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wingless Signaling Acts through *zeste-white 3*, the Drosophila Homolog of *glycogen synthase kinase-3*, to Regulate *engrailed* and Establish Cell Fate

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Summary

Intrasegmental patterning in the Drosophila embryo is regulated by cell–cell communication. One of the signaling pathways that operates to specify positional information throughout the segment is mediated by the wingless (wg) protein, which is the homolog of the proto-oncogene *Wnt-1*. The early role of *wg* is to stabilize *engrailed* (*en*) expression by initiating a phase of *en* autoregulation in the adjacent more posterior cells. Here, we report that the segment polarity gene *zeste-white 3* (*zw3*; also known as *shaggy*) acts as a repressor of *en* autoregulation. Genetic epistasis experiments indicate that *wg* signaling operates by inactivating the *zw3* repression of *en* autoactivation. In addition, we demonstrate that *zw3* encodes the Drosophila homolog of mammalian glycogen synthase kinase-3.

Introduction

The Drosophila embryo is composed of repeating metameric units along the anteroposterior axis. The number, position, and polarity of these units are controlled by a hierarchy of maternal and zygotic genes (Akam, 1987; Ingham, 1988). The gap and pair rule genes act to subdivide the embryo into segmental units, whereas the segment polarity genes control patterning within each segmental unit. In contrast with the early subdivisions of the embryo, which occur in a syncitial blastoderm, intrasegmental patterning occurs in the cellularized gastrulating embryo. The cells of the ectoderm demarcate the segmental borders and assume specific cell fates within the segments that can be visualized in the cuticle of the emergent larvae. This process of segmental patterning is believed to occur through cell–cell interactions that determine cell identity (Ingham, 1991; Ingham and Martinez-Arias, 1992; Peifer and Bejsovec, 1992). The molecular nature of some of the segment polarity genes is consistent with this interpretation; *wingless* (*wg*) encodes a secreted factor homologous to the mammalian Wnt-1 protein (Baker, 1987; Rijsewijk et al., 1987; van den Heuvel et al., 1989; Gonzalez et al., 1991), *patched* encodes a transmembrane protein (Hooper and Scott, 1989; Nakano et al., 1989), *engrailed* (*en*) encodes a homeodomain protein (Fjose et al., 1985; Poole et al., 1985), and both *fused* and *zeste-white 3* (*zw3*) encode serine/threonine protein kinases (Preat et al., 1990; Siegfried et al., 1990; Bourouis et al., 1990).

The first indication of segmentation in the embryo is the transient parasegmental border, which defines expression of homeotic genes (Ingham and Martinez-Arias, 1992). The parasegmental border is formed at the juxtaposition of *wg*- and *en*-expressing cells; *wg* is expressed in the cells anterior of the border and *en* is expressed in the cells posterior to it (Baker, 1987; Martinez-Arias et al., 1988). Subsequently, the segmental border forms at the posterior edge of the *en*-expressing cells. Thoracic and abdominal segments can be easily identified in the ventral larval cuticle and are characterized by denticles in the anterior region and mostly naked cuticle in the posterior of each segment (see Figure 1B1).

Studies of segmental patterning have implicated wg as a key molecule that dictates cell fate and positional information of the epidermal cells in each embryonic segment (for review see Peifer and Bejsovec, 1992). *wg* is the homolog of mammalian *Wnt-1* and is a member of a large gene family postulated to encode signaling molecules that regulate developmental processes in Drosophila, Xenopus, and mouse (for reviews see Nusse and Varmus, 1992; McMahon, 1992). *Wnt-1*, which encodes a secreted glycoprotein, has been demonstrated to act via a paracrine mechanism in cell culture (Rijsewijk et al., 1987; Papkoff et al., 1987; Bradley and Brown, 1990; Jue et al., 1992). In Drosophila, there is direct evidence that wg protein acts as a signaling molecule conveying positional information. Although *wg* is expressed in only a subset of the cells in each segmental unit, wg protein has been detected in a graded distribution throughout the segment (Baker, 1987; van den Heuvel et al., 1989; Gonzalez et al., 1991). Using a temperature-sensitive allele of *wg*, two distinct roles for *wg* in patterning and cell fate determination have been demonstrated (Bejsovec and Martinez-Arias, 1991). The early function of *wg* is to maintain *en* expression in the adjacent cells posterior to the source of wg protein. Later, wg protein specifies positional information in cells that give rise to the naked cuticle (Bejsovec and Martinez-Arias, 1991). What remains unclear is how wg protein is secreted and the signal transduction pathway it initiates that regulates the transcription of other genes, such as *en*.

Pivotal to the establishment of cell fate and polarity within each embryonic segment is the expression of *en*, which is necessary for the commitment of cells to the posterior compartment of each segment (Kornberg, 1981; Vincent and O'Farrell, 1992). *en* expression is initiated by the pair rule genes, but its maintenance is dependent on *wg* signaling from adjacent cells (Howard and Ingham, 1986; DiNardo and O'Farrell, 1987; Frasch et al., 1988; Martinez-Arias et al., 1988; DiNardo et al., 1988; Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991). Hence in *wg* mutant embryos the border fails to form and *en* expression is lost in the adjacent cells (Perrimon and Mahowald, 1987; Martinez-Arias et al., 1988; DiNardo et al., 1988). Recently, using a novel technique to label lineages, it has been demonstrated that stable *en* expression and commitment of specific cells to the posterior compartment is not clonally inherited but is determined by cell signaling (Vin-

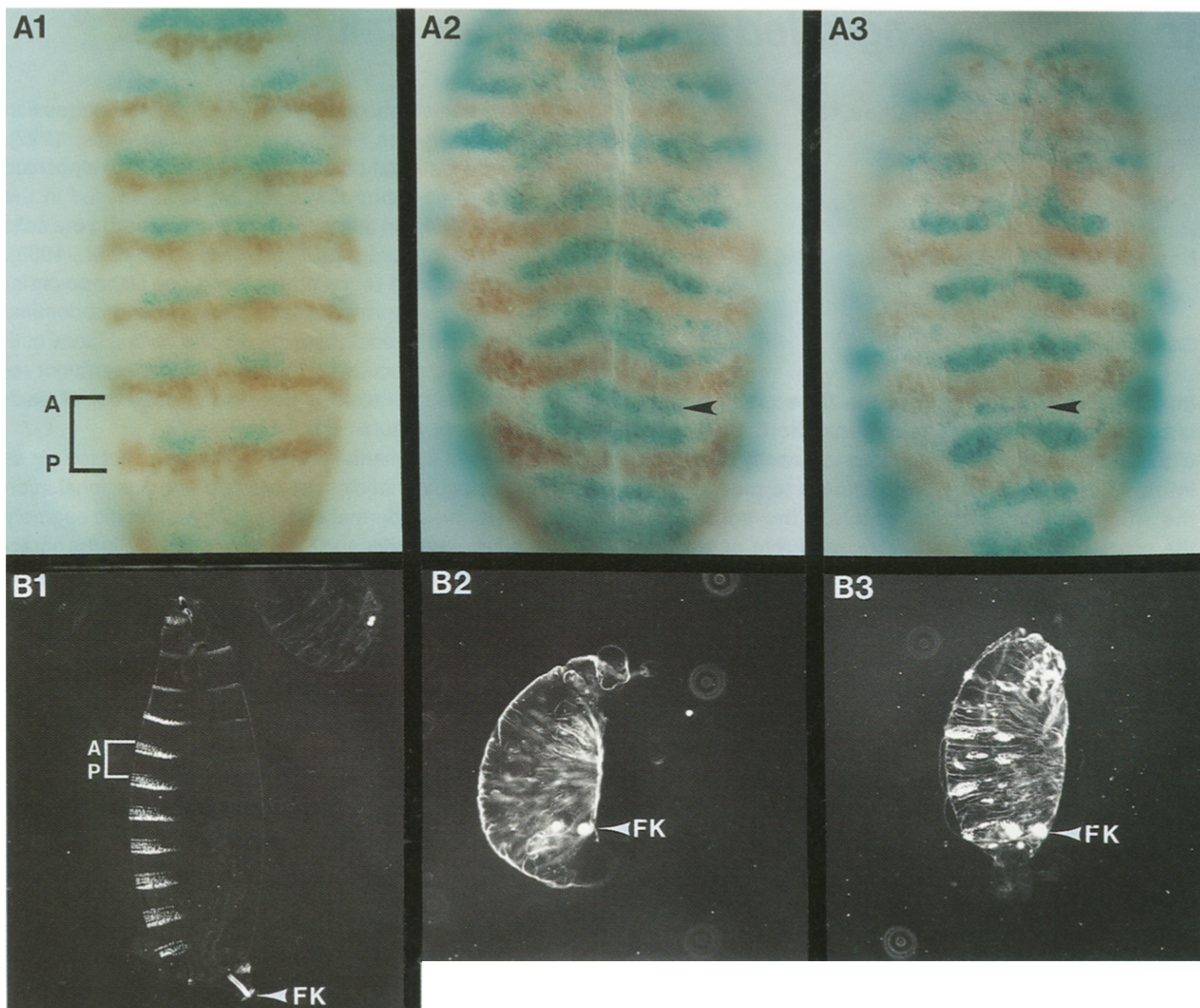

Figure 1. Phenotype of *zw3* Mutant Embryos

(A) en protein (brown) and *wg* expression (blue) in stage 11 wild-type embryo (1); stage 11 *zw3* null mutant embryo (2); and stage 11 *zw3* rescued mutant embryo (3).

