boxes indicate predicted transmembrane domains.

(Figures 2C, 2D, 2G, and 2H). The dorsal cuticle defects seen in *sac1* mutant embryos are similar to those seen in embryos mutant for genes encoding participants in DC of the epidermis (reviewed recently in [2]). DC involves the migration of the lateral epidermal flanks over the amnioserosa, a squamous epithelium occupying the dorsal hole. The movement of the epidermis is driven in part by an actomyosin contractile apparatus in the leading edge cells that constricts them along the anterior-posterior axis of the embryo, resulting in elongation along the dorsal-ventral axis of cells throughout the lateral epidermis. The cells of the amnioserosa also undergo a dramatic change in morphology during DC, constricting apically and shifting from a squamous to columnar morphology; this morphogenesis is required for normal DC to occur [11–14].

To investigate further the DC phenotype of *sac1* mutants, we stained embryos with anti-phosphotyrosine antibodies to assess morphogenesis and the status of the leading edge cytoskeleton. Anti-phosphotyrosine antibodies reveal epithelial cell outlines and mark specialized adherens junctions participating in assembly of the leading edge cytoskeleton. In *sac1* mutant embryos, the leading edge adherens junctions are intact, and epidermal cells show normal dorsal-ventral elongation (data not shown). In stage 13 embryos bearing the *sac1*^{2107} allele as a homozygote or hemizygote, cells located toward the ends of the amnioserosa have a smaller apical surface area than cells in the middle of the tissue (Figures 3C and 3D). This is in contrast to wild-type embryos at this stage in which apical cell surface area is fairly uniform throughout the tissue (Figure 3A). Comparisons of the average apical surface area of amnioserosa cells in wild-type and *sac1*^{2107} mutant embryos at the onset of DC indicate that the “end” cells in *sac1*^{2107} embryos are significantly more apically constricted than normal, and the “middle” cells are significantly less constricted. This phenotype could be due to excessive constriction

