THE DROSOPHILA GENE *NEMO* REGULATES MULTIPLE DEVELOPMENTAL PATHWAYS

By

Andrea C. Uetrecht

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Name: **Andrea Uetrecht**

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Examining Committee:

Chair: **Fiona Brinkman**
Assistant Professor, Department of MBB

Esther Verheyen
Senior Supervisor
Assistant Professor, Department of MBB

Nick Harden
Supervisor
Assistant Professor, Department of MBB

Nancy Hawkins
Supervisor
Assistant Professor, Department of MBB

Lynne Quarmby
Internal Examiner
Associate Professor, Department of Biology

Date Defended/Approved: July 9, 2004

SIMON FRASER UNIVERSITY

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Abstract

Drosophila *nemo* (*nmo*) encodes a serine/threonine kinase similar to the MAP kinases. It was first identified as an epithelial planar polarity gene involved in ommatidial rotation in the developing eye. It has since been shown to be a negative regulator of Wingless signaling and is essential for proper development. Nemo-like kinases (NLKs) have been identified in *C. elegans,* Xenopus, mice and humans, and have been shown biochemically to bind and/or phosphorylate Armadillo (Arm) homologues and members of the T-Cell factor/Lymphocyte enhancing factor (TCF/LEF) family of transcriptional regulators. xNLK has also been shown to bind the SOX11 transcriptional regulator.

In Drosophila, loss of *nmo* results in reduced adult viability. Those that survive exhibit eye defects, mild wing patterning defects and are sterile. Loss of both maternal and paternal *nmo* contribution through the induction of mutant clones in the germ-line results in ~80% embryonic lethality characterized by loss or fusion of denticle belts. I have characterized this embryonic phenotype and find that defects are consistent with a role for *nmo* in pair-rule gene patterning. This is based on the observations that denticle belt fusions occur in a pair-rule-like pattern, and stripes of Wingless (Wg) and Engrailed (En) protein expression are disrupted in alternating stripes. Additionally, I found expression of the *nmo[P]* reporter construct to be enriched in those regions of the embryo in which patterning defects usually manifest when *nmo* is lost. Embryos derived from *nmo*$^{-}$ germ-lines were also examined for CNS and PNS defects, as the *nmo* reporter construct is also highly expressed in these tissues. Finally, I have examined a role for *nmo* in modulating expression of several Wingless target gene constructs in the embryo and wing disc.

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Introduction

Development of a multicellular organism occurs in a highly orchestrated series of events. Cell identities must be established in the correct time and place, and morphogenic movements must occur in a precise and tightly regulated manner. Paramount to these events is the ability of cells to sense their environment and communicate with one another. Many of the ways in which cells respond to cues or signals from their environment are still poorly understood, particularly because the presence of multiple signals confounds what otherwise might be a relatively simple or linear series of events. However, the importance of correct signaling events is obvious; failure can lead to gross abnormalities in the adult or, more commonly, lethality earlier in development. Deregulation of signaling events in the adult may also lead to cancers or other diseases.

One of the most heavily utilized and highly conserved signaling cascades is the Wingless (Wg or Wnt) pathway. Wnt signaling occurs in such diverse species as nematodes, urchins, frogs, flies, fish, and mammals, and is required for the development of multiple tissues in these organisms, from limbs, to the nervous system, skin, gut, and respiratory and circulatory systems. Deregulation of Wnt signaling has been implicated in various cancers.

The pathway was named for the founding members Wingless (Wg) in flies and Integrated (Int) in mice. Wingless and other related Wnt molecules act in a multitude of developmental contexts as morphogens, organizers, and pro-survival signals. To date numerous *wnt* genes have been identified; all encode secreted glycoproteins that associate

with the cell surface and extracellular matrix (ECM) (Smolich et al., 1993) and are the signaling molecules responsible for the activation of Wnt pathways. Wnt proteins can activate a number of different signaling pathways, the best characterized of which is the canonical Wnt pathway (outlined in Figure 1). There appear to be at least two additional non-canonical pathways (outlined in Figure 2): the planar cell polarity (PCP) pathway, and calcium-dependent Wnt pathway. How these pathways relate to one another is presently unclear, and there is some question as to how distinct each really is (for example, see Veeman et al., 2003). For simplicity, and because much of the work to delineate the pathway was done in *Drosophila*, Wnt signaling will be discussed with reference to the fly homologues where possible. A list of some of the pathway members and their vertebrate and worm homologues is presented in Table 1.

The canonical Wnt pathway has been extensively reviewed (see for example Lustig and Behrens, 2003, Wodarz and Nusse, 1998). A brief outline is given below and diagramed in Figure 1. Secretion of Wg by the signaling cell depends on correct N-linked glycosylation mediated by the *porcupine* (*porc*) gene product (Tanaka et al., 2002). Secreted Wg then binds to the receptor complex on the responsive cell, comprised of a member of the multi-pass trans-membrane Frizzled (Fz) protein family, and the single-pass trans-membrane protein Arrow (Arr) (reviewed in He et al., 2004). In the absence of Wg signaling, cytosolic levels of the Armadillo (Arm) protein are kept low. This is mediated by the destruction complex, comprised of Axin, Adenomatous Polyposis Coli (APC), and two kinases, Casein Kinase I (CKI) and Shaggy (Sgg). Arm is phosphorylated at multiple sites by Sgg following a 'priming' phosphorylation by CKI (Amit et al., 2002),

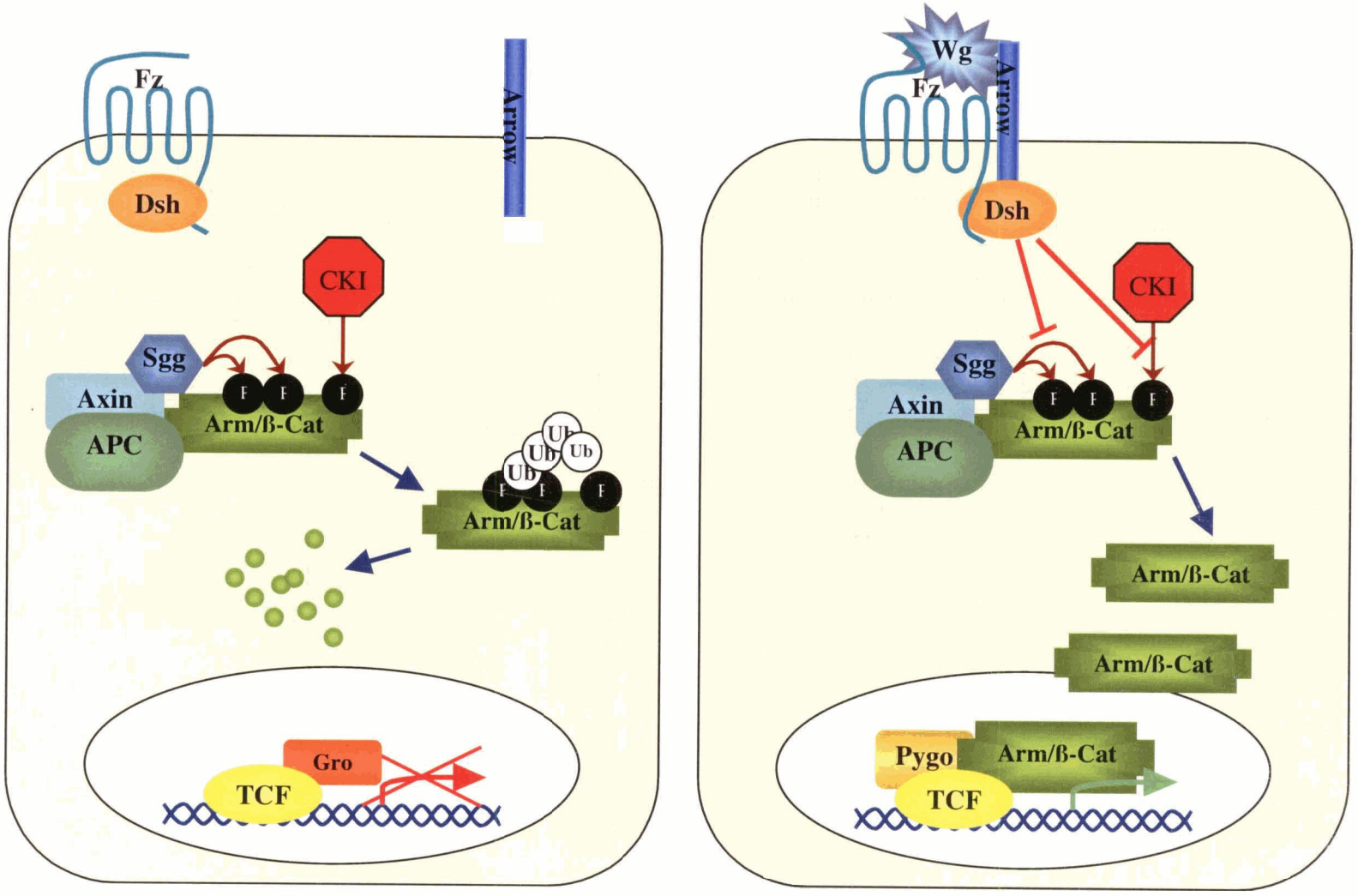


Figure 1: Model for the canonical wingless pathway in *Drosophila*. Activites of the various components are depicted in the absence (left) and presence (right) of the Wg ligand. See text for a detailed description.