(B) Cuticle phenotype of wild-type embryo (1), *zw3* null mutant embryo (2), and *zw3* rescued mutant embryo (3).

In *zw3* mutant embryos, en protein distribution is expanded to approximately one half the segment width. Subsequent to the expansion of *en* expression there is an ectopic stripe of *wg* expression (indicated by the arrowhead) at the posterior edge of the en stripe. The en stripe is identical in *zw3* null and *zw3* rescued embryos. *zw3* null mutant embryos have a stronger and more consistent ectopic stripe of *wg*. This correlates with the completely naked cuticle of *zw3* null embryos. *zw3* rescued mutant embryos have a sparse ectopic *wg* stripe and have a small but variable number of denticles on the ventral cuticle.

wg expression is detected with the enhancer trap line *CyO,wg^{en11}*. Brackets indicate the orientation of a segmental unit; A, anterior; P, posterior. Arrowheads indicate ectopic *wg* expression. FK indicates filzkörper material. Stained embryos in (A) are shown in a ventral view with anterior up. Embryos in (B) are shown anterior up and ventral to the left. Staging is according to Campos-Ortega and Hartenstein, 1985.

cent and O'Farrell, 1992). Examination of marked clones revealed that the cells at the anterior edge of the stripe of *en* expression, closest to the *wg*-expressing cell, maintain stable *en* expression and respect clonal boundaries whereas the cells at the posterior edge of the *en* stripe do not.

The stabilization of *en* expression and the subsequent cell fate determination is a multistep process requiring both cell-extrinsic and cell-intrinsic mechanisms. Experiments using the temperature-sensitive *wg* allele and ectopic expression of *en* have defined four phases of *en* regulation (Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991). The domain of *en* expression is first defined by the pair rule genes. Thereupon, maintenance of *en* expression requires wg protein secreted from the adjacent more anterior cells. The *wg*-dependent phase of *en* expression is transient and coincident with the early phase of *en* autoregulation. *en* autoregulation gradually becomes independent of *wg* signaling, and subsequently *en* expression is regulated in an *en*-independent fashion, possibly under the control of *Polycomb*-like genes (Martinez-Arias et al., 1988; DiNardo et al., 1988; Heemskerk et al., 1991).

Concurrent with mechanisms that stabilize *en* expression, there is selective repression of *en*. Transient global

expression of *en*, under the control of a heat shock promoter, resolves into a distinct pattern rather than uniform expression of endogenous *en* (Heemskerk et al., 1991). This suggests the presence of repressor activity in wild-type embryos that restricts *en* autoactivation. The segment polarity gene *naked* has been identified as a potential repressor of *en* autoactivation, since in *naked* mutant embryos transient global expression of *en* does result in uniform expression of endogenous *en* (Heemskerk et al., 1991).

The phenotype of embryos derived from *zw3* mutant germlines is similar to *naked* mutant embryos; the denticle hairs on the ventral epidermis are missing, leaving mostly naked cuticle (Perrimon and Smouse, 1989). The underlying cellular basis for this phenotype is the altered pattern of *en* expression (Martinez-Arias et al., 1988; DiNardo et al., 1988; Perrimon and Smouse, 1989). The similarity of *zw3* and *naked* mutant phenotypes suggests that these two genes may function in a common pathway of repression of *en* expression.

We have tested the role of *zw3* as a repressor of *en* expression and examined its function in the *wg* signaling pathway. Data are presented to support a model for *wg* signaling as a mechanism to stabilize *en* expression by inactivating the *zw3* repression of *en* autoactivation.

Results

zw3 Is Essential in Many Developmental Processes

zw3 is expressed throughout development and is required for several different developmental processes in the embryo, larvae, and adult (Simpson et al., 1988; Perrimon and Smouse, 1989; Siegfried et al., 1990; Bourouis et al., 1989, 1990; see Experimental Procedures). Homozygous or hemizygous *zw3* mutant individuals die during larval stages with some defects in development of the nervous system (Shannon et al., 1972; Bourouis et al., 1989). Generation of mosaic individuals has revealed that *zw3* is also required for adult epidermal differentiation and correct embryonic patterning. Patches of homozygous mutant tissue in the wing and notum give rise to ectopic bristles, indicative of a transformation from epidermal to neural fate (Simpson et al., 1988; Perrimon and Smouse, 1989).

Embryos derived from homozygous *zw3* mutant female germlines, *zw3* mutant embryos, have a phenotype similar to embryos mutant for *naked*, characterized by the absence of denticles on the ventral cuticle. This phenotype is a consequence of the altered expression of *en* and *wg*. *zw3* mutant embryos lacking both maternal and zygotic product, *zw3* null mutant embryos, are completely naked, devoid of all denticles on the ventral cuticle (Figure 1B2). In these mutant embryos, *en* expression is expanded and there is a strong ectopic stripe of *wg* expression posterior to the expanded *en* stripe (Figure 1A2). In contrast, *zw3* rescued mutant embryos, which lack maternal zw3 product but have received a wild-type copy of the gene from their father, have sparse ectopic *wg* stripes and have some denticles on the ventral cuticle (Figure 1A3 and 1B3). The expanded *en* expression is identical in the *zw3* null and *zw3* rescued mutant embryos. These results indicate that zygotic zw3 product can partially rescue the *zw3* maternal effect.

zw3 Is a Repressor of *en* Autoregulation

Initiation and maintenance of *en* expression are differentially controlled phases of *en* regulation (see Introduction). This early and late regulation of *en* expression is reflected in the cis regulatory regions of *P(en/lac)*, a transgenic strain containing a fusion of part of the *en* promoter to the Escherichia coli β-galactosidase gene (DiNardo et al., 1988). This transgenic strain does not reiterate the complete *en* expression pattern, but rather discrete phases of *en* expression: the initiation of even-numbered stripes of *en* expression by pair rule genes and the maintenance of *en* expression in both even- and odd-numbered stripes by cell signaling through *wg*. The early pattern of *P(en/lac)* expression is 7 stripes identical to the even-numbered endogenous en stripes (Figure 2A1). After germband elongation, *P(en/lac)* expression shifts to 11 stripes that are coincident with the even- and odd-numbered en stripes 3–13, corresponding to the labial, 3 thoracic and first 7 abdominal stripes (Figure 2A2). The 11 stripes of *P(en/lac)* expression are unaffected by the pair rule genes but are regulated by *wg* and other segment polarity genes (DiNardo et al., 1988).

The expression of *P(en/lac)* was examined in *zw3* mutant embryos to test whether the expanded *en* expression in *zw3* mutant embryos is due to ectopic initiation or incorrect maintenance. In *zw3* mutant embryos, the initial pattern of *P(en/lac)* is identical to wild-type embryos (Figure 2B1). However, the late phase of *P(en/lac)* expression is altered in *zw3* mutant embryos (Figure 2B2). The 11 stripes in *zw3* embryos are broader, approximately twice as wide as in wild-type embryos (compare the width of stripe 7 in Figures 2A2 and 2B2). We conclude that *en* expression is initiated normally in *zw3* mutant embryos but is not maintained correctly. This result, which we have confirmed by direct examination of en protein in *zw3* mutant embryos (data not shown), is consistent with a role for *zw3* in the late phase of *en* regulation that is *wg* dependent.

A further analysis of the regulation of *en* expression has indicated that *en* autoactivation is a necessary step in the evolution of stable *en* expression (Heemskerk et al., 1991). A transgenic strain, *hs-en*, expressing the *en* cDNA through the Drosophila *hsp70* promoter, was used to demonstrate *wg*-dependent *en* autoregulation. To monitor *en* autoactivation, the distribution of endogenous en protein was examined following transient global expression of *hs-en*. Heemskerk et al. (1991) demonstrated that *en* autoregulation is both activated by *wg* and restricted by another segment polarity gene, *naked*. In wild-type embryos endogenous en protein is expressed in slightly expanded stripes following transient expression of *hs-en* (Figure 3A). This is in contrast with *naked* mutant embryos in which endogenous en can be detected uniformly throughout the embryos subsequent to transient expression of *hs-en* (Heemskerk et al., 1991). These results lead to the suggestion that *naked* is a repressor of *en* autoactivation. To test whether *zw3* is such a repressor, expression of *hs-en* was used to activate endogenous *en* in *zw3* mutant embryos.