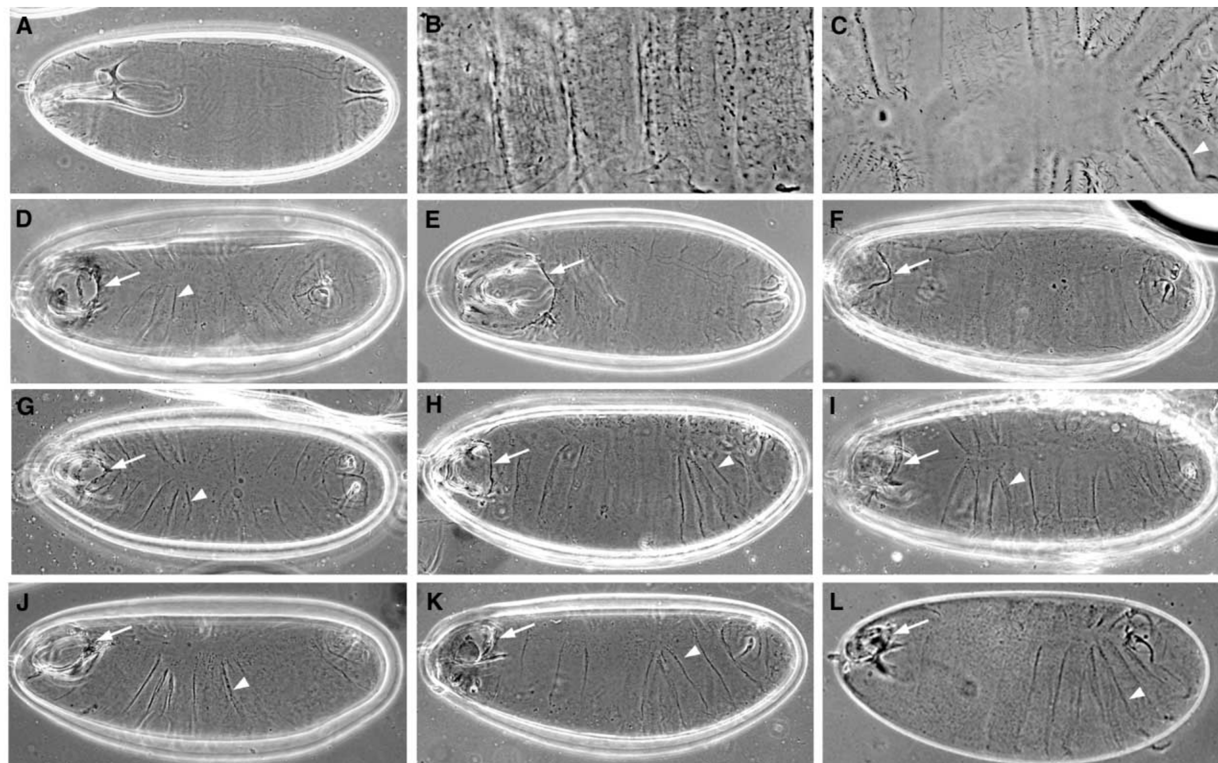

Figure 2. Cuticle Phenotypes of *sac1* Mutant Embryos
Anterior is to the left in this and all subsequent embryo figures. Dorsal anterior openings in cuticle are marked with arrows; arrowheads indicate puckering. (A) Wild-type embryo. (B) Enlarged view of dorsal cuticle of wild-type embryo. (C) Enlarged view of dorsal cuticle of *sac1*^{L2F}/*sac1*^{L2F} embryo showing naked cuticle along dorsal midline and puckering. (D) *sac1*^{L2F}/*sac1*^{L2F}. (E) *sac1*^{2107}/*sac1*^{2107}. (F) *sac1*^{BG02228}/*sac1*^{BG02228}. (G) *sac1*^{L2F}/*sac1*^{BG02228}. (H) *sac1*^{L2F}/*Df(3L)B67*. (I) *sac1*^{2107}/*Df(3L)Fpa2*. (J) *sac1*^{L2F}/*puc*^{E69}. (K) *sac1*^{L16C}/*sac1*^{L16C}. (L) *sac1*^{L16C}/*puc*^{E69}.