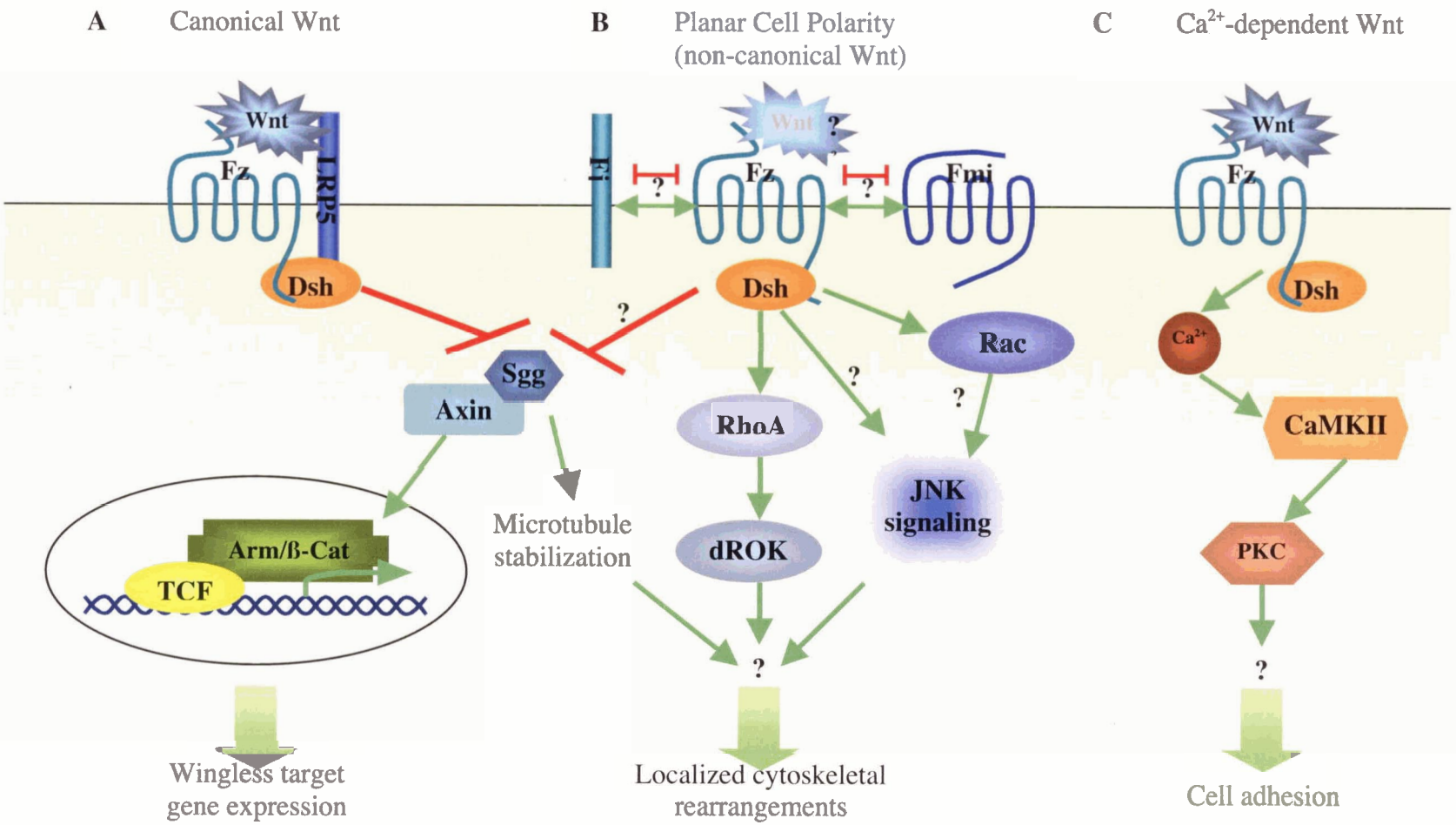


Figure 2: Three distinct signaling cascades are triggered by Wnt: canonical Wnt (A), Planar Cell Polarity (PCP) or non-canonical Wnt (B), and the calcium/Protein Kinase C (PKC) pathway (C). See text for details.

Table 1:

Canonical Wg pathway members and their vertebrate homologues.

	Drosophila	Vertebrate	Pathway
Canonical Wnt pathway members	Wingless (Wg)	Wnt-1	Canonical
	Frizzled (Fz)	Fzd1	Canonical
	Arrow (Arr)	LRP5/6	Canonical
	Dishevelled (Dsh)	Dvl	Canonical
	Shaggy (Sgg)	GSK3	Canonical
	Armadillo (Arm)	ß-catenin	Canonical
	Pangolin (Pan) Or dTCF	TCF Or LEF	Canonical
Non-canonical Wnts	DWnt5	Wnt5A Wnt5B	Ca^{2+}
	No orthologue	Wnt8A Wnt8B	PCP?
	No orthologue	Wnt11	Convergent Extension

which leads to the subsequent ubiquitylation and degradation of Arm in the proteasome. Under these conditions, transcription of Wg target genes is repressed by Pangolin (Pan), a member of the T-cell factor (TCF) family of transcriptional regulators, acting in concert with co-repressors such as Groucho (Gro) or CREB-binding protein (CBP). The interaction between Wg and the receptor complex leads to the activation of the cytoplasmic Dishevelled (Dsh) protein. Activation of Dsh leads, via an as yet undefined, but hotly debated mechanism, to the inhibition of Arm phosphorylation. This probably involves either the inhibition of CKI and/or Sgg, or the degradation of Axin (reviewed in Tolwinski and Wieschaus, 2004), but how this is accomplished is unclear. Unphosphorylated Arm is not degraded, but becomes stabilized in the cytoplasm. Stabilized Arm can complex with Pan/TCF, and other co-factors such as Pygopus (Pygo) and Legless (Lgs) (Thompson, 2004). This complex can enter the nucleus, displace co-repressor proteins, and effect transcription of Wg target genes.

Due to the importance of Wnt signaling, and the implications of deregulation for development, multiple checkpoints must be in place to ensure that Wg target expression is only active at the appropriate time and place. Regulation of the pathway can also integrate inputs from other pathways to ensure coordinated signaling. Because Arm stability and the binding of the Arm/TCF complex to the target gene promoter are the most crucial events in canonical Wnt signaling, the greatest potential for regulation also occurs at these steps. One can imagine that signaling events of another pathway could play a role in regulating Wg signaling in either a global manner, such as influencing Arm stability, or in a target-specific manner through influencing the binding of the Arm/TCF complex at a specific promoter. ICAT, for example, can act as a global inhibitor of Wnt

signaling by binding ß-catenin and preventing its interaction with TCF (Tago et al., 2000). Likewise, the transcriptional co-repressor Gro binds TCF (or other Gro-dependent repressors) to form a repressor complex at the enhancer region (reviewed in Courey and Jia, 2001, Brantjes et al., 2002). The histone actyltransferase CBP normally acts as a general transcriptional activator by remodeling chromatin, rendering it more accessible to transcription factors. However, in *Drosophila* CBP can also acetylate TCF, thereby reducing its affinity for Armadillo and antagonizing Wg signaling (Waltzer and Bienz, 1998). C-terminal binding protein (CtBP) also binds TCF to mediate transcriptional repression, possibly by recruiting histone deacetylases (Courey and Jia, 2001). The transcriptional repressor Brinker acts in an enhancer-specific manner to inhibit Wg target gene expression only under high levels of signaling through recruiting the co-repressors CtBP and Teashirt (Tsh) to the enhancer region at sites distinct from the TCF binding sites (Saller et al., 2002).

The Planar Cell Polarity (PCP) or non-canonical Wnt pathway (reviewed in Mlodzik, 2002; see Figure 2) requires the Fz receptor and Dsh, but not Arrow. The analogous pathway in vertebrates, convergent extension (CE), is triggered by a Wnt molecule, Wnt11, but no such Wnt has been identified in flies (if one indeed exists). What is crucial for PCP is the asymmetric localization of Fz and Dsh molecules at the plasma membrane, and subsequent polarization of the cell. While the upstream components remain elusive, and the exact mechanism(s) used to establish polarity are still unclear, some important components of the pathway have been identified. For example, graded expression of *four-jointed* in the pupal wing may be important in establishing localized differences in activity of Fat (Ft) and Dachsous (Ds) in

neighbouring cells (Ma et al., 1998, Strutt et al., 2004). Strabismus (Sb) appears to be required to recruit the cytoplasmic protein Prickle (Pk) to the membrane and exclude the Fz/Dsh complex from one side of the polarized cell (Jenny et al., 2003). The serpentine receptor Flamingo (Fmi) is also important in PCP, though its role in the process is unclear. Cytoskeletal rearrangements in response to PCP signaling are mediated by RhoA GTPases and dROK, and have also been shown to involve transcriptional regulation mediated by the c-Jun N-terminal Kinase (JNK) signaling pathway (Strutt et al., 1997).