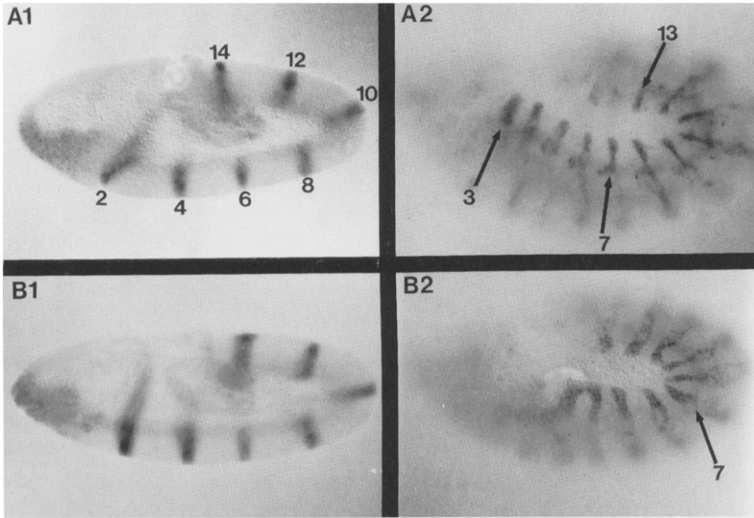

Figure 2. *P(en/lac)* Expression in Wild-Type and *zw3* Mutant Embryos

(A) β-Galactosidase expression in stage 8 *P(en/lac)* embryo (1) and stage 12 *P(en/lac)* embryo (2).

(B) β-Galactosidase expression in stage 8 *zw3; P(en/lac)* mutant embryo (1) and stage 12 *zw3; P(en/lac)* mutant embryo (2). Note (in B2) only 10 stripes can be seen in this embryo, since the most posterior stripe is out of focus. The stripes are numbered relative to the endogenous en stripes. There is no detectable difference in *P(en/lac)* expression in *zw3* null and rescued mutant embryos.

The 7 early stripes correspond to the even-numbered endogenous en stripes. Later, there are 11 stripes that correspond to endogenous even and odd en stripes; the labial, first, second, and third thoracic, and abdominal stripes 1–7. Note that the 11 *P(en/lac)* stripes do not fill the entire *en*-expressing region but are restricted to a dorsolateral position (DiNardo et al., 1988). In *zw3* mutant embryos, the early pattern of *P(en/lac)* expression is normal, but by stage 12 it has expanded; compare the width of stripe 7 in A2 and B2. *P(en/lac)* expression is expanded in a similar fashion to the endogenous en protein in *zw3* mutant embryos.

Embryos are shown anterior to the right and dorsal to the top.

zw3; hs-en embryos were heat shocked between 3 and 5 hr after egg laying (AEL, stages 7–9), allowed to recover, and the distribution of the endogenous en protein was examined (see Experimental Procedures). Whereas in wild-type embryos endogenous *en* expression is only slightly altered (Figure 3A), in *zw3* mutant embryos en protein is detected in almost all cells subsequent to transient expression of *hs-en* (Figure 3B). These results indicate that *zw3* does function as a repressor of *en* autoactivation.

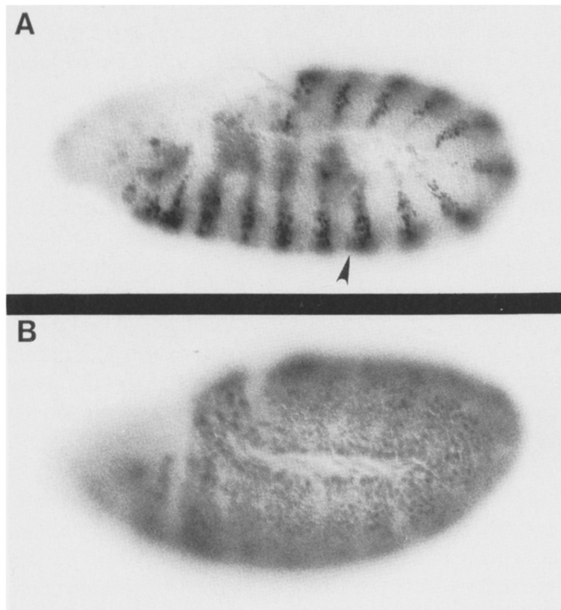

Figure 3. Activation of Endogenous *en* by *hs-en* in Wild-Type and *zw3* Mutant Embryos

(A) Distribution of endogenous en protein in stage 11 *hs-en* embryo following transient expression of *hs-en* (see Experimental Procedures).
(B) Distribution of endogenous en protein in stage 11 *zw3;hs-en* mutant embryo following transient expression of *hs-en*.

In wild-type embryos endogenous *en* is not uniformly activated; rather en protein is detected in a slightly altered stripe that is expanded anteriorly (indicated by the arrowhead; Heemskerk et al., 1991). Note that the expansion is in the ventral region of the en stripes. In *zw3* mutant embryos endogenous *en* is activated in almost all cells of the embryonic epidermis. Arrowhead indicates ectopic *en* expression. Orientation of the embryos is the same as in Figure 2.

wg Is an Antagonist of *zw3* Repression of *en*

As described above, *en* autoactivation is both positively regulated by *wg* and negatively regulated by *zw3*. To determine how *zw3* functions in the *wg* signaling pathway, en protein was examined in *zw3-wg* double mutant embryos (see Experimental Procedures). In *wg* mutant embryos, *en* expression fades by stage 10 and the larval cuticle pattern is perturbed, resulting in a lawn of denticles on the ventral epidermis (Figures 4A3 and 4B3; DiNardo et al., 1988; Martinez-Arias et al., 1988; Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991). In contrast, in *zw3* mutant embryos, *en* expression is maintained in an expanded fashion resulting in the absence of denticles on the ventral cuticle (see above, Figures 1A2 and 1B2). If *zw3* acts downstream of *wg* signaling, then *zw3-wg* mutant embryos should resemble *zw3* mutant embryos. In *zw3-wg* mutant embryos en protein can be detected at stage 10 and is expanded posteriorly in every segment, as observed in *zw3* mutant embryos (Figure 4A2). This indicates that in the absence of maternal zw3 product *en* expression is maintained in a *wg*-independent fashion. These results demonstrate that *zw3* is epistatic to *wg*, and they suggest that in wild-type embryos the early role of *wg* signaling is to release *zw3* repression of *en* autoregulation.

Initially, all the *zw3-wg* mutant embryos (*zw3* rescued-*wg* and *zw3* null-*wg*) express *en* in a stable and expanded fashion. However, the *zw3* rescued-*wg* mutant embryos do not maintain an expanded domain of *en* expression, and en protein is no longer detectable in the epidermis