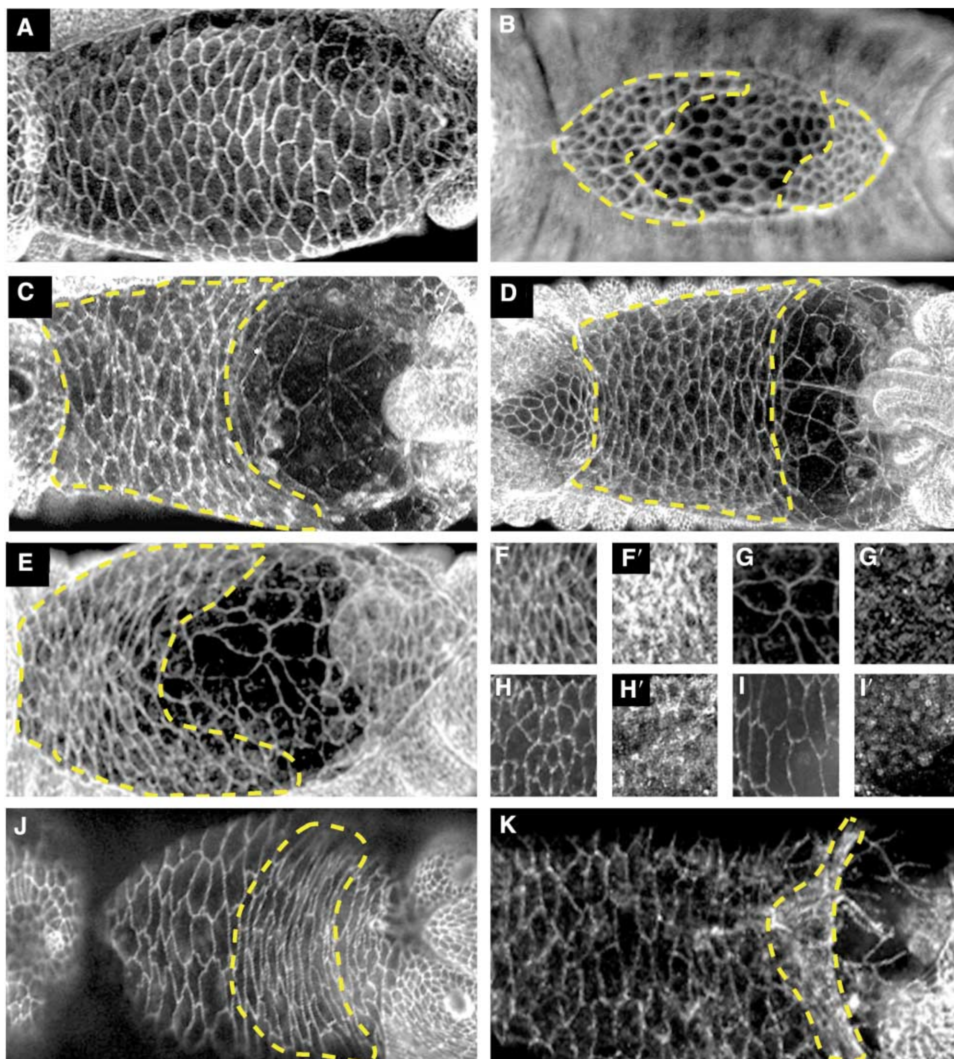

Figure 3. Dorsal Views of the Amnioserosa

(A–K) Anti-phosphotyrosine staining. (F′– I′) Anti-non-muscle myosin staining. Dashed yellow lines demarcate clusters of cells showing pronounced apical constriction. (A) Stage 13 wild-type embryo prior to commencement of DC showing amnioserosa with cells of fairly uniform apical surface area. (B) Late stage 14 embryo midway through DC showing apical constriction of cells at ends of amnioserosa. (C–E) Stage 13 embryos showing apical constriction of cells at anterior end of amnioserosa. The posterior amnioserosa cells are often difficult to distinguish due to staining in underlying hindgut and Malpighian tubules. (C) *sac1*^{2107}/*sac1*^{2107}. (D) *sac1*^{2107}/*Df(3L)Fpa2*. (E) Embryo in which *UAS-crb* had been expressed using the *GAL4*$^{332.3}$ amnioserosa-specific driver [21]. (F and F′) Double staining of apically constricted anterior end cells in the amnioserosa of the embryo in (E) showing high myosin levels. (G and G′) Double staining of middle cells in the amnioserosa of the embryo in (E) showing lower myosin levels than end cells. (H and H′) Double staining of apically constricted anterior end cells in the amnioserosa of *sac1*^{2107}/*sac1*^{2107} embryo showing high myosin levels. (I and I′) Double staining of middle cells in the amnioserosa of the embryo in (H) showing lower myosin levels than end cells. (J and K) Stage 13 *sac1*^{L2F}/*sac1*^{L2F} (J) and *sac1*^{L2F}/*Df(3L)Fpa2* (K) embryos showing pronounced constriction of cells near the middle of amnioserosa.

of the end cells (leading to "pulling" of middle cells into a large apical surface area) or expansion of the middle cells (leading to "pushing" of end cells into a small apical surface area), or a combination of both. The distinction between end and middle amnioserosa cells in terms of degree of apical constriction seen in *sac1*^{2107} mutant embryos at the onset of DC is similar to what is seen in wild-type embryos midway through DC (Figure 3B). This suggests that in *sac1*^{2107} mutant embryos, morphogenesis of amnioserosa cells is occurring prematurely. This phenotype is very similar to what we previously observed following overexpression of Crumbs (Crb), a determinant of epithelial apical-basal polarity, in the amnioserosa [13](Figure 3E). In these Crb-overexpressing embryos, the constricted end cells show greatly elevated levels of myosin compared to the unconstricted middle cells, and we proposed that Crb was involved in establishing an apically localized actomyosin contractile apparatus driving amnioserosa cell constriction during DC [13] (Figures 3F and 3G). We checked myosin levels in the amnioserosa of *sac1*^{2107} mutant embryos by staining with anti-non-muscle myosin antibodies, and found that myosin levels are similarly elevated in the end cells (Figures 3H and 3I). These results suggest that Sac1 is