Cells can mediate localized microtubule stability in response to Wnt signaling through the inhibition of GSK3 by Dsh and Axin (Ciani et al., 2003). Ectopic expression of either Dsh or Axin in neuronal cells can lead simultaneously to both stabilization of cytoplasmic ß-Catenin and localized stabilization of microtubules. Axin can bind and stabilize microtubules, but requires Dsh and GSK3 to do so. This activity was found to be independent of ß-Catenin, TCF or any other transcriptional activity. The authors propose a model where canonical Wnt signaling bifurcates at GSK3/Axin/Dsh, leading to both ß-Catenin-mediated transcriptional regulation, and the inhibition of GSK3, which in turn prevents the phosphorylation and inactivation of microtubule stabilizing proteins such as MAB-1B and Tau. What is not clear is whether this is a true bifurcation of the canonical Wnt pathway, or simultaneous activation of both canonical and PCP signaling. It is clear that this activity depends on several components of the canonical Wnt pathway, but is independent of ß-Catenin, and a requirement for the canonical LRP5/6 co-receptor was not demonstrated. Regardless, a mechanism by which Wnt signaling can induce localized changes in cytoskeletal stability has now been demonstrated.

A third Wnt-mediated signaling pathway results in an intracellular increase in Ca^{2+} ions the subsequent activation of Calmodulin-dependent Kinase II (CaMKII) and Protein Kinase C (PKC). In vertebrates, this pathway is activated by specific Wnt/Fz combinations (specifically Wnt-5a and rat Fz-2) and has recently been shown to require Dsh (Sheldahl et al., 2003). Activation of this pathway is thought to mediate the adhesive properties of cells, though the mechanism for this is unclear.

A discussion of Wnt signaling would not be complete without the discussion of a certain kinase known as Nemo. Drosophila *nemo* (*nmo*) encodes the founding member of the Nemo-like kinase (NLK) family of proline-directed serine/threonine kinases. Homologues have been identified in *C. elegans* (*LIT-1*), Xenopus (*xNlk*), mice (*mNlk*) and humans (*Dres-16*). The catalytic domains of Nmo and mNlk are most closely related to a family of mitogen activated protein kinases (MAPKs) that include murine ERK1 and yeast CDC28 (Choi and Benzer, 1994, Brott et al., 1998). Nemo and NLKs have been implicated in all three Wnt pathways (see below and Figure 3)

The first evidence that NLKs might be involved in Wnt signaling came from genetic studies in *C. elegans*, where Wnt/ß-catenin signaling occurs in a rather unorthodox manner (See Korswagen, 2002). A ß-catenin homologue, WRM-1, mediates Wnt signaling by exporting a TCF homologue, POP-1, from the nucleus, rather than acting as a transcriptional co-activator. Thus POP-1 acts as a dedicated repressor in the absence of Wnt, and is exported from the nucleus in response to Wnt to allow activation of target gene expression. Wnt signaling is required early in embryogenesis to mediate asymmetric cell divisions along the anterior/posterior (A/P) axis. Anterior daughter cells inherit higher levels of nuclear POP-1. Loss of POP-1 results in posteriorization of the

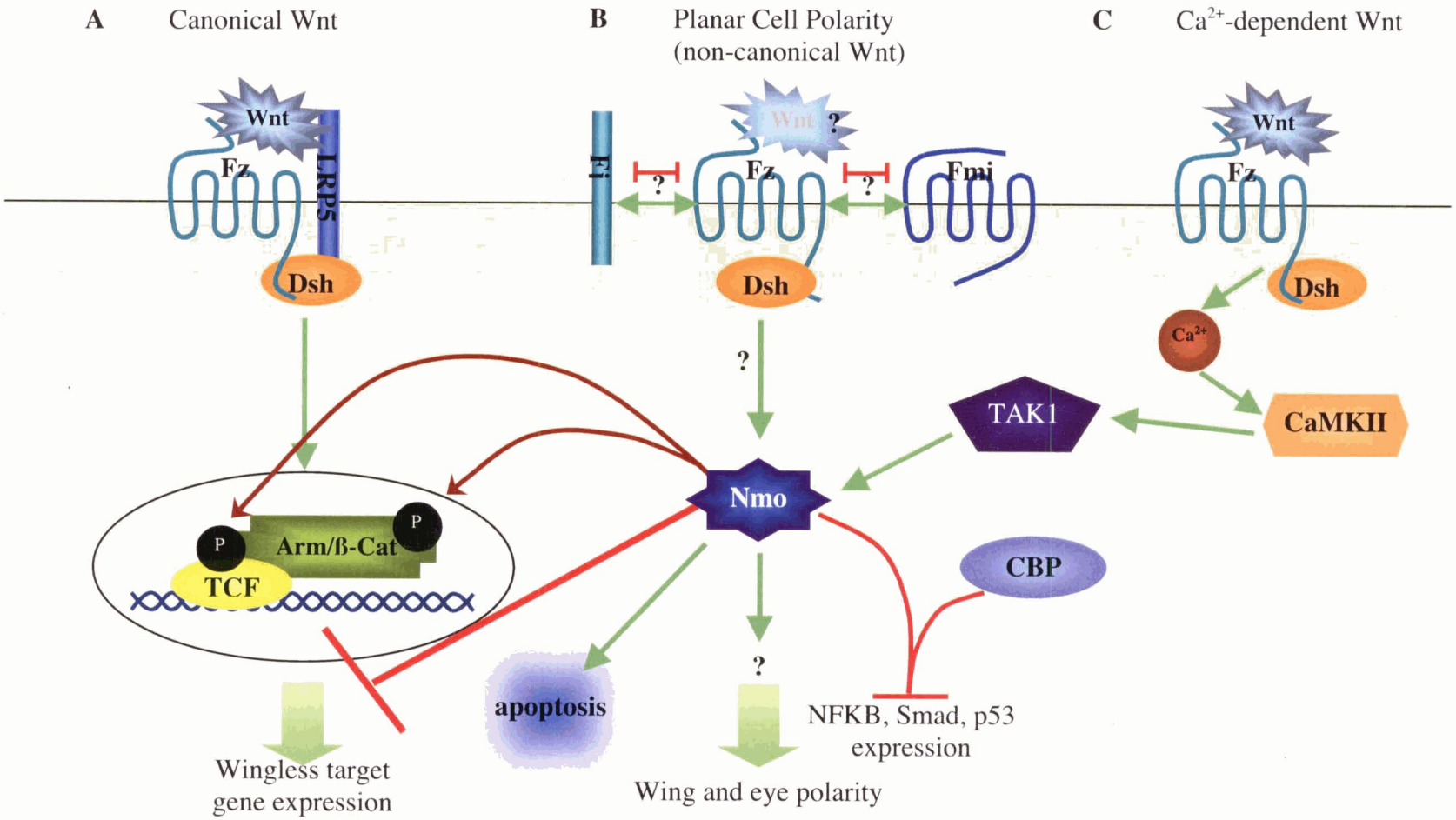


Figure 3: NLKs are involved in all three Wnt pathways, as well as having other roles in apoptosis, and the inhibition of gene expression. See text for details.

embryo, while loss of WRM-1, results in an anteriorization of the embryo. The *nmo* homologue, *lit-1*, and a homologue of TGF-ß Activated Kinase-1 (TAK1), *mom-4*, are involved in this process. Loss of *lit-1* or *mom-4* results in the symmetric localization of POP-1 in daughter cells, and they have been shown to be required for the down-regulation of POP-1 in posterior daughter cells (Meneghini et al., 1999). Indeed, LIT-1 binds WRM-1 directly, and the complex is responsible for phosphorylation of POP-1 (Rocheleau et al., 1999). This interaction is thought to lead to the nuclear export of POP-1 and the subsequent transcription of Wnt targets. It is thought that MOM-4/TAK1 is responsible for the activation of LIT-1/NLK. Thus, in worms, the MOM-4/LIT-1 MAPK pathway appears to cooperate with WRM-1/ß-catenin to transduce Wnt signaling.

In contrast, vertebrate NLKs have been shown to inhibit Wnt signaling in vertebrate systems, although through a similar mechanism. In vertebrate systems, transfection of either TAK1 or NLK inhibits TCF/ß-catenin-dependent reporter expression in a kinase-dependent manner (Ishitani et al., 1999). NLK binds ß-catenin only indirectly via LEF-1 or TCF-4, and this interaction enhances NLK-dependent phosphorylation of TCF (Ishitani et al., 2003). Additionally, NLK-dependent phosphorylation of LEF-1 is thought to inhibit the transcriptional activity of the Wnt activated ß-catenin/LEF-1 complex through preventing the complex from binding DNA (Ishitani et al., 2003). However, NLK seems to have little effect on the DNA binding ability of TCF-4 in the absence of ß-catenin (Ishitani et al., 1999). Thus NLKs appear to either activate or inhibit Wnt-dependent gene expression (depending on the context) through interfering with the DNA-binding ability of TCF homologues or TCF/ß-catenin complexes. These studies also establish that TAK1 can activate vertebrate NLK.