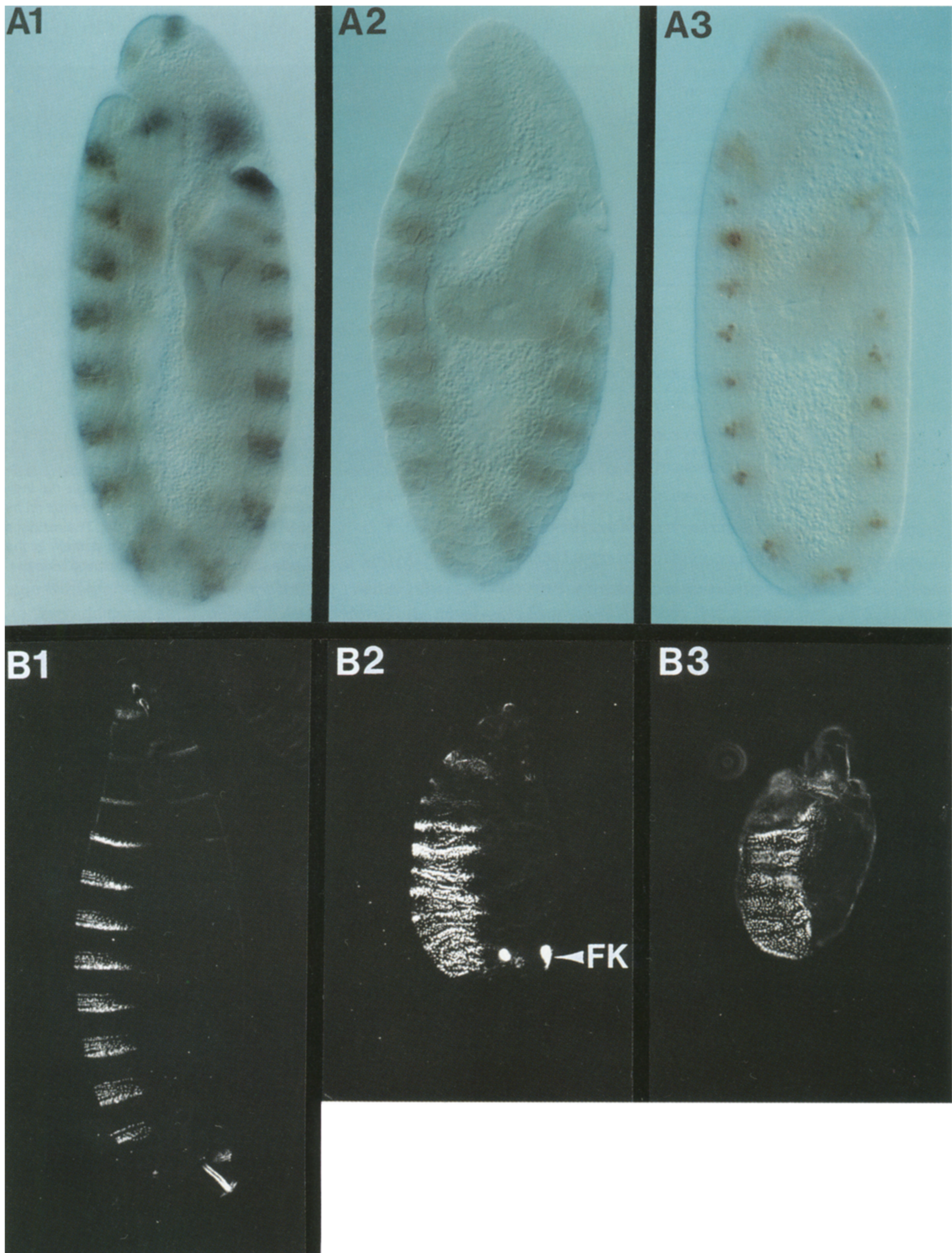

Figure 4. En and Wg Protein Distribution in Wild-Type, *zw3-wg* Mutant, and *wg* Mutant Embryos

(A) En protein (brown) and wg protein (blue) in stage 11 wild-type embryo (1), stage 10 *zw3-wg* mutant embryo (2), and stage 10 *wg* mutant embryo (3).

(B) Cuticle phenotypes of wild-type embryo (1), *zw3 svb* rescued-*wg* (*zw3 svb/+; wg/wg*) mutant embryo (2), and *wg* mutant embryos (3).

In (A) the *wg* mutant embryos are identified by the absence of wg immunostaining (see Experimental Procedures). To be certain that the wg immunostaining was a reliable marker for the mutant genotype, we examined 226 embryos between stages 10 and 12; 173 embryos had wg staining and 53 embryos had no wg staining. This is consistent with the lack of wg immunostaining indicating *wg* mutant embryos. The residual en protein detected in *wg* mutant embryos (A3) is due to expression in the nervous system. For the examination of the cuticle phenotype of *zw3* rescued–*wg* (B2) and *zw3* null–*wg* mutant embryos, *svb* was used as a marker to distinguish the genotypes. *zw3 svb* rescued–*wg* mutant embryos have a *wg*-like mutant phenotype with wild-type denticles indicating that they are paternally rescued for the X chromosome. *zw3* rescued–*wg* mutant embryos differ from *wg* mutant embryos in that filzkörper material (FK) is present. Embryos are shown anterior to the top and ventral to the left.

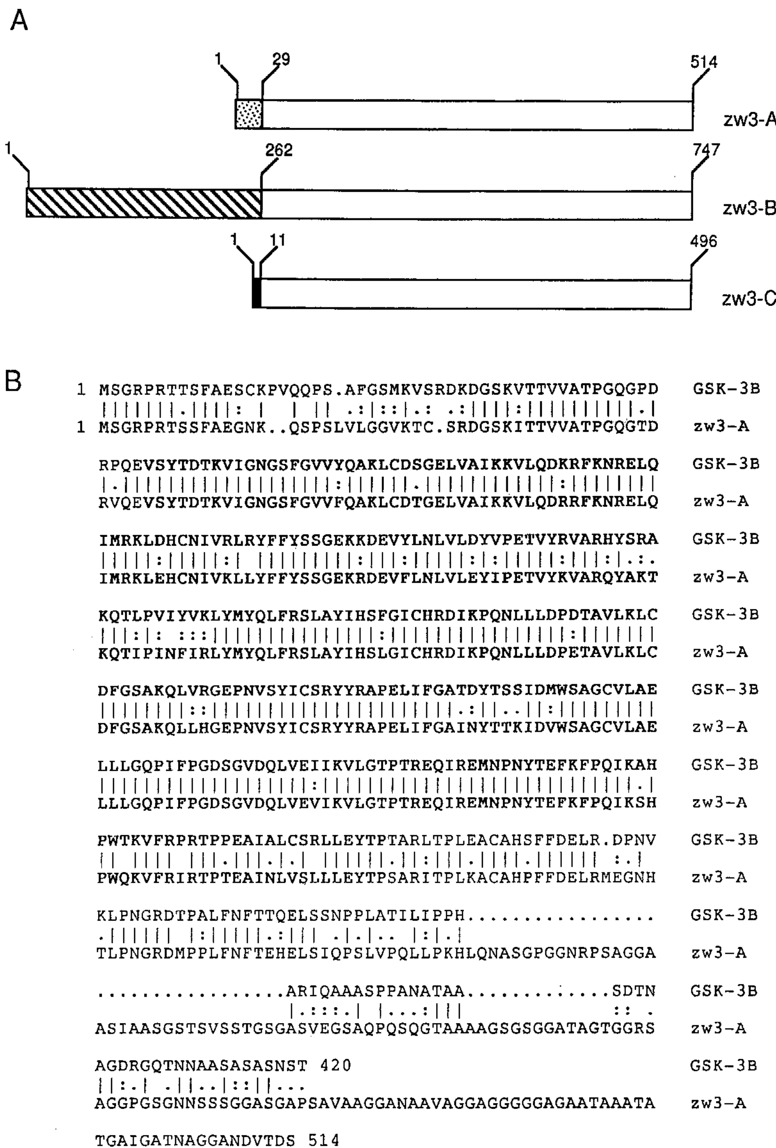

Figure 5. The *zw3*-Encoded Proteins

(A) Comparison of three *zw3*-encoded proteins. The predicted proteins encoded by *zw3* are represented schematically with the amino acid residues indicated. The open region is the sequence identical in all of the *zw3*-encoded proteins. The stippled region is sequence that is unique to zw3-A and is similar to GSK-3β (see [B]). The striped region is sequence unique to zw3-B (Siegfried et al., 1990), and the closed region indicates sequence unique to zw3-C. Zw3-A is described in Siegfried et al., 1990 (cOV7) and Bourouis et al., 1990; zw3-B is described in Siegfried et al., 1990 (cKZ5). Zw3-C is described in this work (Experimental Procedures).

(B) Comparison of GSK-3β and zw3-A. Overall identity between the two proteins is 76%, with as much as 85% identity within the catalytic kinase domain. The lengths of the two predicted proteins are indicated. A vertical line indicates identity and a colon or single dot indicates conservative changes as determined by BESTFIT (Devereux et al., 1984). The residues in bold are within the catalytic domain of the kinase, starting with valine at position 55 to proline at position 326 (Hanks et al., 1988).

following germband retraction (data not shown). These embryos develop a *wg*-like mutant cuticle phenotype (Figure 4B2) that is in contrast to their siblings, *zw3*-null, *wg* mutant embryos, which maintain *en* expression and have a phenotype similar to *zw3* null mutant embryos shown in Figure 1B2 (data not shown). These results indicate that although zygotic zw3 product is not able to rescue the maternal effect completely, it is sufficient to render *en* expression *wg* dependent. Interestingly, the *zw3* rescued-*wg* mutant embryos have filzkörper material that is missing in *wg* mutant embryos. This indicates that in *zw3* null and *zw3* rescued mutant embryos the development of filzkorper material is *wg* independent.

