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 10. Sympathetic neurons were isolated from superior cervical ganglia of neonatal rats (21). Cells were plated in compartmentalized chambers (Tyler Research Products) as described (8) and grown in growth medium [Dulbecco's modified Eagle's medium containing fetal bovine serum (10%), 5 μ M arabinosylcytosine (Ara-C), and NGF (200 ng/ml)]. Medium was replaced every 3 days. After 4 to 7 days, medium within the chamber containing cell bodies was replaced with medium lacking NGF. This procedure resulted in the death of those neurons that had not extended processes into adjacent compartments. Neurons grown in center-plated chambers were used 2 to 3 weeks after plating. Neurons grown in side-plated chambers required a longer time to project through two barriers and were used 3 to 4 weeks after plating. For P-CREB immunocytochemistry experiments, medium was changed to contain a low concentration of NGF (2 ng/ml) for 48 hours before stimulation with NGF. Neurons were treated with NGF (200 ng/ml) and then fixed with acetone: methanol (1:1) for 3 min. Fixed cells were rinsed with phosphate-buffered saline (PBS) and permeabilized with PBS containing Triton X-100 (0.1%). After blocking with PBS solution containing horse serum (3%) and bovine serum albumin (BSA, 3%) at room temperature for 2 hours, cells were incubated with anti-P-CREB (1:1000 dilution) in the above solution at 4°C for 18 hours. Immune complexes were detected with an avidin biotin detection system (Vector Laboratories).
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 12. NGF was covalently coupled to 1 μ m-diameter microspheres by means of a carbodiimide cross-linking method. Amine-modified FluoSpheres (2% solids; Molecular Probes) were washed three times with solution 1 [2(N-morpholino)ethanesulfonic acid (0.1 M, pH 6.0)] using centrifugation and gentle resuspension. The FluoSpheres were then resuspended in solution 1 containing NGF (100 μ g/ml) to a final concentration of 1% microspheres. EDAC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; Molecular Probes], which was freshly dissolved in solution 1, was then added to this suspension, and the mixture was rotated at room temperature for 2 hours. The cross-linking reaction was quenched by the addition of glycine (0.1 M, pH 6.0). Beads were then washed four times with a high-salt buffer (10 mM sodium phosphate, 1.8 mM potassium phosphate, 1 M sodium chloride, and 2.6 mM potassium chloride, pH 4.0) for 30 min per wash. The beads were next incubated overnight in the high-salt buffer (pH 7.4), washed four times the next day with high-salt buffer (pH 10.0) to remove adsorbed NGF, and then resuspended in PBS (pH 7.4) at a final concentration of 0.25% solids. To ensure that all adsorbed NGF was removed from the FluoSpheres, we subjected a set of FluoSpheres (control beads) to a similar procedure, except EDAC was left out of the cross-linking step. The bioactivity of NGF-coupled FluoSpheres was found to depend on the concentration of NGF and EDAC used. At the concentration of NGF used, we found that 2 to 4 μ M EDAC was optimal for obtaining bioactive NGF-coupled beads; we also found that 4 μ l/ml of the bead solution was sufficient to cover all cell surfaces.
 13. TrkA was immunoprecipitated with anti-panTrk (22) from PC12 cells (7×10^6 cells per plate) treated with control medium, control beads (4 μ l/ml), NGF (100 ng/ml), or NGF-coupled beads (4 μ l/ml) (13), and phosphotyrosine protein immunoblotting was done as described (22). Similar results were obtained by immunoprecipitating TrkA from primary cultures of sympathetic neurons (14).
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Hypermethylated *SUPERMAN* Epigenetic Alleles in *Arabidopsis*

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Mutations in the *SUPERMAN* gene affect flower development in *Arabidopsis*. Seven heritable but unstable *sup* epi-alleles (the *clark kent* alleles) are associated with nearly identical patterns of excess cytosine methylation within the *SUP* gene and a decreased level of *SUP* RNA. Revertants of these alleles are largely demethylated at the *SUP* locus and have restored levels of *SUP* RNA. A transgenic *Arabidopsis* line carrying an antisense methyltransferase gene, which shows an overall decrease in genomic cytosine methylation, also contains a hypermethylated *sup* allele. Thus, disruption of methylation systems may yield more complex outcomes than expected and can result in methylation defects at known genes. The *clark kent* alleles differ from the antisense line because they do not show a general decrease in genomic methylation.