In vitro evidence that NLKs inhibit ß-catenin-mediated Wnt signaling is supported by *in vivo* evidence. One of the classic *in vivo* assays for identifying components of canonical Wnt signaling is the axis-formation assay in Xenopus embryos. Wnt is required to signal from the ventral to the dorsal side of the embryo at the four-cell stage to mediate formation of the embryonic axis. Injection of mRNAs for inhibitory intracellular components of Wnt signaling into the dorsal side of the embryo results in the failure of axis formation. Conversely, injection of mRNAs for intracellular components that activate Wnt signaling (for example, ß-catenin) into the ventral side of the embryo results in the duplication of the embryonic axis. Injection of *NLK* mRNA into the dorsal side of Xenopus embryos inhibits endogenous axis formation, while co-injection of NLK and ß-catenin mRNA into the ventral side of the embryo inhibits secondary axis formation caused by ß-catenin alone (Ishitani et al., 1999). This indicates that NLK negatively regulates canonical Wnt signaling downstream of ß-catenin.

The non-canonical Wnt/Ca^{2+} pathway is thought to inhibit canonical Wnt signaling, and this appears to be mediated by the TAK1/NLK pathway (Ishitani et al., 2003). TAK1 activity is increased on binding activated CaMKII, and leads to increased NLK-dependent phosphorylation of LEF-1. Additionally, endogenous TAK1 and NLK activity is stimulated by Ca^{2+} influx, and this stimulation depends on CaMKII. This is thought to be significant *in vivo* as well, as co-injection of either Wnt-5a (the Wnt/Ca^{2+} ligand) or a constitutively active CaMKII with ß-catenin blocks secondary axis formation caused by injection of ß-catenin alone. Thus, as previously demonstrated for NLK alone, Wnt/Ca2+ signaling interferes with canonical Wnt signaling through the TAK1/NLK pathway downstream of ß-catenin.

While it is clear that NLKs can mediate transcriptional regulation via interference with the DNA binding activity of TCF homologues, NLKs have also been shown to interact with high mobility group (HMG)-domain-containing proteins other than TCF/LEF. HMG-domain proteins are DNA binding proteins that are thought to mediate the formation of large transcriptional regulatory complexes (see Yamada et al., 2003). In Xenopus embryos, xNLK physically interacts with xHMG2L1, which can also negatively regulate canonical Wnt signaling, both *in vitro* and *in vivo* (Yamada et al., 2003). xNLK also interacts with xSOX11, a member of the SOX family of HMG-domain containing proteins (Hyodo-Miura et al., 2002). Interestingly, TCF, HMG2L1 and SOX proteins are all members of the same HMGB subfamily which bind specific DNA sequences, rather than binding DNA non-specifically as other subfamilies do (Yamada et al., 2003).

The roles for NLKs in mediating transcriptional regulation do not appear to be limited to Wnt signaling. NLK also binds CREB binding protein (CBP) and suppresses the activities of a number of transcription factors including NF-κB, Smad, and p53, which all rely on CBP (Yasuda et al., 2004). Specifically, the authors show that NLK can phosphorylate the C-terminus of CBP, but has no effect on either the histone acetyltransferase activity of CBP or the nuclear localization of NF-κB.

While physical interactions between NLK and TAK, TCF and Arm homologues, and the roles that these complexes play in signaling in cell culture are well documented, the functional significance of these interactions during development are less so. Early in *Xenopus* development, xNLK is expressed in the ectoderm, and by the tadpole stage transcripts are restricted to the central nervous system (CNS), eye, and head neural crest cells (Hyodo-Miura et al., 2002). It is thought that in this context, NLK cooperates with

the xSOX11 to induce neural development, as co-injection of xNLK and xSOX11 mRNA into animal caps can induce expression of neural markers and this activity depends on the kinase activity of NLK (Hyodo-Miura et al., 2002).

The role of Nlk in mice is even less clear (Kortenjann et al., 2001). *Nlk* knock-out (KO) mice exhibit variable reductions in viability, ranging from death during the third trimester of pregnancy to death at 4-6 weeks, that depends on the genetic background. Those *Nlk* KO mice that survive to birth exhibit severe growth retardation, cerebellar ataxia and defects in bone marrow stromal cell differentiation. Defects appear to be restricted to the stromal cells not hematopoietic cells, as *Nlk* KO bone marrow could be used to reconstitute SCID mice.

Thorpe and Moon (2004) have recently cloned a zebrafish *nlk*. Injection of *nlk* mRNA into one-cell embryos results in loss of eyes and forebrain. This phenotype is similar to injection of *wnt8* or loss of the repressors, *tcf3a* and *tcf3b,* and depends on the presence of the TCF interaction domain and kinase activity. However, while injection of *nlk* morpholino (MO) was able to enhance the ventrolateral defects caused by *wnt8* MO, injection of *nlk* MO alone had only moderate effects on ventrolateral development. Thus it appears in zebrafish that *nlk* functions in a manner analogous to LIT-1 in worms through inhibition of TCF-mediated repression of Wnt targets.

Perhaps the best characterized role for NLK in development is in Drosophila. Drosophila *nmo* was first identified as a modulator of epithelial planar polarity in eye development, where loss of *nmo* results in incomplete rotation of the photoreceptors (Choi and Benzer, 1994). Subsequent studies have identified *nmo* as a dominant modifier of eye phenotypes in a Notch gain-of-function mutant background (Verheyen et al.,

1996). More recently *nmo* has been implicated as a modulator of various signaling pathways including Dpp, Wingless, and EGFR (Verheyen et al., 2001). Homozygosity for *nmo* loss of function mutations results in reduced adult viability, and escapers exhibit a variety of wing phenotypes in addition to the polarity defects initially observed in the eye.(Choi and Benzer, 1994). Wings are held out from the body at an angle, are short and rounded, and exhibit both polarity defects in the wing hairs and ectopic vein at the posterior cross-vein (PCV) (Verheyen et al., 2001). Ectopic epidermal expression of *nmo* throughout the wing disc using the 69B-Gal4 driver results in longer, narrower wings with ectopic vein above L2 and often loss of the PCV. Recent evidence indicates that *nmo* expression is induced in response to Wingless signaling at the dorsal/ventral (D/V) boundary of the wing disc, and that it acts as a feedback inhibitor of Wingless in this context (Zeng and Verheyen, 2004).

Wild-type *nmo* transcript is maternally loaded, and loss of both maternal and paternal *nmo* results in ~80% embryonic lethality characterized by loss or fusion of denticle belts and loss of denticle diversity (Mirkovic et al., 2002). *nmo* is also required for correct levels of apoptosis in the embryo (Mirkovic et al., 2002).

Two *nmo* isoforms have been cloned, NemoI (c4-2) and NemoII (c5-1), that differ in their C-terminus (Choi and Benzer, 1994). Biochemically, Nemo has been found to bind Armadillo, ß-catenin, and dTCF, and phosphorylates dTCF and Arm (D Bessette, Y Zeng and EM Verheyen, unpublished data). Whether this phosphorylation affects the DNA binding of the dTCF/Arm complex or TCF alone is currently unknown. Thus, *nmo* appears to be acting as an inhibitor of Wg signaling in a manner similar to the role of most vertebrate NLKs.

The major findings in the following study demonstrate a role for *nmo* in embryonic development outside of the established role in Wnt signaling. Loss of maternal *nmo* results in a moderate pair-rule-like phenotype in the embryo including fusion of alternating denticle belts and loss of alternating stripes of Wg and En protein. I suggest that *nmo*, possibly in concert with the xSOX11 homologue SOX70D, mediates the activity of the pair-rule gene *even-skipped* throughout embryonic development. I also present here the results of two additional projects aimed at understanding *nmo* function during development. First I present preliminary data that initially indicated that *nemo* does not play a role in mediating cross-talk between Wingless and Notch at the D/V boundary in wing discs. Second, the results of a deficiency screen to identify dominant modifiers of a phenotype resulting from ectopic expression of *nmo* are reported here.

Materials and Methods

Fly stocks and culture

Flies were maintained on standard cornmeal/molasses/yeast/agar media. The following stocks were used: *fsh*^{87}*/TM3* and *fsh*^{96}*/TM3* (Nambu and Nambu, 1996), *UbxB-lacZ,24ß-Gal4* (Riese et al., 1997), *BE-lacZ* (Yang et al., 2000). The following stocks are described in (Mirkovic et al., 2002):

nmo^{adk1}*/TM6B*
nmo^{adk2}*/TM6B*
nmo^{P}*/TM6B*
nmo^{adk1}*, FRT79D/TM6B* or *TM3*
UAS-nmo[C5-1e] (nmoII)
UAS-nmo[C4-2](nmoI)

The following stocks, as well as deficiencies, were obtained from the Bloomington Stock Center:

en^{1}*/Cyo* (BL265)
en^{4}*/Cyo* (BL1817)
en^{7}*/Cyo* (BL1820)
sax^{4}*/SM6a* (BL5404)
mae^{k06602}*/Cyo* BL10633)
sbb^{04440}*/Cyo* (BL11376)
pk^{1}*/Cyo* (BL367)
wg^{CX4}*/Cyo* (BL2980)
Df(2r)eve/Cyo (BL1545)
ftz^{11}*/TM3* (BL1841)
slp^{05965}*/Cyo* (BL11463)
odd^{01863}*/Cyo* (BL11111)
red^{1}*,e*^{1}*,mbc*^{C1}*/TM3,ftz-lacZ* (BL1671)
69B-Gal4
wg^{Gla}*/Cyo,twi-GFP* (BL6662)
Dr^{Mio}*/TM3,twi-GFP* (BL6663)

Dll-lacZ (Zecca et al., 1996) and *vg(quadrant)-lacZ* (Kim et al., 1996) were gifts from Masahiro Go, *en-Gal4* a gift from Norbert Perrimon and *UAS-sSpi, UAS-DERDN* (Schweitzer et al., 1995) were donated by Matthew Freeman.