These results demonstrate that in the absence of both maternal and zygotic zw3 product *en* expression is *wg* independent. However, if no maternal but some zygotic zw3 product is present in *wg* mutant embryos, *en* expression is repressed and will fade prematurely, resulting in a *wg*-like mutant phenotype. This supports our conclusion that *wg* is required to stabilize *en* expression because *wg* signaling is a means of inactivating *zw3* repression of *en*.

zw3 Encodes Multiple Protein Kinases Homologous to Mammalian Glycogen Synthase Kinase-3

The sequence of *zw3* is consistent with its role as a molecule involved in a signal transduction pathway. *zw3* generates several developmentally regulated transcripts that encode multiple forms of a serine/threonine protein kinase (Siegfried et al., 1990; Bourouis et al., 1990). Previous work has identified two different predicted protein products: a putative protein of 514 amino acids, zw3-A, and one of 747 amino acids, zw3-B (Figure 5A; Siegfried et al., 1990). These proteins share a common region of 485 amino acids that encodes a putative catalytic domain of a

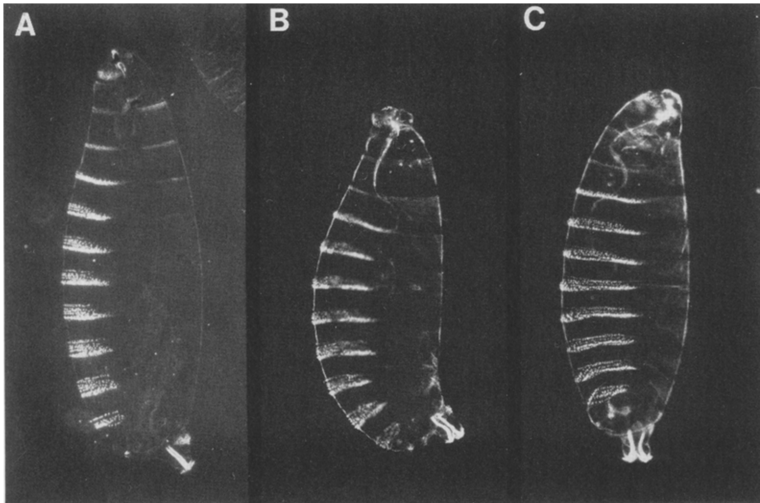

Figure 6. Rescue of *zw3* Maternal-Effect Phenotype through Expression of *zw3-A* and *GSK-3β*

Wild-type embryo (A), *zw3; hs-zw3-A* rescued embryo (B), and *zw3; hs-GSK-3β* rescued embryo (C). Transient expression of *hs-zw3-A* or *hs-GSK-3β* during embryonic development can rescue *zw3* mutant embryos to a wild-type segmental pattern (see Experimental Procedures). Orientation of the embryos is the same as Figure 4.

serine/threonine protein kinase (Hanks et al., 1988), but completely diverge at their amino termini. We have characterized additional embryonic cDNAs and identified a novel protein, zw3-C. This predicted protein is 496 amino acids in length and contains the identical catalytic domain of zw3-A and zw3-B (Figure 5A). However, zw3-C diverges from both zw3-A and zw3-B at the amino terminus, which is 11 amino acids in length (Figure 5).

When the *zw3* sequence was determined, searches of the available data bases revealed the greatest homology to yeast *cdc2/CDC28*; however, recently a striking similarity between *zw3* and mammalian *glycogen synthase kinase-3* (*GSK-3*) was detected (Woodgett, 1990, 1991). The greatest similarity exists between zw3-A and GSK-3β (Figure 5B). The overall comparison reveals 76% identity and 85% similarity at the amino acid level (see Experimental Procedures). The respective kinase domains of these two proteins share 85% identity and 94% similarity. GSK-3 is a kinase implicated in the regulation of glycogen synthase as well as the regulation of transcription factors *c-jun*, *myb*, and *L-myc* (see Discussion, Boyle et al., 1991; Woodgett, 1991; Saksela et al., 1992).

Ubiquitous Expression of a Single Zw3 Protein Rescues All Aspects of the *zw3* Mutant Phenotypes

The complexity of the *zw3* locus raises the possibility that each unique protein could function specifically and independently in the development of the organism. We have tested the ability of one cDNA, *zw3-A*, expressed under the control of the Drosophila *hsp70* promoter (*hs-zw3-A*) to rescue the various *zw3* mutant phenotypes.

Transient zygotic expression of a single copy of *hs-zw3-A* in embryos (0–4 hr AEL) derived from *zw3* mutant germlines is sufficient to restore the mutant embryos to a wild-type phenotype (Experimental Procedures; Figure 6B). Ubiquitous expression of *zw3-A* can also rescue the lethality of *zw3* mutant larvae. *zw3* mutant individuals, carrying a single copy of *hs-zw3-A*, were subjected to daily heat shocks throughout larval development and examined for the presence of rescued *zw3* adult males (Experimental Procedures). From this experiment, *zw3/Y; hs-zw3-A/+* males were recovered that were normal and fertile. Occasionally, rescued males with ectopic bristles on the notum or wings were observed. In these animals, a sufficient level of zw3 activity was presumably provided to rescue the larval lethality but not the bristle phenotype associated with loss of zw3 activity in imaginal cells (Simpson et al., 1988; Perrimon and Smouse, 1989).

In conclusion, the zw3-A protein is sufficient to rescue all aspects of the *zw3* mutant phenotype: the maternal effect segment polarity phenotype, the larval lethality, and the imaginal epidermal function. In addition, under the experimental conditions described (Experimental Procedures) we do not detect any phenotypes as a consequence of the expression of *hs-zw3-A* in wild-type embryos or larvae, suggesting that increased levels of zw3 protein in wild-type cells does not alter cell fate (data not shown).

Mammalian GSK-3β Can Rescue the *zw3* Maternal Effect Phenotype

The sequence similarity between zw3-A and GSK-3β suggests that the mechanism of *zw3* regulation of *en* expression may be homologous to the mechanism of *GSK-3β* regulation of transcription factors. To examine this possibility, we tested whether expression of *GSK-3β* can rescue the *zw3* maternal effect phenotype. Transgenic strains were generated that express a cDNA encoding GSK-3β (Woodgett, 1990) under the control of the Drosophila *hsp70* promoter (*hs-GSK-3β*). Embryos derived from *zw3* mutant germlines and carrying a single copy of *hs-GSK-3β* can be rescued to a wild-type phenotype if they are subjected to a heat shock during embryonic development (Experimental Procedures; Figure 6C). Under the experimental conditions described (Experimental Procedures), expression of *hs-GSK-3β* does not rescue the *zw3* larval lethality. These results indicate that at least in the embryo, zw3-A and GSK-3β share substrate specificity.

Discussion

The Role of *wg* Signaling Is to Alleviate the *zw3* Repression of *en* Autoregulation

Intrasegmental patterning and cell fate determination in the embryonic epidermis occur through a signal transduction pathway mediated in part by the wg protein. Although *wg* is expressed in a subset of the cells of each segment, wg protein is detected in a graded fashion in neighboring cells (van den Heuvel et al., 1989; Gonzalez et al., 1991). *wg* signaling functions early in embryogenesis to stabilize *en* expression in more posterior cells, which is required for cells to become committed to the posterior cell fate (Heemskerk et al., 1991; Vincent and O'Farrell, 1992). The signal transduction mechanism and transcriptional regulation initiated by *wg* signaling are unknown. However, we offer an insight into the mechanism of *wg* signaling and suggest that *wg* regulation of *en* expression is mediated through *zw3*. Our results indicate that *wg* signaling stabilizes *en* expression by inactivating zw3 kinase activity, which is a repressor of *en* autoactivation.

In *zw3* mutant embryos, *en* expression is expanded posteriorly, to approximately twice the number of cells as in wild-type embryos. Subsequently, an ectopic stripe of *wg* expression is observed just posterior to the *en*-expressing cells. The expanded en stripe in *zw3* mutant embryos is indicative of a role for *zw3* as a repressor of *en* expression. The expanded *en* expression does not spread throughout the segment but is restricted to approximately half of the segment, the cells of the proposed *en* equivalence group (Ingham, 1991; Ingham and Martinez-Arias, 1992). This restriction reflects the competency of cells within each segment to express either *en* or *wg*, which is presumably established by the pair rule genes. In *zw3* mutant embryos *en* expression is expanded to fill the entire *en* equivalence group, and the ectopic stripe of *wg* expression is induced in cells of the *wg* equivalence group.

en expression evolves through a progression of regulatory mechanisms: initiation occurs at the blastoderm stage, activated by the pair rule genes (Howard and Ingham, 1986; DiNardo and O'Farrell, 1987; Frasch et al., 1988), and is subsequently stabilized through *wg* signaling, triggering an *en* autoregulatory phase (Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991). In *zw3* mutant embryos, the initiation of *en* expression is normal; however, the *wg*-dependent maintenance of *en* expression is perturbed (Figure 2). We have demonstrated that *zw3* is a repressor of *en* autoactivation. In *zw3* mutant embryos, transient global expression of *en* can induce endogenous *en* expression uniformly, unlike wild-type embryos, in which *en* autoactivation is restricted (Figure 3).

In wild-type embryos *en* expression is maintained in the cells of the *en* equivalence group closest to the *wg*-expressing cells, which receive high levels of wg protein. The more posterior cells, farthest from the source of *wg* signal, receive insufficient wg protein to stabilize *en* expression and hence their cell fate is not restricted (Vincent and O'Farrell, 1992; Ingham and Martinez-Arias, 1992). However, in *zw3* null mutant embryos, *en* expression occurs in all cells of the *en* equivalence group and is independent of *wg* signaling. This is demonstrated by the identical phenotypes of *zw3* null mutant embryos and *zw3* null–*wg* mutant embryos (Figure 4). These results suggest that the function of *wg* signaling is to inactivate the *zw3* repression of *en* autoregulation. *zw3* represses *en* autoactivation in all the cells of the *en* equivalence group; this repression can, however, be overcome by the delivery of sufficient amounts of wg protein. Hence, cell fate is determined by *zw3* repression of *en* autoregulation and the selective inactivation of this repression by wg protein (see Figure 7).