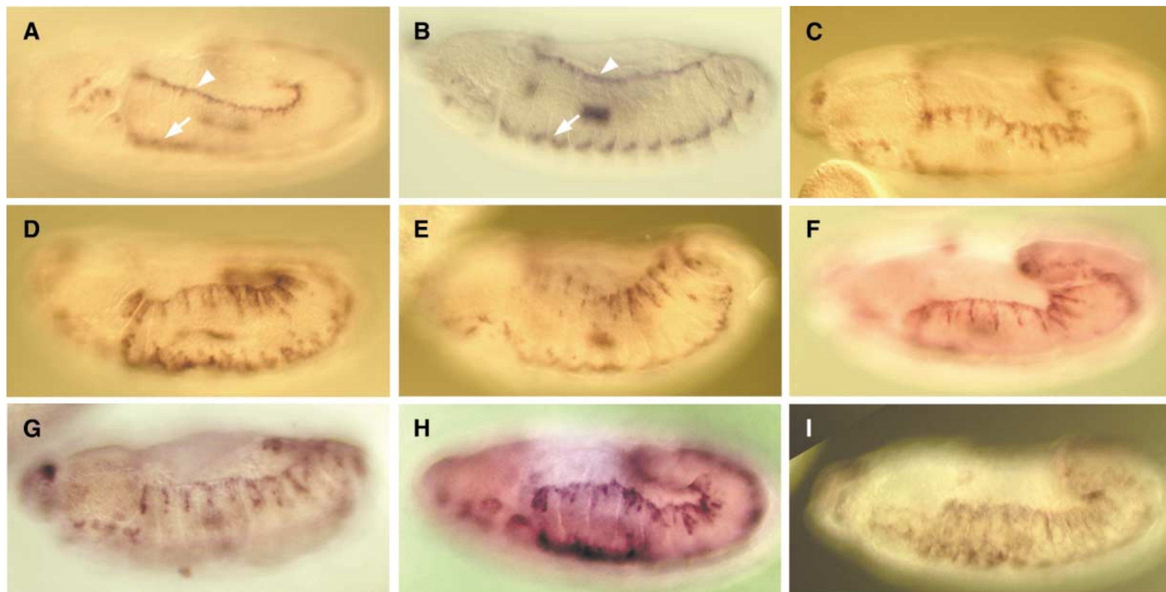

Figure 4. Ectopic *dpp* Expression in Epidermal Cells Located Ventral to the Leading Edge in *sac1* Mutant Embryos Indicating Ectopic Activation of the JNK Cascade

Embryos were hybridized with a *dpp* riboprobe. (A and B) Stage 12 (A) and 13 (B) wild-type embryos showing JNK-dependent *dpp* expression along the leading edge (arrowheads). Arrows mark a ventrolateral band of *dpp* expression that is not JNK dependent. (C) Stage 12 *sac1^{L2F}/sac1^{L2F}*. (D) Stage 13 *sac1^{L2F}/sac1^{L2F}*. (E) Stage 13 *sac1^{L2F}/Df(3L)Fpa2*. (F) Stage 12 *sac1^{L2F}/sac1^{BG02228}*. (G) Stage 13 *sac1^{L2F}/sac1^{2107}*. (H) Stage 12 *sac1^{2107}/Df(3L)Fpa2* . (I) Stage 12 *sac1^{L2F}/puc^{E69}*.