The female-sterile *ovo*^{D}*,FRT79D/TM3* stock was maintained by crossing males to male-sterile *Tubulin/TM3* virgins (Bloomington Stock Center).

The following stocks were generated according to standard genetic techniques:

nmo^{adk1}, fsh^{96}/TM6B
UAS-nmo[C4-2], 69B-Gal4/TM6B
ftz^{11}/TM3,twi-GFP
nmo^{adk2}, fsh^{96}/TM3nmo^{adk2}/TM6B
fsh^{87}/TM3,twi-GFP
Df(2r)eve/Cyo,Twi-GFP

Generation of germ-line clones and collection of embryos

hsFLP;nmo^{adk1}, FRT79D/TM6B females were crossed to *ovoD,FRT79D/TM3* males and transferred every 2-3 days. Progeny were heat-shocked at 37°C for two hours on two consecutive days at the beginning of the third larval instar to induce clones in the germline. Female progeny carrying both *nmo^{adk1}, FRT79* and *ovoD, FRT79* were selected and crossed to the appropriate males for embryo collection.

For all embryonic analyses, embryos were collected on standard apple-juice agar plates for 4, 6, 8, 16 or 24 hours. For cuticle preparations, plates were aged at least 24 hours to assess lethality and, in some cases, to allow removal or sorting of embryos carrying green balancers. Embryos were dechorionated in 50% commercial bleach for 5 minutes, rinsed thoroughly, mounted in 1:1 Hoyers:lactic acid and incubated at 65°C overnight. For cuticle preparations where examination of individual denticles was desired, embryos were fixed as if for antibody staining (below) before being mounted as above. Embryos to be used for *in situ* hybridization or antibody staining were fixed according to Patel (1994) and stored in MeOH at –20°C for at least 16 hours before staining.

Embryo Staining

Antibody staining was done according to Patel (1994), unless otherwise indicated. The following antibodies were used: anti-En (1:5), anti-Wg (1:50), BP102 (1:100), anti-elav (1:100) anti-FasII (1:100)(DSHB), rabbit-anti-ß-Gal (1:200), HRP-conjugated goat-anti-mouse, HRP-conjugated goat-anti-rabbit (Jackson ImmunoResearch Laboratories Inc.). Antibody staining against BP102 was carried out essentially as described, except that biotinylated anti-mouse (1:200; Vector Laboratories) was used as a secondary, followed by incubation with streptavidin-HRP (1:1000; Pierce) and detection with 3,3'-diaminobenzidine (DAB) + nickel.

Digoxygenin-labeled sense and antisense RNA probes for *in situ* hybridizations were made according to standard procedures using the DIG labeling kit (Roche) using either the T7 polymerase supplied or T3 polymerase (Roche). The cDNAs for *eve* (in pUAST) and *run* (in Bluescript) were a gift from Steven Russell. An EcoRI-XbaI fragment containing *eve* was sub-cloned into pBluescript for generation of probe. Template for antisense *eve* probe was generated by cutting the resulting plasmid with EcoRI and using T7 polymerase and the sense control was generated by cutting with XbaI and using T3 polymerase. Template for the antisense *run* probe was generated by cutting with XbaI and using T3 polymerase.

Larval disc staining

3rd instar larvae were dissected in phosphate-buffered saline (PBS), fixed in 1% glutaraldehyde in PBS for 5 minutes, rinsed 2x in PBS + 0.3% Triton-X (PT) and washed 2x 15 minutes in PT. Tissues were then incubated in 1:50 8% X-Gal in DMSO:X-Gal

staining solution for 30 minutes. Following staining, tissues were washed 2x in PT and mounted in 75% glycerol.

Analysis of adult wings

Wings were dissected from flies, dehydrated in ethanol, and mounted in Aquatex (EM Science).

The role of *nmo* in modulating expression of wingless target genes

Part 1: nemo *and the D/V boundary in wing discs*

Wingless and Notch signaling act to organize the D/V boundary in the developing wing disc. Serrate is expressed in the dorsal compartment of the wing disc. Notch signaling is activated at the boundary of Serrate expression, and directs expression of Wg at the D/V boundary (Diaz-Benjumea and Cohen, 1995). Notch signaling also directly results in the expression of *vestigial* (*vg*) at the D/V boundary, but the subsequent expansion of *vg* expression throughout the wing pouch depends on Wingless (Neumann and Cohen, 1996). Notch and Wg signaling co-operate to direct expression of *cut* at the boundary (Neumann and Cohen, 1996). Expression of Wingless in the narrow stripe that defines the D/V boundary of the disc is thought to define the wing margin. Target genes such as *achaete-scute* and *distalless* (*Dll*), in addition to *cut* and *vg*, are expressed in a dose-dependent manner in response to Wingless signaling (Neumann and Cohen, 1997) and thus their expression can be used as a direct readout of Wingless signaling. Because Nemo is proposed to act antagonistically to Wg signaling and has also been implicated in Notch signaling (Verheyen et al., 1996), I thought this might be an interesting context in which to study the possible role for Nemo in regulating the cross-talk between the two pathways.

Using lac-Z reporter strains, I attempted to determine if altered levels or patterns of *nmo* expression resulted in altered expression of two reporter constructs: a *Dll-lacZ* reporter and a reporter for the quadrant enhancer of *vg* (*vg(quadrant)-lacZ*). Examination of reporter expression patterns in a wild-type background showed variability in staining, likely due to slight differences in the developmental stage of the larvae. Similar

variability was observed in discs from *nmo* mutant larvae and in discs in which *nmo* had been misexpressed using the 69B-Gal4 driver, making comparison between the different genotypes difficult. Therefore, expression pattern of both constructs in wild-type discs was considered to be indistinguishable from discs from either *nmo* mutant larvae or discs in which *nmo* had been misexpressed using the 69B-Gal4 driver (Figure 4).

Part 2:* nemo *and Wingless signaling in the visceral mesoderm

Another tissue in which Wg reporter genes are well studied is the embryonic mesoderm. Parasegmental identities in the visceral mesoderm (VM) are established through a series of complex regulatory interactions. In parasegment 7 (PS7), mesoderm induction is initiated by the homeotic gene *Ultrabithorax* (*Ubx*) (see Riese et al., 1997). *Ubx* expression induces expression of the TGFß homologue *decapentaplegic* (*dpp*) within PS7. *dpp*, in turn, leads to expression of *wingless* (*wg*) in the neighboring cells in PS8, while also reinforcing *Ubx* expression in PS7. Similarly *wg* and *dpp* expression define the identities of PS2 and PS3, respectively, although no homeotic genes are expressed in PS3 (Yang et al., 2000)(although I question this statement as there appears to be *Ubx* expressed there too – see Waltzer and Bienz, 1998).

The VM enhancer region of *Ubx* contains a Wg response sequence (WRS) to which the TCF homologue LEF-1 can bind to promote transcription *in vitro* (Riese et al., 1997). Expression of a lacZ reporter under control of this WRS (the Ubx-B reporter, Riese et al., 1997, and Figure 5) occurs in the VM in a pattern similar to that of *Ubx*. Additionally, expression of this construct requires Wg signaling, as reporter expression is lost in *wg[CX4]* and *arm[XM19]/Y* embryos, and is expanded under conditions of