DNA methylation is emerging as an important component of cell memory, the process by which dividing cells inherit states of gene activity. In mammals, methylation appears to play a key role in processes such as genomic imprinting and X-chromosome inactivation, and in plants methylation is correlated with a number of phenomena, including silencing of duplicated regions of the genome (1).

Arabidopsis mutants at the *DDM1* and *DDM2* loci have a reduced overall level of cytosine methylation and display a number of developmental defects (2). Transgenic *Arabidopsis* plants expressing an antisense cytosine methyltransferase RNA also exhibit abnormalities including a number of floral defects resembling the phenotypes of known floral homeotic mutants (3, 4). These experiments suggest a direct cause

and effect relation between DNA methylation and proper regulation of developmentally important genes. We describe here a class of epi-mutations in *Arabidopsis* that appear to be caused by overmethylation of the flower development gene *SUPERMAN* (*SUP*).

Seven independent mutants were identified [*clark kent* (*clk*) 1 through 7] with phenotypes similar to but weaker than that of the known *sup* mutants (5, 6). Wild-type *Arabidopsis* flowers (Fig. 1A) contain six stamens (the male reproductive organs) and two central carpels that fuse to form the female reproductive structure. The *sup-5* allele (Fig. 1B) (7), which contains a nearly complete deletion of the *SUP* gene (8), produces an increased number of stamens [12.3 ± 0.3 (mean $\pm$ SE)] and carpels (2.9 ± 0.1) on the first 10 flowers produced on the plant. The *clk-3* allele (Fig. 1C) has an average of 7.8 ± 0.3 stamens and 3.4 ± 0.1 carpels, whereas the weaker *clk-1* allele has an average of 6.4 ± 0.1 stamens and

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Fig. 1. (A) Wild-type Landsberg erecta (Ler) flower showing the normal number of six stamens and the central gynoecium, which consists of two fused carpels. (B) Flower from a *sup-5* homozygote containing 11 stamens and three incompletely fused carpels. (C) Flower from a *clk-3* homozygote containing nine stamens and three incompletely fused carpels. (D)



An F_1 flower from a cross between a *sup-5* homozygote and a *clk-3* homozygote containing 10 stamens and three incompletely fused carpels. (E) Flower from a transgenic *clk-3* plant containing a 6.7-kb wild-type *SUP* genomic fragment (12), containing the normal number of six stamens and two fused carpels. (F) An F_1 flower from a cross between the AMT line and a *clk-3* homozygote containing 10 stamens and three incompletely fused carpels.

3.2 ± 0.1 carpels.

In F_1 complementation tests, *clk* mutants fail to complement *sup* mutants (Fig. 1D). However, these tests are complicated by the fact that both *clk* and *sup* mutants are semi-dominant (9). Also, in F_2 progeny of these crosses, about 1 to 3% of the plants are wild type. These data suggested that *CLK* might define a separate gene linked to *SUP*. However, further analysis shows that the *clk* mutants are *SUP* alleles.

First, *clk* and *sup* are very closely linked. *clk-3* perfectly segregated with a Hind III restriction fragment length polymorphism detected with a 5.5-kb Eco RI *SUP* genomic fragment in 45 meiotic progeny (10, 11). In addition a cis-trans test was performed; if *sup* and *clk* are different genes, they should have the same double-heterozygote phenotype in cis or trans. *clk* and *sup* homozygotes were crossed to make trans *clk-3* $+/+$ *sup-5* heterozygotes, which were then crossed to wild-type plants. These F_1 progeny were analyzed for plants with *sup*-like cis *clk* *sup*/ $+$ heterozygote phenotypes. However, all 7863 F_1 progeny from this cross appeared wild type. Because this number of plants represents a recombinant every 0.025 cM, or one about every 3.3 kb, the results of this test suggest either that *clk* and *sup* are allelic, or that if *clk* and *sup* are two different genes, they are very closely spaced in the genome.