involved in the regulation of Crb-mediated amnioserosa cell constriction. To investigate the possibility that loss of Sac1 is dependent on Crb in the generation of DC defects, we compared the frequency of dorsal embryonic cuticle defects in the *sac1^{2107}* mutant line with the frequency of dorsal embryonic cuticle defects in the progeny of a cross between the *sac1^{2107}* mutant line and a line in which a weak *crb* allele, *crb^{S010409}* [13], had been recombined onto the *sac1^{2107}* mutant chromosome. In both cases, a quarter of the embryos were homozygous mutant for *sac1^{2107}*. In the *sac1^{2107}* mutant line, 29% of embryos showed a dorsal anterior hole; this was reduced to 4% in the progeny of the cross in which the *sac1^{2107}* homozygous mutant embryos were heterozygous for *crb^{S010409}*. Embryos homozygous mutant for both *sac1^{2107}* and *crb^{S010409}* do not show a dorsal anterior open phenotype but do exhibit the typical *crb^{S010409}* phenotype [13] of a dorsal posterior hole and germband retraction defect (data not shown). *sac1^{BG02228}* mutant embryos do not show premature apical constriction of amnioserosa cells (data not shown), whereas *sac1^{L2F}* mutant embryos surprisingly show apical constriction of the middle amnioserosa cells at the onset of DC (Figure 3J). In wild-type embryos, the middle amnioserosa cells are the last to show apical constriction during DC [13]. One possibility is that the middle amnioserosa cells respond to the same signal as the end cells, but have a higher response threshold that is not normally reached until late in DC. As *sac1^{L2F}* is likely the most severe loss-of-function *sac1* allele, any signal upregulated by loss of Sac1 would be strongest for this allele and could be sufficient to trigger constriction of the middle cells. Once these cells have constricted, they may impede constriction of the end cells. That this is a strong loss-of-function phenotype is supported by the finding that *sac1^{L2F}/Df(3L)Fpa2* embryos also show constriction of the middle cells (Figure 3K).

A central component of DC is a JNK MAPK cascade required for a number of events in the leading edge cells, including assembly of the actomyosin contractile apparatus, cell shape change, and transcriptional upregulation of several genes including *decapentaplegic* (*dpp*), which encodes a member of the transforming growth factor-β (TGF-β) superfamily [2]. The cuticle puckering and loss of dorsal hairs along the midline seen in *sac1^{L2F}* mutant embryos are identical to the cuticle phenotype seen in embryos in which the JNK cascade has been ectopically activated. For example, this phenotype is seen in embryos mutant for *puckered* (*puc*), which encodes a MAPK phosphatase negatively regulating the JNK cascade, and when JNK signaling is upregulated through ectopic activation of Wingless signaling [15–17]. This striking similarity prompted us to check our *sac1* mutants for effects on the JNK cascade during DC. We assessed the status of the JNK cascade by looking at the transcription of *dpp*. From stage 11, expression of *dpp* at the leading edge of the epidermis is dependent on the JNK cascade and is restricted to this single row of cells through the actions of a variety of negative regulators of the JNK cascade including Puc, Anterior open, Raw, and Notch. Loss-of-function mutations in the genes encoding these negative regulators result in ectopic *dpp* expression in the epidermis [2]. The distribution of *dpp* transcripts in embryos homozygous for *sac1^{2107}* or *sac1^{BG02228}* is normal (data not shown), however, embryos bearing the *sac1^{L2F}* allele as a homozygote, hemizygote,

or in a heteroallelic combination show ectopic *dpp* expression in lateral epidermal cells located ventrally to the leading edge (Figures 4C–4G). This ectopic *dpp* expression is distinct from that caused by mutations in other genes in that it occurs in stripes in a segmented pattern. *sac1* mRNA is uniformly expressed in the epidermis (data not shown), suggesting that the ectopic *dpp* expression induced by loss of Sac1 is dependent on another protein or proteins expressed in a segmented pattern. That ectopic JNK activation is a strong loss-of-function *sac1* phenotype is confirmed by the finding that $sac1^{2107}$/*Df(3L)Fpa2* and $sac1^{BG02228}$/*Df(3L)Fpa2* embryos have puckered cuticles and ectopic *dpp* expression (Figures 2I and 4H, and data not shown). The above results indicate that disruption of Sac1 allows JNK signaling to occur in regions of the epidermis where it is normally repressed and suggest that Sac1 negatively regulates JNK activity in the lateral epidermis.