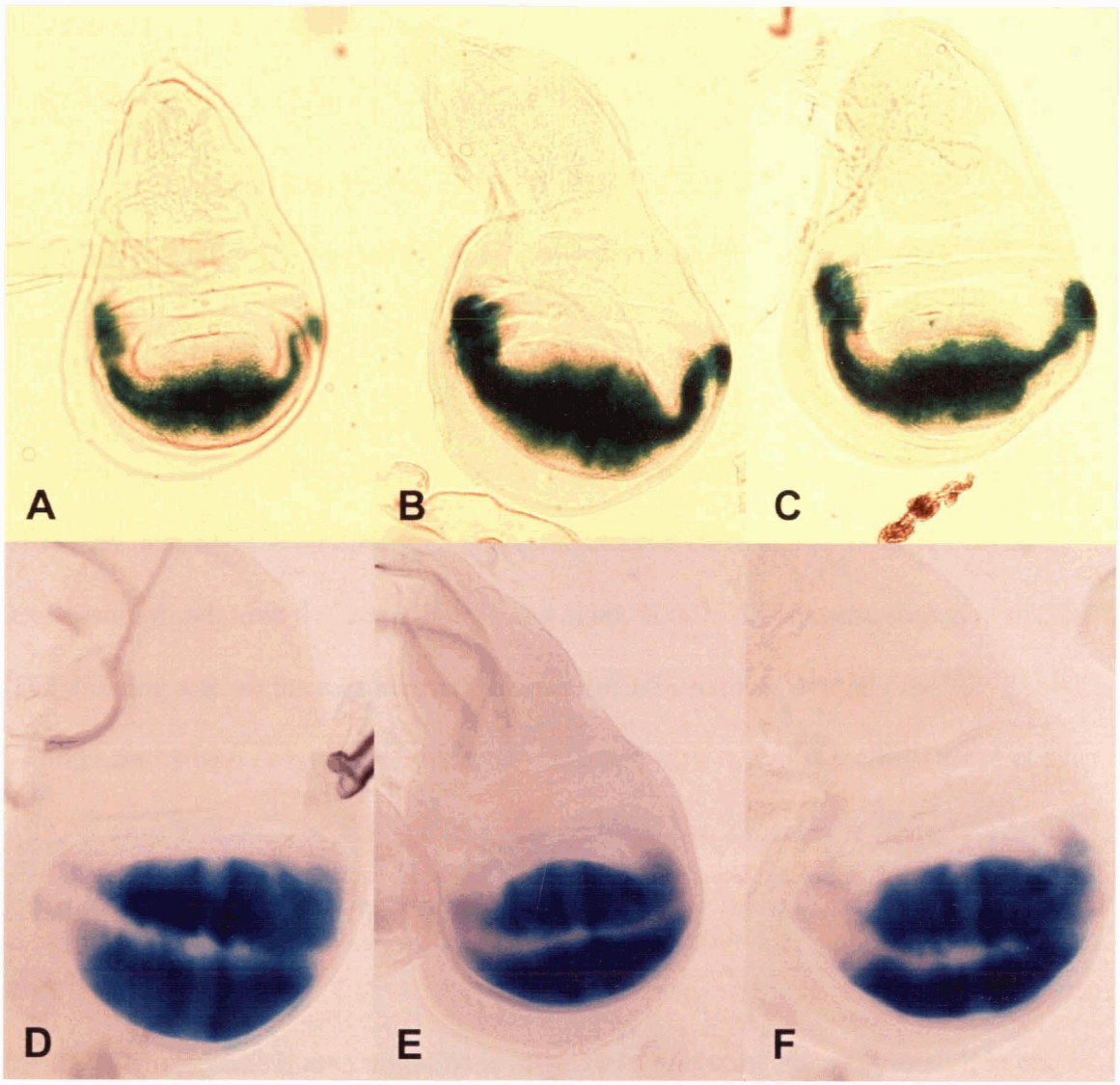


Figure 4: Expression of the Dll-lacZ (A-C) and vg(quadrant)-lacZ (D-F) reporter constructs as detected by X-Gal staining. Expression in wild-type discs (A, D) is indistinguishable from expression in *nmo*^{adk1} discs (B, E) and discs in which *nmo* is ectopically expressed using the 69B-Gal4 driver (C, F).

increased Wg signaling. Interestingly, the increase in reporter expression mediated by increased LEF-1 is also dependent on Dpp signaling (Riese et al., 1997). Additionally, this reporter expression is inhibited by *Drosophila* CREB binding protein (dCBP), a known transcriptional activator (Waltzer and Bienz, 1998).

Similarly, the mesodermal enhancer region of *dpp* contains regulatory sequences that bind TCF. An 812 bp BamHI-EcoRI fragment (the BE fragment, Yang et al., 2000, and Figure 6) can direct expression of lacZ in the visceral mesoderm in a pattern similar to that of *dpp*. A reduction in Wg signaling results in the decrease or loss of reporter expression, indicating that Wg signaling is required for *dpp* expression in the VM. However, loss of TCF binding sites in the enhancer leads to an expansion of reporter expression throughout the mesoderm (Yang et al., 2000). In contrast, mutation of the TCF binding sites in the mesodermal enhancer of *Ubx* reduces overall reporter expression, as well as rendering the enhancer insensitive to Wg signaling (Riese et al., 1997). Thus TCF may be acting differently on these two enhancer regions, repressing reporter expression in one the context of one enhancer, while enhancing expression in the context of the other.

To determine if *nmo* might be involved in modulating gene expression in one or both of these contexts, expression of these constructs was examined in embryos lacking the maternal *nmo* contribution through induction of *nmo* clones in the germ-line, but receiving a wild-type paternal *nmo* allele. Embryos of this genetic background exhibit ~60% embryonic lethality, and therefore should represent a valid system in which to study the role of *nmo* in gut development. Both BE (Figure 5) and UbxB (Figure 6) reporter expression appeared to be unaffected by loss of maternal *nmo*. Additionally, gut

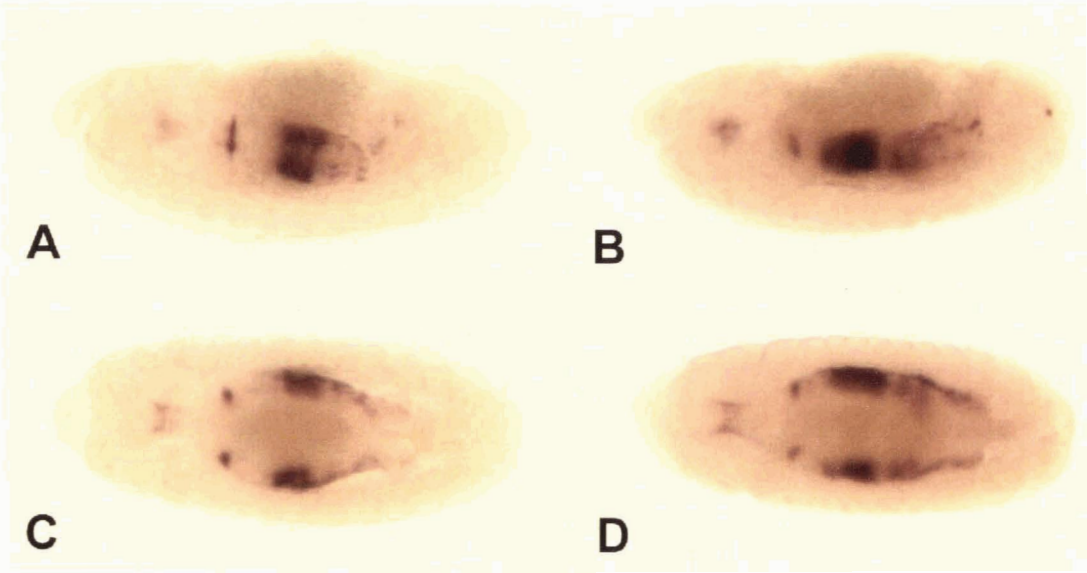


Figure 5: BE-lacZ reporter detected by anti-ß-Gal antibody in wild type (A,C) and *nmo* GLC (B,D) embryos. Both lateral views (A,B) and ventral views (C,D) are shown. Reporter expression in stage 15 embryos is unaffected by loss of maternal *nmo*.