Second, a 6.7-kb genomic clone containing only the *SUP* coding region and ~5 kb of upstream sequence, which has been shown to rescue the *sup* mutant phenotype (11), complements the *clk-3* phenotype in transgenic plants (Fig. 1E) (12). However, other genomic clones spanning 40 kb on the distal side of *SUP* and 25 kb on the proximal side fail to complement *clk-3* (13), apparently precluding the possibility that *CLK* is a separate gene very closely linked to *SUP*.

In situ hybridization experiments show that *SUP* RNA expression is reduced in *clk-3*. In wild type, expression of *SUP* RNA occurs early during floral meristem development in the incipient stamen primordia (11). In *clk-3* homozygotes, however, this expression was reduced in some floral meristems and undetectable in others (Fig. 2).

Despite the evidence that *clk* and *sup* are allelic, sequencing of the *SUP* coding region from *clk-1*, -2, -3, and -5, and the entire 6.7-kb *SUP* genomic region from the *clk-1* and -3 alleles revealed no nucleic acid sequence differences from the wild type. In addition, the cloned *SUP* gene from a *clk-3* genomic library complements the *clk-3* and *sup-5* mutants in transgenic plants (12), as if cloning the *clk-3* allele restores it to wild type. Together these results suggest that the *clk* alleles represent an alternative epigenetic state of the *SUP* gene (14).

To examine whether the *clk* mutants exhibit the genetic instability that is characteristic of many other epigenetic phenomena, we constructed a *clk-3 gl-1* double mutant and analyzed the selfed progeny [*gl-1* maps 10 cM from *SUP* and eliminates epidermal hairs (15)]. Of 586 plants, 17 had a wild-type or nearly wild-type phenotype,

Table 1. Sequence context of the methylated cytosines in the *SUP* region in *clk-3*.

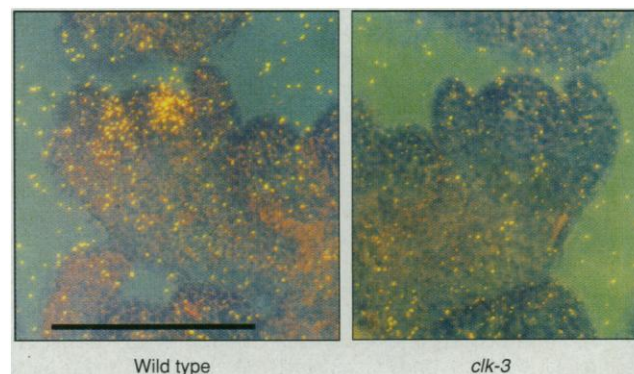
Se- quence	Total number	Number methyl- ated	Number unmethyl- ated	Percent methyl- ated
CX	383	211	172	55
CA	129	79	50	61
CT	156	93	63	60
CG	15	10	5	67
CC	83	29	54	35
CXA	110	71	39	65
CXT	124	39	85	31
CXG	56	54	2	96
CXC	93	47	46	51

but were still hairless. Three of these lines were complete revertants; the selfed progeny from these plants segregated 3:1 for wild type:*clk* plants, and in subsequent generations they segregated homozygous *clk* and wild-type lines. The other 14 lines appeared to contain partially reverted alleles. Analysis of one complete revertant (number 6) by in situ hybridization showed that wild-type levels of *SUP* RNA expression were restored (16).

We analyzed methylation patterns within the *SUP* gene in different genotypes using bisulfite genomic sequencing (17). Although there was no cytosine methylation detected in the wild-type or in a *sup* nonsense allele [*sup-1* (11)], extensive methylation was found in the *clk* alleles (shaded regions, Fig. 3A), covering the start of transcription and most of the transcribed region. The *clk-3* allele contained six more methylcytosines (a total of 211 detected) than the weaker *clk-1* allele (Fig. 3B), possibly providing an explanation for the slight difference in phenotypic strength of these alleles. In *clk-3* revertant 6, only 14 of the original 211 methylcytosines remained. Thus, phenotypic reversion is correlated with both a restoration of the wild-type RNA expression level and a decrease in cytosine methylation of the *SUP* DNA.