This interpretation is supported by the results of experiments examining the effects of ectopic *wg* expression. When *wg* is globally expressed during embryogenesis, *en* expression is expanded and embryos develop a *zw3/naked* mutant phenotype (Noordermeer et al., 1992). Presumably this is due to sufficiently high levels of wg protein uniformly distributed in the embryo that can completely antagonize *zw3* repression and stabilize *en* expression. In contrast, the overexpression of *zw3* in wild-type embryos appears to have no effect on cell fate determination. Moreover, transient overexpression of zw3-A or GSK-3β is sufficient to restore *zw3* mutant embryos to a wild-type pattern. These results are consistent with the proposed role for *zw3* as a constitutive repressor of *en* autoregulation.

Although normal zygotic expression of *zw3* can not completely rescue the *zw3* maternal effect, it is sufficient to render *en* expression *wg* dependent. In *zw3* null–*wg* mutant embryos, *en* expression is maintained in a similar fashion to *zw3* null mutant embryos. However, if some zw3 product is introduced (*zw3* rescued–*wg* mutant embryos), *en* expression is initially expanded but becomes *wg* dependent and prematurely fades, resulting in a *wg*-like mutant phenotype (Figure 4). This transition from *wg* independent to *wg* dependent is presumably due to the accumulation of zw3 protein, which must reach some threshold level to repress *en* autoregulation effectively. Alternatively, it may be the requirement for specific forms of zygotic zw3 proteins that accounts for the delay in *zw3* repression of *en*. This is unlikely, given that all aspects of the *zw3* mutant phenotype can be rescued by the expression of a single *zw3*-encoded protein.

The reduced level of zw3 activity in *zw3* rescued mutant embryos also accounts for the less severe mutant phenotypes of these embryos (Figure 1B3). We propose that in these mutant embryos zw3 activity is low enough to allow *en* expression in all cells of the *en* equivalence group; however, *en* expression is somewhat reduced in the more posterior cells, which would result in weaker induction of ectopic *wg* expression (Figure 1A3). The correlation of the phenotypes of *zw3* null and rescued mutant embryos with the different levels of ectopic *wg* expression is consistent with the observation that the mutant phenotype of *naked* is due to *wg* expression (Dougan and DiNardo, 1992).

We propose that cell fate is regulated by the constitutive *zw3* repression of *en* autoactivation and the selective delivery of wg protein, which inactivates this repression and stabilizes *en* expression. How the secretion of wg protein occurs is unknown, but it has been suggested that it is delivered from one cell to another via an intracellular receptor generating a gradient of protein throughout the seg-

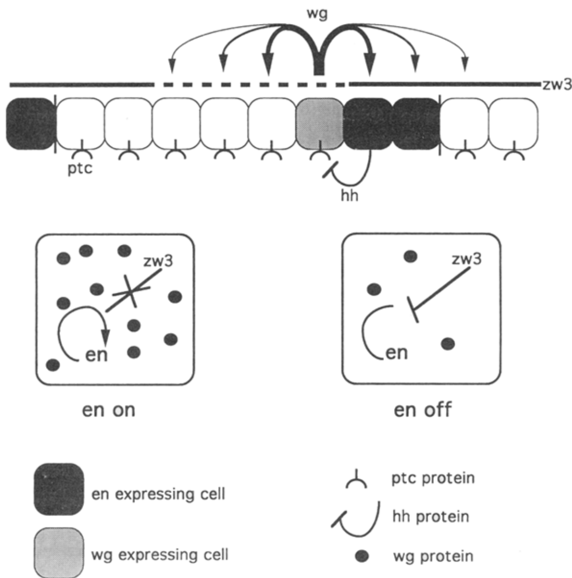

Figure 7. *wg* Signaling Functions to Dismantle *zw3* Repression of *en* Autoregulation and Stabilize *en* Expression

Depicted in this schematic drawing are the cells in one segment of a stage 9 wild-type embryo. *wg*-expressing cells are indicated by the stippled cells and *en*-expressing cells by the closed cells. The segmental border (indicated by vertical lines) forms at the posterior edge of *en*-expressing cells. Wg protein is secreted in a graded fashion (indicated by the arrows) centered on the *wg*-expressing cell.

The initiation of *wg* and *en* expression is regulated by the pair rule genes, but their continued expression at this stage of development requires intercellular communication. *wg* signaling is required to maintain *en* expression in the adjacent posterior cells by initiating an *en* autoregulatory phase. *zw3* functions as a constitutive repressor of *en* autoregulation in the cells of the segment that are competent to express *en*, the *en* equivalence group (indicated by the solid line). *wg* signaling stabilizes *en* expression by inactivating the *zw3* repression of *en* autoactivation. Stable *en* expression and subsequent cell fate determination occur through selective inactivation of *zw3* repression by *wg* signaling. Although *zw3* is uniformly expressed in the embryo, we have not detected any requirement for *zw3* in the *wg* equivalence group (indicated by the broken line). It is possible that in some mutant background there is a function for *zw3* in these cells.

The cells of the *en* equivalence group can either be expressing *en* (en on) or repressing *en* (en off) (Ingham, 1991; Ingham and Martinez-Arias, 1992). The posterior cells closest to the source of wg protein receive high levels of wg protein (indicated by the closed dots). In these cells, *zw3* repression of *en* autoregulation is blocked and *en* expression is maintained (en on). However, in more posterior cells that receive low levels of wg protein, *zw3* repression of *en* autoactivation is unaffected and *en* expression fades (en off).

In addition to *wg* signaling to maintain *en* expression, there is reciprocal signaling from the *en*-expressing cells to the *wg*-expressing cells required to maintain *wg* expression (Ingham et al., 1991). The transmembrane patched (ptc) protein is a repressor of *wg* expression in all the cells of the *wg* equivalence group. Signaling from the anterior *en*-expressing cell to the *wg*-expressing cell is required to block this repression and maintain *wg* expression. It has been postulated that this signaling occurs through *hedgehog*, which encodes a putative transmembrane protein (Mohler and Vani, 1992; Lee et al., 1992). This is consistent with mosaic analysis, which indicates that clones of *hedgehog* behave nonautonomously (Mohler, 1988).

ment (Gonzalez et al., 1991). Mutations in several segment polarity genes have *wg*-like mutant embryonic phenotypes. Some of these genes presumably function in the *wg* signaling pathway and include *armadillo*, *porcupine*, and *dishevelled* (Perrimon et al., 1989; Klingensmith et al., 1989; Riggleman et al., 1990; M. van den Heuvel et al., unpublished data). Mosaic analysis has revealed that both *armadillo* and *dishevelled* are cell autonomous and may enable cells to receive or interpret the *wg* signal (J. Klingensmith and N. P., personal communication). *armadillo* encodes a protein with similarities to plakoglobin and β-catenin, indicating an association with the cell membrane (Peifer and Wieschaus, 1990; Peifer and Bejsovec, 1992). *dishevelled* encodes a novel protein of unknown function (J. Klingensmith and N. P., personal communication). Double mutant combinations with these *wg*-like mutations, similar to the analysis of *zw3-wg* mutant embryos, will help elucidate the function of these genes in mediating *wg* signaling.

Are *zw3* and *naked* Acting in the Same Pathway?

Another proposed repressor of *en* autoregulation is the zygotic segment polarity gene, *naked* (Heemskerk et al., 1991). The similarity of the *zw3* and *naked* mutant cuticle phenotypes indicates that these two gene products may act in the same signaling pathway. *en* and *wg* expression are altered in *naked* mutant embryos similar to *zw3* mutant embryos (DiNardo et al., 1988; Martinez-Arias et al., 1988; Perrimon and Smouse, 1989). Despite the similarities between *naked* and *zw3*, there is one significant difference between these two genes: *zw3-wg* double mutant embryos exhibit the *zw3* mutant phenotype, whereas *naked-wg* mutant embryos exhibit the *wg* mutant phenotype (Ingham, 1991; M. van den Heuvel et al., unpublished data; S. DiNardo, personal communication). If *wg* signaling is mediated through *zw3*, then these observations would indicate that *naked* and *zw3* function independently in the regulation of *en* expression. One possibility is that *naked* interacts with and enhances *zw3* activity such that in a *naked* mutant embryo residual zw3 activity, sufficient to render *en* expression dependent on *wg*, would be present. Finally, it is not clear if the strongest of the *naked* alleles are null mutations or if there is any maternal contribution of *naked* to the embryonic phenotype, which would result in residual naked activity in early embryos. In either case, if some naked activity were still present in the *naked-wg* mutant embryos, *en* expression would be *wg* dependent, similar to the situation observed in *zw3* rescued–*wg* mutant embryos.

zw3 Is a Homolog of Mammalian *GSK-3*

zw3 is a Drosophila homolog of the mammalian kinase *GSK-3*. The overall identity between the zw3-A and GSK-3β is 76%, with as much as 85% in the kinase domain. It has been demonstrated that in vitro GSK-3 can specifically phosphorylate and repress the activity of the proto-oncogenes *c-jun*, *myb*, and L-*myc* (Boyle et al., 1991; Woodgett, 1991; Saksela et al., 1992). The role of GSK-3 in the regulation of *c-jun* and AP-1 activity has come into question owing to recent studies indicating that casein kinase II is the primary kinase that phosphorylates and inactivates c-Jun DNA binding activity in vivo (Lin et al., 1992).