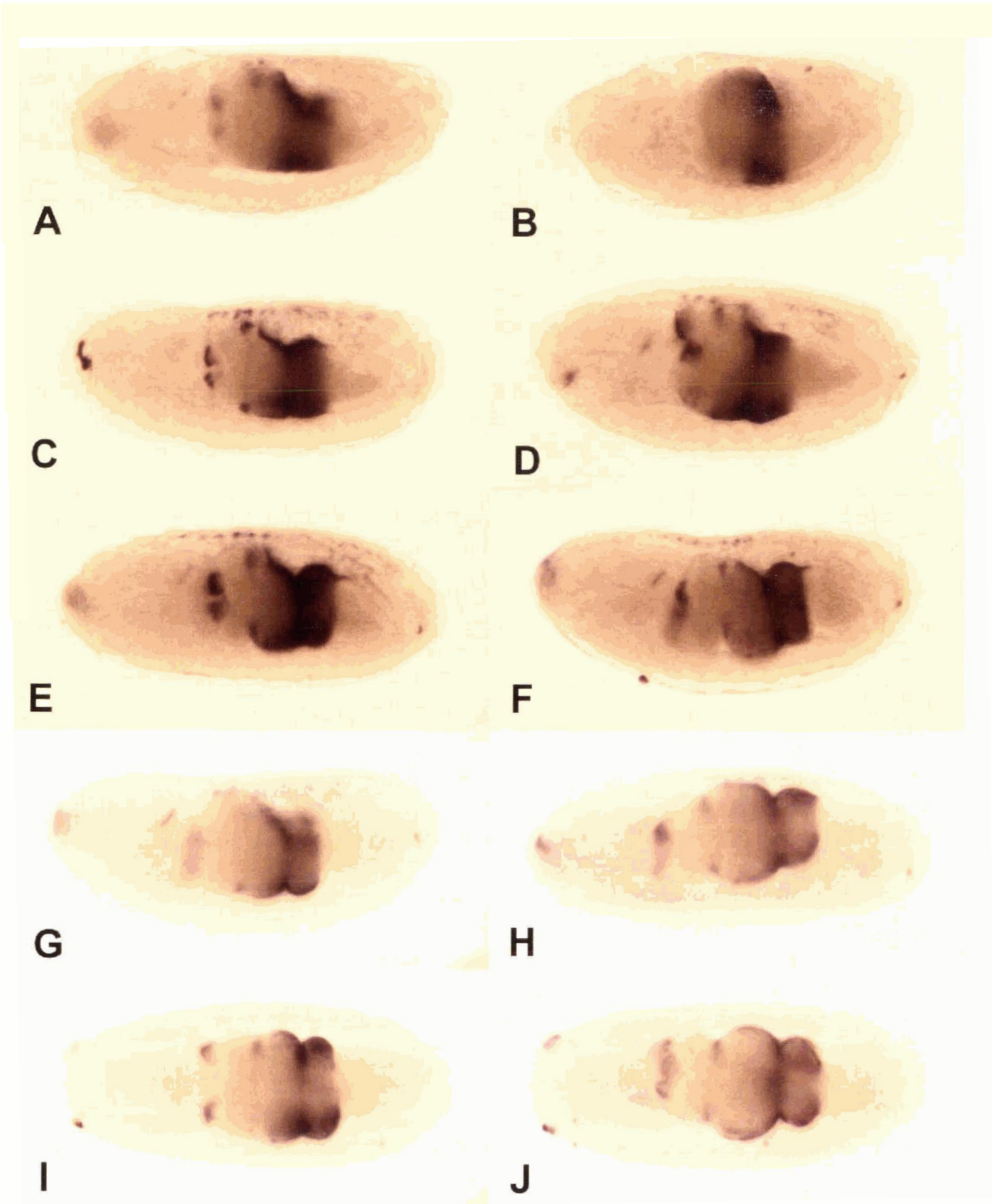


Figure 6: UbxB reporter expression detected by anti-ß-Gal antibody in wild-type (A,C,E,G,I), *nmo* GLC (B,D,F), and *UAS-nmo*/+; *24ß-Gal4*/+ (H,J) embryos. Reporter expression is unaffected by either loss of maternal *nmo* or ectopic expression of *nmo* using the 24ß-Gal4 driver.

morphology appeared to be relatively normal in these embryos. These results indicate that *nmo* does not normally play a role in gut development, either because it is unable to influence *dpp* or *Ubx* expression in this context, or because it is not normally expressed in the visceral mesoderm. To distinguish between these two possibilities, gut morphology and *Ubx* reporter expression were also examined in embryos in which *nmo* was misexpressed using the mesodermal driver 24ß-Gal4. In this context, gut morphology and UbxB staining were indistinguishable from wild-type (Figure 6 G-J). Taken together, these results indicate that *nmo* does not play a role in mediating Wingless target gene expression in the visceral mesoderm.

Discussion

In the absence of any obvious morphological defects in the adult wing margin in *nmo* flies, it would not be expected that loss of *nmo* would have obvious effects on patterning of the D/V boundary in the disc. Refinement of the adult wing vein pattern does not occur until pupal development, thus relatively subtle wing phenotypes such as those exhibited by *nmo* mutants would not be detectable in the wing disc. Similarly, *69B-Gal4>UAS-nmo[C4-2]* flies exhibit a very mild wing phenotype, and likewise should also have no obvious defects in D/V boundary patterning in the disc. However, in the absence of internal controls for staining, the possibility that *nmo* might modulate expression of Wg targets in the wing margin could not be ruled out. The induction of somatic *nmo* clones could address this issue, as the surrounding wild-type tissue provides an internal staining control. Indeed, others in our lab have recently found that *nmo* does play a role in patterning of the wing margin through effects on Wg reporter expression downstream of wingless signaling (Zeng and Verheyen, 2004). The main differences

between these studies were the use of the relatively strong apterous-Gal4 driver, which gives a severe wing phenotype when used to ectopically express *nmo*, and also the use of somatic clones, which provided an internal control for staining. These findings highlight the utility of not only using much stronger Gal4 lines, but also using mosaic studies for the analysis of gene expression.

nmo appears to play no role in development of the embryonic midgut, as loss of maternal *nmo* or ectopic expression of *nmo* in the gut has no obvious effects on either gut morphology or the expression of Wg target genes. However, because the effects of loss of *nmo* on expression of Wg targets in the wing disc appear to be quite subtle, it is not surprising that no effects on Wg target gene expression in the gut were observed, and the possibility that *nmo* might regulate gut development cannot be ruled out. Further studies could potentially be carried out to discern whether *nmo* plays a role in Wg or dpp inhibition using ectopic *nmo* in combination with ectopic *dTCF*, *wg* or *dpp*. However, taken together, these results indicate that *nmo* does not normally play a role in modulating *dpp* or *Ubx* expression in the visceral mesoderm.

Identifying novel *nmo* interactors

To identify potential *nmo* interactors our lab carried out a deficiency screen (described in Figure 7) for regions on the second chromosome that could dominantly modify the *69B-Gal4>UAS-nmo[C5-1]* wing phenotype . This phenotype is characterized by a small amount of ectopic vein above L2 and loss of the PCV. I further examined five regions previously identified as interactors by others, taking advantage of a *69B-Gal4>UAS-nmo[C4-2]* recombinant. The phenotype of this recombinant is characterized by a small amount of ectopic vein above L2, but no loss of the PCV (See middle inset in Figure 7). The regions I examined were 42B3-4;43F5-8, 48A;48F, 51A1;51B6, 55A;55F and 60E6;60F1-2.

Heterozygous deficiency for 42B3-4;43F5-8 demonstrated suppression of the *69B-Gal4>UAS-nmo* phenotype (Figure 8). Two promising candidate genes, a Dpp receptor *saxophone* (*sax*), and the PCP pathway member *prickle* (*pk*) were identified and tested. Both sax^{4} and pk^{1} seemed to moderately suppress the *69B-Gal4>UAS-nmo[C4-2]* phenotype.

Heterozygous deficiency for the 48A;48F region suppressed the extra vein above L2, but also resulted in extra vein at the end of L5. The *engrailed* (*en*) gene is located within this region and represents a good candidate. However, none of the *en* alleles tested mimicked this interaction. I also looked for suppression of a more severe phenotype, *ap-Gal4>UAS-nmo*, by different *en* alleles, but was unable to distinguish between *en*/+ flies and their balanced sibs because the wing phenotype was so severe it did not allow for identification of the Cyo marker.