The methylation pattern in *clk* was dense and essentially non-sequence specifi-

Fig. 2. *SUP* RNA expression in wild-type and *clk-3*. Longitudinal sections of the inflorescences of 14-day-old plants of wild-type Ler or the *clk-3* mutant were mounted on the same slide and hybridized with a *SUP* antisense probe as described in (11). Medial sections of stage 4 flower primordia (19) were photographed with bright-field-dark-field double exposure. Yellow spots represent silver grains exposed by the ^{35}S -labeled *SUP* probe, after a 7.5-week exposure. Bar, 120 μM .



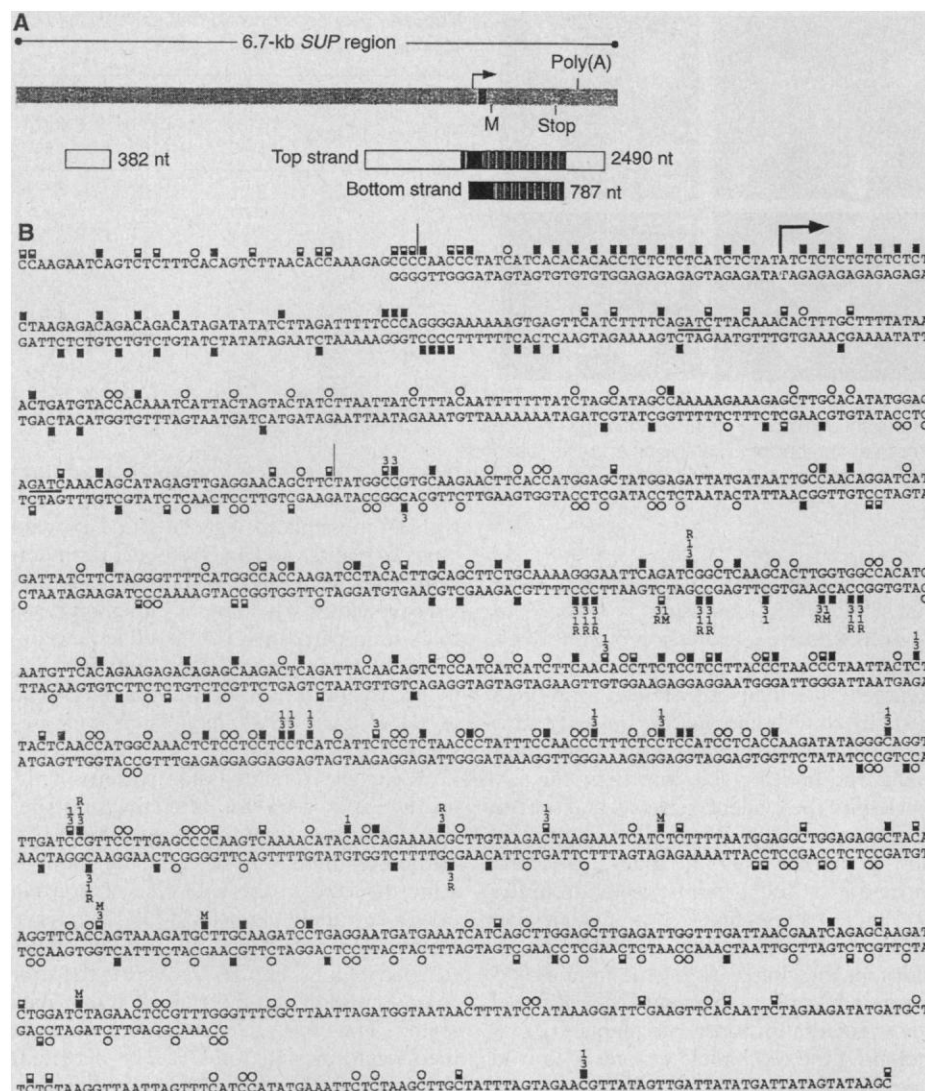


Fig. 3 (above). (A) Summary of the methylation pattern observed in the *SUP* genomic region from the *clk-1* and *clk-3* alleles and from the AMT line. Gene diagram shows the start of transcription (arrow), the single intron (shaded area), the putative start of translation (M), the stop codon, and the poly(A) addition site (11). Boxes below the gene show the regions of either the top or bottom strand analyzed by bisulfite genomic sequencing; open boxes denote no methylation detected in all three genotypes, solid boxes indicate that all of the cytosines in the region were methylated, and hatched boxes indicate that only some of the cytosines in the region were methylated. Numbers indicate the length in nucleotides (nt) of each region analyzed. (B) Exact pattern of methylation detected in six different genotypes: *clk-1* (1), *clk-3* (3), the AMT line (M), revertant 6 (R), wild-type Ler, and wild-type C24. Open circles indicate that cytosine methylation was not detected in any of the six genotypes. Boxes with no accompanying symbol indicate that methylation was detected at this site in *clk-1*, *clk-3*, and the AMT line, but not in either of the wild-type controls or in revertant 6. All exceptions to this general pattern are indicated by symbols above or below the box. For example, 3,1,R indicates that methylation was detected in *clk-3*, *clk-1*, and revertant 6, but not in the other genotypes. Because the data were obtained by direct sequencing of PCR-amplified genomic DNA (17), an average level of methylation was determined for each cytosine. Thus, solid boxes indicate that more than 50% of the cytosine at this position was methylated, whereas half-shaded boxes indicate that less than 50% of the cytosine was methylated. The region between the vertical lines on the top strand was analyzed from other genotypes as described in the text. Arrow indicates the beginning of the *SUP* RNA as deduced from the longest cDNA detected and corresponds to nucleotide -202 relative to the putative start codon as described in (11). Methylation at the two underlined GATC restriction sites was confirmed with the methylation-sensitive restriction enzyme Sau 3AI and its methylation-insensitive isoschizomer Mbo I. **Fig. 4 (right).