We have demonstrated that GSK-3β and zw3-A are func-

tionally homologous in vivo; expression of GSK-3β in *zw3* mutant embryos is sufficient to restore these embryos to a wild-type phenotype (Figure 6C). It is unlikely that this is due to a nonspecific effect of overexpression of a kinase, since the expression of *hs-GSK-3β* in wild-type embryos has no phenotype (data not shown). This result suggests that zw3 and GSK-3β have similar kinase activity and that they share substrate specificity. This is consistent with the observation that in vitro zw3 and GSK-3β can phosphorylate a common substrate, *c-jun* (de Groot et al., 1992). Given the homologous structure and activity of zw3 and GSK-3β, it is possible that *GSK-3* may function in pathways of vertebrate development that are mediated by *Wnt* signaling in a similar fashion to the proposed role of *zw3* in *wg* signaling.

Conclusions

The establishment of cell fates within the embryonic segments is a complex process requiring cell signaling to determine cell identity. Wg protein is one of the crucial signaling molecules in this signal transduction pathway. We propose that *wg* signaling is mediated through *zw3* and that *wg* signaling functions to dismantle *zw3* repression of *en* autoregulation to stabilize *en* expression and establish the correct cell fates within each embryonic segment (Figure 7). This is a novel mechanism for the signal transduction pathway of *wg* signaling and may have significant relevance for the study of signal transduction pathways in vertebrates. Like the Drosophila homolog *wg*, vertebrate *Wnt*s have also been implicated in the establishment of pattern in embryogenesis (Nusse and Varmus, 1992; McMahon, 1992). The *wg*-dependent maintenance of *en* expression may be analogous to the interaction of *Wnt-1* and *En-1/En-2* in specifying cell identities in the mouse embryonic brain (McMahon et al., 1992). Finally, the *wg* signaling mechanism we have proposed is likely to be conserved in vertebrates, since the mammalian homolog of *zw3*, *GSK-3*, can function in place of *zw3*.

How *zw3* represses *en* autoactivation is unknown. However, given the homology between *zw3* and *GSK-3*, it is tempting to speculate that *zw3* phosphorylates and inhibits the DNA binding activity of a transcription factor, possibly *en* itself. *en* is known to be phosphorylated by a serine/threonine protein kinase (Gay et al., 1988), and this phosphorylation may be important for autoregulation. It is possible that *wg* signaling may antagonize *zw3* repression and stabilize *en* expression by triggering a signal transduction cascade that results in the dephosphorylation of en protein and activation of *en* autoregulation. Further genetic and biochemical studies of *zw3* will be required to elucidate the mechanism of signal transduction in intrasegmental patterning.

Experimental Procedures

zw3 Mutations

The data presented in this paper were generated using *zw3*$^{M11\text{-}1}$. Similar results were obtained with two additional *zw3* mutations, *zw3*^{k22} (Perrimon and Smouse, 1989) and *zw3*^{sggD127} (Simpson and Carteret, 1989). We have examined embryos derived from homozygous mutant germlines for all three alleles and observe a similar segment polarity phenotype. It has been reported that maternal *zw3* is also required for proper differentiation of the embryonic neuroectoderm. In the absence of maternal *zw3*, most of the embryonic cells assume a neural fate (Bourouis et al., 1989). Using several antibodies that recognize the central and peripheral nervous systems (anti-horseradish peroxidase [anti-HRP], BP102, and 22C10), we were unable to detect the neurogenic phenotype previously described.

In addition, Bourouis et al. (1989) reported that embryos lacking both maternal and zygotic *zw3* product arrest in preblastoderm stages and fail to cellularize correctly. We have found that this phenotype is most severe for *zw3*^{sggD127}. Females with germlines that are homozygous mutant for this allele lay a large number of unfertilized eggs, approximately 70%. Among the unfertilized eggs, 30% appear to be arrested in preblastoderm stages with abnormal nuclear division patterns. This is similar to the maternal effect of *fused*, which gives rise to defects in oogenesis (Preat et al., 1990). Among the fertilized eggs we have detected an equal number of *zw3* rescued and *zw3* null mutant embryos, using the *FM7, ftz-lacZ* chromosome to mark the genotype of the mutant embryos (see below). This indicates that the cellularization defect is due to the absence of maternal zw3 product only, and not to maternal and zygotic interactions.

Drosophila Strains Used

The duplication of *zw3* we used is *Dp(1;Y)w*$^{+303}$ (Perrimon and Smouse, 1989).

FM7, ftz-lacZ was used to distinguish the genotype of mutant embryos (Kania et al., 1990). This *FM7* chromosome carries an insertion of the *fushi tarazu* promoter fused to the E. coli β-galactosidase gene. Staining in the epidermis can be detected as early as stage 6 and persists through stage 11.

P(en/lac) is homozygous for an insertion of part of the *en* promoter fused to the E. coli β-galactosidase gene on the third chromosome (*31R-1G*) (DiNardo et al., 1988).

hs-en is a homozygous insertion on the third chromosome of the *en* cDNA fused to the Drosophila *hsp70* promoter (Heemskerk et al., 1991).

CyO,wg^{en11} is a *CyO* balancer that includes an allele of *wg* that does not produce wg protein in the embryo (Kassis et al., 1992; A. Martinez-Arias, personal communication; E. S. and N. P., unpublished data). This line expresses β-galactosidase in the same pattern as *wg* and this pattern can still be detected in mutant embryos.

Germline Mosaic Analysis

Mosaic germlines were generated by the FLP–DFS technique (Chou and Perrimon, 1992). Females with germline clones will be referred to as GLC females. In brief, FLP recombinase catalyzes site-specific recombination through recombination target sites (FRT) in the genome (Golic and Lindquist, 1989; Golic, 1991). Germline mosaics were induced in females homozygous for an FRT insertion on the X chromosome, *FRT*^{101}, and heterozygous for the dominant female sterile mutation, *ovo*^{D1}, as well as the mutation(s) to be studied. The source of recombinase is a second chromosome insertion of a fusion of the *hsp70* promoter to the FLP coding sequence (*FLP*^{38}).

Females of the genotype *zw3*$^{M11\text{-}1}$ *FRT*^{101}*/FM7* and *zw3*$^{M11\text{-}1}$ *FRT*^{101}*/FM7; CyO,wg*^{en11}/+ were mated to *ovo*^{D1} *FRT*^{101}*/ Y; FLP*^{38}*/ FLP*^{38} males. The progeny of these crosses were heat shocked at 37°C for 2 hr during the third larval instar stage to induce FLP-directed recombination. Under these conditions, 80%–90% of the *zw3*$^{M11\text{-}1}$ *FRT*^{101}*/ovo*^{D1}*FRT*^{101} females recovered have mosaic germlines. This technique allows the production of a large number of mosaic females and consequently we were able to examine a large number of mutant embryos. The exceptions are the *zw3*$^{M11\text{-}1}$ *svb*^{YP17b}*; CyO,wg*^{en11}/+ GLC females that were generated by X-ray-induced dominant female sterile technique (Perrimon et al., 1984).

Marking Embryos to Determine the Zygotic Genotype

The embryos derived from *zw3* mutant germlines fall into two genotypic classes: those that have received a wild-type copy of the gene from the father, referred to as rescued embryos, and those that lack both maternal and zygotic wild-type product, referred to as null embryos. The *FM7, ftz-lac Z* chromosome was used to distinguish between *zw3* rescued and *zw3* null mutant embryos. *zw3* GLC females were mated to *FM7, ftz-lac Z/Y* males; the embryos derived from this cross were

stained for X-gal to detect the *FM7* chromosome. The distribution of en and wg protein in these embryos was determined by immunostaining. Whereas the en pattern is indistinguishable in *zw3* rescued and *zw3* null mutant embryos, the wg pattern is reproducibly different between the two classes of embryos. This difference is so consistent and easy to distinguish that the pattern of *wg* expression was later used to determine the genotype of the mutant embryos.