We looked for a genetic interaction between *sac1* and another negative regulator of the JNK cascade, *puc*, by making embryos transheterozygous for $sac1^{L2F}$ and a loss-of-function *puc* allele, puc^{E69} [15], and evaluating them with cuticle preparations and *dpp* RNA in situ hybridizations. Embryos heterozygous for either one of these mutations alone do not show cuticle defects or ectopic *dpp* expression (data not shown). In a cross between the $sac1^{L2F}$ and puc^{E69} lines, 12% of the progeny (an estimated 48% of the transheterozygotes) had defects in the dorsal surface in the form of anterior head holes and/or puckering, and 8% of the progeny (an estimated 32% of the transheterozygotes) showed ectopic *dpp* expression in the epidermis (Figures 2J and 4I). This result indicates that Sac1 and Puc cooperate in restricting JNK cascade activation to the leading edge during DC. Heterozygosity for puc^{E69} can suppress the dorsal-open phenotype associated with reduced JNK signaling [16]. We tested $sac1^{L2F}$ for the same ability by comparing the frequency of dorsal holes in embryos homozygous mutant for $basket^{1}$ (bsk^{1}), a weak allele of the gene encoding *Drosophila* JNK [18, 19]. In a control cross where half of the bsk^{1} homozygous embryos were made heterozygous for the third chromosome balancer from the $sac1^{L2F}$ line, 20% of embryos (an estimated 80% of the bsk^{1} homozygotes) had a large dorsal hole. When half of the bsk^{1} homozygous embryos were made heterozygous for $sac1^{L2F}$, the frequency of large dorsal holes fell to 11% (an estimated 44% of the bsk^{1} homozygotes). Thus, a reduction in Sac1 function is able to suppress the phenotypic effects resulting from impairment of JNK signaling, presumably by increasing the degree of activation of the residual JNK protein. That the ectopic *dpp* expression seen in $sac1^{L2F}$ mutant embryos is JNK dependent was confirmed by the finding that it could be suppressed by heterozygosity for the bsk^{1} allele (data not shown). The $sac1^{2107}$ and $sac1^{BG02228}$ alleles show no genetic interactions with puc^{E69} or bsk^{1}, but we have recently found that another *sac1* allele, $sac1^{L16C}$ [9], affects JNK signaling. $sac1^{L16C}$ mutant embryos have a puckered cuticle and exhibit elevated *dpp* expression (Figure 2K and data not shown), and $sac1^{L16C}$ genetically interacts with puc^{E69} (Figure 2L and data not shown).

We have shown that a reduction in Sac1 function leads to the initiation of two key events in DC: apical constriction of the amnioserosa cells and activation of the JNK cascade. It is possible that morphogenesis of the amnioserosa and JNK cascade activation in the epidermis are a response to a single upstream signal, given the communication known to exist between these two tissues in regulating DC events [2, 20]. A likely route for generation of such a signal would be alteration of PI levels. Unraveling the roles of PIs in DC will require determining PI levels in *sac1* mutant embryos and addressing the DC roles of other regulators of PIs such as the lipid kinases. Further study of Sac1 in DC should prove useful in identifying the routes used by PIs in the regulation of the cytoskeleton and JNK signaling in the context of epithelial morphogenesis.

Supplemental Data

Supplemental Data including experimental procedures are available online at http://www.current-biology.com/cgi/content/full/13/21/1882/DC1/.

Acknowledgments

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