** Repetitive DNA methylation in *clk*. Genomic DNA of the AMT line (1), wild-type Ler (2), and *clk-3* (3) was digested with the methylation-sensitive enzyme Hpa II, fractionated on an agarose gel, and probed with a 180-bp centromeric repeat clone (2). Undermethylated DNA in the AMT line is detected as a low molecular weight ladder, not present in the wild type or *clk-3*.

ic; both symmetric (CG and CXG) and nonsymmetric cytosines were methylated (Table 1). However, the pattern of methylation was nearly identical in different *clk* alleles (Fig. 3B). For example, *clk-1* and *clk-3* shared 204 methylcytosines; seven sites were methylated in *clk-3* but not *clk-1*, and one site was methylated in *clk-1* but not *clk-3*. Also, in the most densely methylated region on the top strand (Fig. 3B), *clk-2*, *clk-5*, and *clk-6* had a hypermethylation pattern very similar to that seen in *clk-1* and *clk-3*. The reproducibility of this pattern in independently isolated *clk* lines suggests that the mechanism by which these sequences become methylated is rather specific. In addition, the sequences methylated in the *SUP* gene appear to be single copy (18). This suggests that the *SUP* hypermethylation could be produced or maintained by a mechanism other than that responsible for most of the methylation in plants, which is mostly found at repetitive sequences and primarily consists of symmetric sites (1, 2).

A number of *Arabidopsis* antisense cytosine methyltransferase (AMT) lines exhibit phenotypes resembling *sup* mutants (3, 4). Crosses between an AMT homozygote exhibiting a *sup* floral phenotype (line 10) (3) and either *clk-3* or *sup-5* plants yielded F_1 plants with a *sup* phenotype (Fig. 1F), whereas F_1 plants resulting from crosses of the AMT line to a wild-type Ler plant had a wild-type floral phenotype (16). This suggests that the AMT line contains a defective *SUP* allele. Hypermethylation of the *SUP* gene was found in the AMT line in a pattern similar to that seen in the *clk* lines. One hundred and eighty-six methylcytosines were detected, all but three of which were in the same positions as those in *clk-3* (Fig. 3B). Thus, although overall methylation in this AMT line is decreased by up to 90% (3), the *SUP* gene has become hypermethylated. These results challenge the original interpretation of the AMT phenotype, in that the various phenotypes in these lines were predicted to be caused by demethylation of specific genes (3, 4).