P(en/lac) Expression

This line gives an early pattern of 7 stripes of expression in the even-numbered endogenous en stripes, regulated by the pair rule genes. Later a pattern of 11 dorso-lateral stripes in the labial, 3 thoracic, and first 7 abdominal segments appears, corresponding to the endogenous en stripes, which are regulated by the segment polarity genes (DiNardo et al., 1988). *zw3; P(en/lac)* embryos were derived from *zw3* GLC females mated to *+/Y; P(en/lac)/P(en/lac)* males. *P(en/lac)* expression was detected in embryos at 0–12 hr AEL by β-galactosidase immunostaining.

hs-en Activation of Endogenous *en*

zw3;hs-en embryos were derived from *zw3* GLC females mated to *+/Y; hs-en/hs-en* males. Embryos were collected between 3–5 hr AEL and heat shocked for 30 min on agar plates floating in a 37°C water bath. Embryos were returned to 25°C for 2 hr and then fixed for en protein immunostaining.

zw3-wg Double Mutant Embryos

To examine the pattern of *en* expression in *zw3-wg* mutant background, embryos were derived from *zw3; CyO,wg*^{en11}*/+* GLC females mated to *+/Y;CyO,wg*^{en11}*/+* males. One-quarter of the embryos from this cross are mutant for *wg* and can be detected by the absence of wg protein. Embryos were doubly stained to detect en and wg proteins simultaneously (see below).

To examine the cuticle phenotype of *zw3 rescued-wg* mutant embryos, *zw3 svb; CyO,wg*^{en11}*/+* GLC females were mated to *+/Y; CyO, wg*^{en11}*/+* males and cuticle preparations were performed on the mutant embryos. *svb* is a recessive mutation that causes denticles to be reduced in number and size (Gergen and Wieschaus, 1986). The allele we used in this study is *svb*^{YP17b}.

Cuticle Preparation

Embryos were dechorionated in 50% Clorox for 3 min and the vitelline membrane removed by a methanol–heptane mixture. The embryos were mounted on Hoyer's medium mixed 1:1 with lactic acid (van der Meer, 1977). Cuticles were viewed and photographed under dark field.

Immunohistochemistry

X-gal staining and antibody staining were performed essentially as described by Klämbt et al. (1991), with the exception of the incubation in 5% fetal calf serum. Embryos were dechorionated in 50% Clorox for 3 min and fixed for 10 to 15 min in 4% formaldehyde in phosphate-buffered saline. Mouse monoclonal antibody to β-galactosidase (Promega) was used at a dilution of 1/1000. Mouse monoclonal antibody directed against en was used at a 1:1 dilution (Patel et al., 1989). Rabbit anti-wg polyclonal serum was used at a dilution of 1:500, after preabsorbing as described previously (van den Heuvel et al., 1989). Monoclonal antibody BP102 recognizes axonal projections of the central nervous system and 22C10 recognizes the peripheral nervous system (Goodman et al., 1984; Klämbt et al., 1991). These antibodies were used at a 1/10 dilution. Anti-HRP recognizes the nervous system of insects (Jan and Jan, 1982). Rabbit anti-HRP was used at a dilution of 1/1000 or 1/10,000. Biotinylated horse anti-mouse antibody (Vector) was used at a dilution of 1/500 and biotinylated goat anti-rabbit antibody (Vector) was used at 1/500, followed by Vectastain Elite (Vector). HRP-conjugated goat anti-mouse (Boehringer-Mannheim) was used at a dilution of 1/300. HRP was detected with 500 μg/ml diaminobenzidine and 0.05% H_2O_2. Embryos were dehydrated in ethanol, mounted in methyl salicylate, and photographed under Nomarski optics.

For the detection of wg and en proteins simultaneously, primary antibodies were incubated together as described above. This was followed by incubation with biotinylated goat anti-rabbit secondary and HRP was detected in the presence of 8% NiCl. The embryos were subsequently reacted with an HRP-conjugated goat anti-mouse secondary, and HRP was detected without NiCl. Wg protein is detected as a blue-purple signal and en protein as a brown signal.

cDNA Analysis

To isolate additional cDNAs, 10^6 recombinant plasmids from a library made to 0–4 hr embryonic mRNA (Brown and Kafatos, 1988) were screened with a ^{32}P-labeled insert of cDNA *zw3-A*. *zw3-C* was identified as a full-length cDNA out of six independently isolated plasmids. Probe preparation by random priming and all hybridizations were performed by standard protocols (Sambrook et al., 1989). The insert of *zw3-C* was isolated by converting the flanking HindIII site in the vector to a NotI site, releasing the insert by a NotI digestion, gel purifying the insert, and cloning it into the NotI site of pBluescript for sequencing (Stratagene). All restriction digests and ligations were performed according to manufacturer's protocols (Boehringer-Mannheim), and the insert was isolated from an agarose gel with Gene-Clean according to the manufacturer's protocol (Bio-101). DNA sequencing of nested deletions (Stratagene) of one strand was performed by dideoxy chain termination method using T7 polymerase and [^{35}S]CTP (Pharmacia). The unique sequence was determined on the second strand using oligonucleotide primers to known sequence. Translations, alignments, and homologies were generated using the program BESTFIT of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984). The sequence predicts a protein with 11 unique amino acids: MLINRGSLLES.

The sequence of *zw3-A* and *zw3-B* originally published as *cOV7* and *cKZ5* (Siegfried et al., 1990) have been corrected. At position 2267 of *zw3-B* (*cKZ5*) an additional G has been included in the sequence, causing a frame shift and a change in the predicted protein. This change occurs in the carboxyl terminus and affects the last 28 residues outside the region encoding a serine/threonine kinase. This error was detected following comparison of our sequence with that published by M. Bourouis and colleagues and has been confirmed by additional sequencing. Other discrepancies between the two published sequences cannot be accounted for by sequencing errors and may reflect polymorphisms in the sequence; these include positions 1806, 2292, and 2460 of *cKZ5*.

Rescue of *zw3* Mutant Phenotypes Using *hs-zw3-A*

Since the ovarian cDNA that encodes *zw3-A* was not a full-length cDNA, a fusion between *cOV7* and *cKZ5* was made to recreate the complete *zw3-A* coding sequence (Siegfried et al., 1990; Figure 5). An EcoRI–HindIII fragment containing the 5′ end of *cOV7* was fused to the HindIII–NotI 3′ end of *cKZ5* and cloned into the EcoRI–NotI sites of *CaSper-hs* (Thummel et al., 1988; C. Thummel, personal communication). This allows the expression of *zw3-A* from the *hsp70* promoter. *hs-zw3-A* was introduced into the genome via P element–mediated transformation following standard techniques (Spradling, 1986). Transformations were performed into *y w/y w; Δ2-3,Sb/TM6* (Robertson et al., 1988).

w zw3^{M11-1}*/FM7* females were mated to *w/Y; hs-zw3-A/hs-zw3-A* males and the progeny were heat shocked at 37°C for 2 hr every day for 5–6 consecutive days, starting at the second larval instar stage. The larvae were heat shocked in glass vials immersed in a circulating water bath. The heat-shocked progeny were examined for the presence of *zw3/Y; hs-zw3-A/+* males, identified as w^+,B^+ males. Using a *hs-zw3-A* insertion on the second chromosome, we can rescue almost all of the *zw3* mutant individuals. In a single experiment performed under the conditions described, we recovered 35 females (*w zw3*$^{M11-1}$*/+ ; hs-zw3-A/+* and *FM7/+; hs-zw3-A/+*), 7 *FM7/Y; hs-zw3-A/+* males and 11 *w zw3*$^{M11-1}$*/ Y; hs-zw3-A/+* males.

The same transgenic strain was used to rescue the *zw3* maternal-effect phenotype. *zw3* GLC females were mated to *w/Y; hs-zw3-A/hs-zw3-A* males and the embryos (0–4 hr AEL) were heat shocked for 1 hr on agar plates floating in a 37°C water bath. The plates were then returned to 25°C to allow the embryos to develop, and cuticle preparations were performed. We examined 78 *zw3 ;hs-zw3-A/+* mutant embryos of which 35 were rescued to a wild-type phenotype. Six of the thirty-five rescued embryos hatched.

Rescue of *zw3* Maternal-Effect Phenotype Using *hs-GSK-3β*

hs-GSK-3β was constructed by the insertion of a Xba–EcoRI fragment from the *GSK-3β* cDNA into the Stu–Xba sites of *CaSper-hs* (Woodgett,

1990). Transgenic lines were established as described above. *zw3* GLC females were mated to *w/Y; hs-GSK-3β/hs-GSK-3β* males, and the embryos were heat shocked as described above. Using an insertion of *hs-GSK-3β* on the second chromosome, we examined 72 *zw3;hs-GSK-3b/+* mutant embryos, of which 20 were rescued to a wild-type phenotype.

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