To examine whether the *clk* lines have general demethylation defects similar to those seen in the AMT lines, we analyzed the methylation status of the 180-bp centromeric repeat (Fig. 4) and the ribosomal DNA loci (16) by DNA blot analysis (2). Whereas in the AMT lines these sequences were hypomethylated (Fig. 4) (3, 4), five *clk* alleles showed normal methylation of these repetitive genes, suggesting that the defects in the *clk* lines are different from those in the AMT line. One possible interpretation of these results is that the AMT lines cause misregulation of a component of the methylation pathway

other than the methyltransferase, resulting in hypermethylation of some genomic regions. If this hypothesized component were mutated in the *clk* lines, this might cause only a portion of the AMT phenotype, namely, hypermethylation of *SUP*.

Note added in proof: We have found a *clk*-like pattern of methylation at the *SUP* locus in *fonl-2* and *fonl-3* [see (14)].

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18. The *SUP* cDNA was used to probe genomic DNA blots at various stringencies. Under high-stringency conditions (10), only the *SUP* gene is detected. At lower washing stringency (2 $\times$ saline sodium phosphate EDTA, 0.5% SDS, 55°C), an additional band is detected. This hybridizing fragment was cloned and found to contain a region of homology limited to a 101-bp sequence, encoding a zinc-finger domain, which shares 76% nucleic acid identity with *SUP*.
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In Situ Activation Pattern of *Drosophila* EGF Receptor Pathway During Development

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Signaling cascades triggered by receptor tyrosine kinases (RTKs) participate in diverse developmental processes. The active state of these signaling pathways was monitored by examination of the in situ distribution of the active, dual phosphorylated form of mitogen-activated protein kinase (ERK) with a specific monoclonal antibody. Detection of the active state of the *Drosophila* epidermal growth factor receptor (DER) pathway allowed the visualization of gradients and boundaries of receptor activation, assessment of the distribution of activating ligands, and analysis of interplay with the inhibitory ligand Argos. This in situ approach can be used to monitor other receptor-triggered pathways in a wide range of organisms.

Receptor-tyrosine kinases participate in diverse biological processes and trigger, by way of Ras, a sequential activation of protein kinases called the mitogen-activated protein (MAP) kinase signaling cascade (1). Several RTKs have been identified in *Drosophila*. Whereas some RTKs control a single developmental decision, DER functions in numerous developmental processes (2–7). Regulation of DER activation is often achieved by the restricted processing of an activating ligand, Spitz (8, 9), and induction of the secreted inhibitory protein Argos (10).

MAP kinase (ERK) is activated by dual phosphorylation of threonine and tyrosine residues by MEK (1). A monoclonal antibody, termed dipospho-ERK (dp-ERK), was raised against a dually phosphorylated 11-amino acid peptide that constitutes the vertebrate ERK activation loop (11–13). All 11 residues are conserved in the single *Drosophila* ERK homolog Rolled (14), raising the possibility of cross-reactivity.

To study recognition specificity, we tested the antibody on *Drosophila* Schneider S2 cells expressing DER. Incubation with se-

creted Spitz (sSpitz) resulted in the detection of a single 44-kD polypeptide by dp-ERK antibody (Fig. 1A). A general polyclonal antibody to ERK detected a similarly sized polypeptide in the presence or absence of Spitz. Immunohistochemical staining of induced cells detected activated ERK (Fig. 1B) (15).

In embryos, activation of DER by ubiquitous sSpitz (HS-sSpi) resulted in the accumulation of a single 44-kD polypeptide detected by dp-ERK antibody (Fig. 1A). Immunohistochemical staining of embryos with the general antibody to ERK displayed ubiquitous staining throughout embryogenesis in accordance with the high maternal contribution of the *rolled* gene. In contrast, dp-ERK displayed a specific staining pattern. Local activation of DER in the central segments led to staining only in this part of the embryo (Fig. 1C) (16).

Because ERK is a common junction for RTK pathways, the dp-ERK staining pattern represents the composite pattern of RTK signaling during development and can be correlated with activity of the known RTKs: Torso, DER, Heartless, and Breathless (17). Here we examine the dynamic DER-induced dp-ERK patterns in the embryo and imaginal discs.

Activation of DER is triggered by processing of Spitz, which is regulated by two membrane-spanning proteins, Rhomboid (Rho) and Star (8, 18, 19). Expression of Rho is tightly controlled (18, 20), and its

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