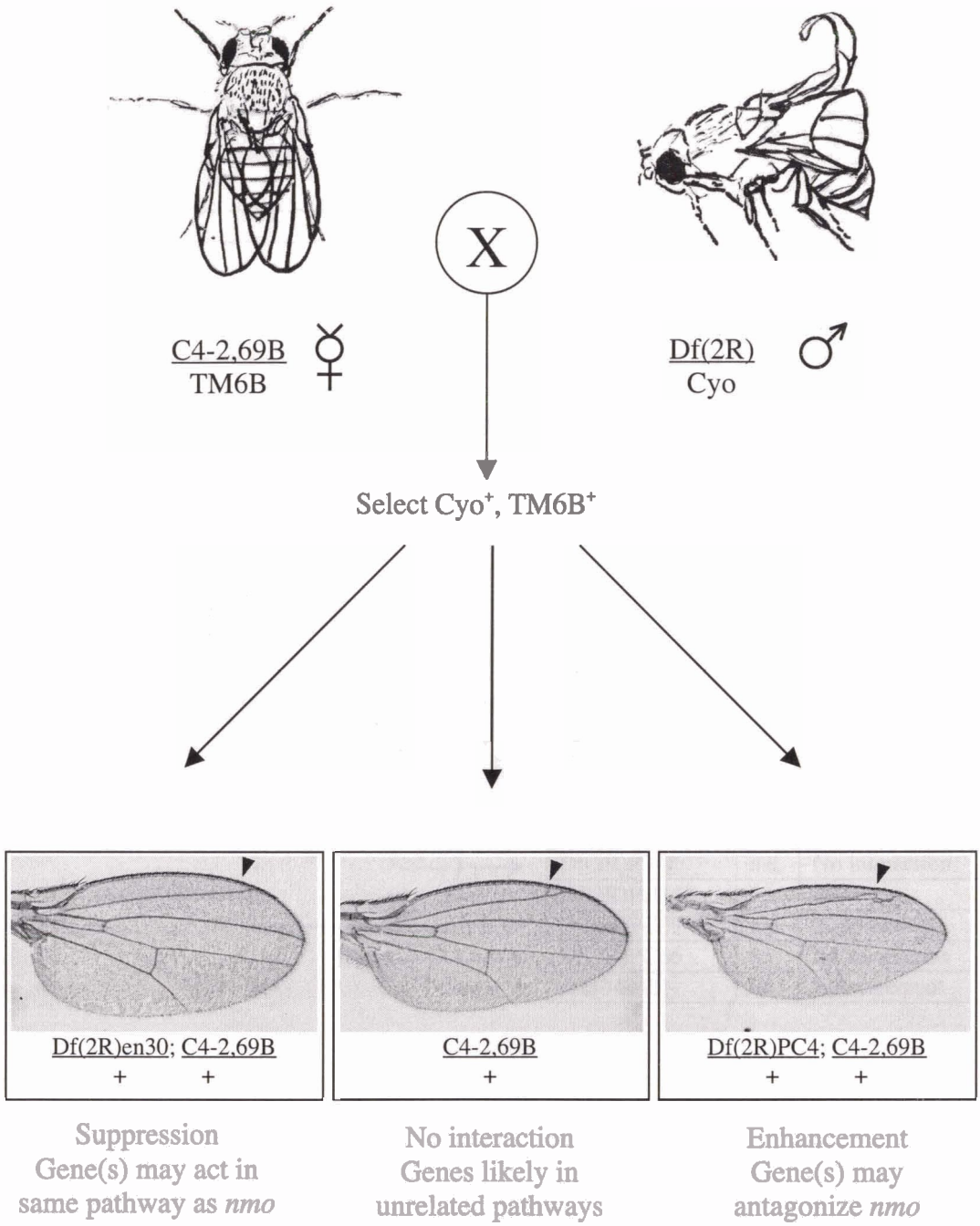
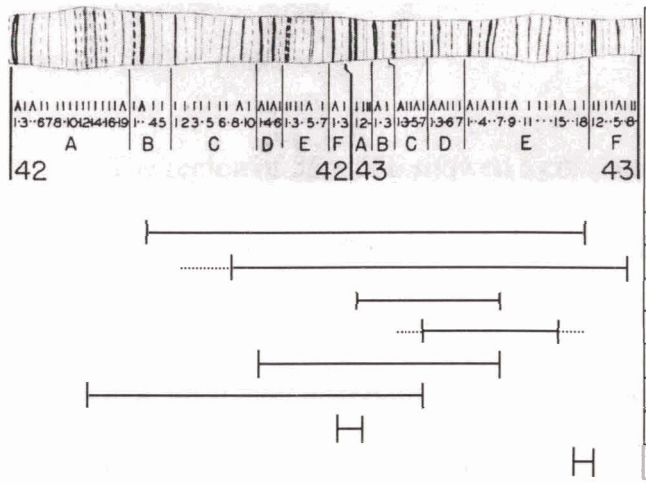
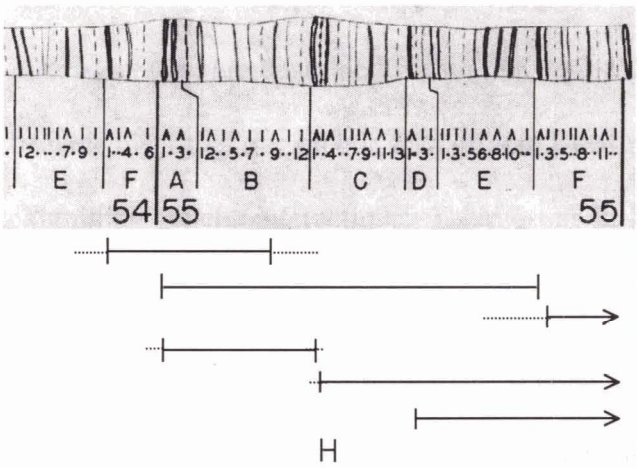


Figure 7: The deficiency screen. 69B-Gal4,UAS-nmo[C2-4]/TM6B virgins are crossed to balanced flies carrying a deficiency for part of the second chromosome and the appropriate progeny are selected. If the deficiency encompasses a gene acting in the same pathway as *nmo* during wing development, it is possible that loss of half of the gene product will result in the enhancement or suppression of the *nmo* mis-expression phenotype (indicated by arrows). Loss of gene products in parallel or unrelated pathways should have no effect on the phenotype (starting phenotype is shown).



Deficiency or mutant	Penetrance (%)	Severity of phenotype in relation to starting phenotype
Df(2R)ST1	7	Suppression
Df(2R)pk78s	37	Suppression
Df(2R)Drl[rv15]	80	No interaction
Df(2R)tor-rx6	32	Suppression
Df(2R)Drl[rv7]	55	n.d.
Df(2R)Drl[rv25]	6	Suppression
pk[1]	31	Mild
sax[4]	20	Mild



Deficiency or mutant	Penetrance (%)	Severity of phenotype in relation to starting phenotype
Df(2R)Pcl7B	n.d.	Enhancement
Df(2R)PC4	n.d.	Enhancement
Df(2R)P34	n.d.	No interaction
Df(2R)Pcl11B	53	Mild
Df(2R)PC29	n.d.	Suppression
Df(2R)PC66	85	No interaction
sbb[04440]	84	Enhancement

Figure 8: Results of a deficiency screen to identify regions of the second chromosome that dominantly modify the *69B-Gal4>UAS-nmo[C4-2]* wing phenotype. The region that each deficiency spans is indicated schematically below the chromosomal map. Dotted lines indicate uncertain breakpoints and arrows indicate that the second breakpoint lies outside of the map.

The region of 51A1;51B6 showed no appreciable interaction upon retesting and was not further pursued.

The region of 55A;55F showed a consistent and strong enhancement (see Figure 8). Several candidate genes are located in this region and were tested for interactions. A *scribbler* mutant (sbb^{04440}) moderately enhanced the phenotype and therefore may represent a novel *nmo* interactor. *sbb* (also known as *brakeless*) encodes a transcription factor involved in axon guidance (Senti et al., 2000).

One other region, 60E6;60F1-2, was also examined and appeared to suppress the *69B-Gal4>UAS-nmo* phenotype. The segment polarity gene *gooseberry* (*gsb*), and *distal-less* (*dll*) are contained within this region, but were not further pursued as it became clear that the genetic background I was using was not a very good one for examining genetic interactions. The reasons for this are discussed below.

Discussion

The *69B-Gal4>UAS-nmo[C4-2]* phenotype exhibits considerable variability in both penetrance (ranging from 50% to more than 80%) and severity from generation to generation. In some cases, most flies exhibit only a small amount of extra vein above L2, while in other cases most flies exhibit considerably more ectopic vein material similar to that observed in the right inset in Figure 7. Therefore it became clear that the *69B-Gal4>UAS-nmo* phenotype does not represent a very good system in which to identify potential genetic interactors for *nmo*. Interactions initially observed for many potential regions were found to be too variable to further characterize. On the other hand, two of the potential *nmo* interactors identified in this screen have been implicated as potential - modulators of *nmo* signaling; Pk may interact with Nemo (M. Mlodzik, unpublished

communication), and the effects of *nmo* on *en* are discussed below. Whether the putative interaction between *nmo* and *sbb* is real remains to be demonstrated.

The role(s) of *nemo* in embryonic patterning and formation of the cuticle

Embryos zygotically mutant for *nmo* exhibit a wild-type phenotype, provided that wild-type maternal *nmo* transcript is present. However, *nmo* germ-line clones (GLCs), which are devoid of maternal transcript, die as embryos (~85%) and often exhibit loss or fusion of denticle belts and decreased levels of apoptosis (Mirkovic et al., 2002). Patterning of the embryonic epidermis is mediated by inputs from both Wg and EGFR pathways, which act to specify specific fates within each segment. Wg signaling is required for naked cuticle in the ventral epidermis, while EGFR specifies denticle fates. Wg also plays a role in specifying distinct denticle identities within each denticle belt. The expression of Wg in the embryo is dictated by the activities of the pair-rule genes (more below). The expression of the pair-rule genes is dependent, in part, on the homologue of xSOX11, SOX70D (Nambu and Nambu, 1996, Russell et al., 1996). Given the established role for *nemo* as a negative regulator of Wingless signaling, its implication in regulation of EGFR signaling, and the physical interaction between NLK and the xSOX11 protein, three plausible explanations for the observed loss or fusion of denticle belts come to mind. 1) Nemo modulates expression or activity of the pair-rule genes in early embryonic development, possibly through interaction with SOX70D. 2) Nemo modulates Wg signaling in determining denticle vs. naked cuticle fate through interaction with dTCF/Arm. 3) Nemo modulates Wg signaling in establishing the diversity of denticle types through interaction with dTCF/Arm.

In an attempt to distinguish between these possibilities, I further characterized the denticle defects in *nmo* GLCs. Embryos from *nmo* GLCs exhibit several characteristic

defects in denticle patterning (see Figure 9 and Table 2), particularly fusion of the third thoracic belt with the first abdominal belt (T3-A1 fusion) and the sixth abdominal belt with the seventh (A6-7 fusion), although A2-3 or A4-5 fusions are frequently observed. Fusions between A1-2, A3-4, A5-6 or A7-8 do not occur. Fusions usually appear to be a result of the loss of either medial or lateral naked cuticle, although we occasionally observe the complete loss of naked cuticle. Thus denticle belt fusion, though not fully penetrant, occurs in a consistent pattern, with fusions occurring in alternating segments. I also noticed a significant amount of defects in head development and failure of germband retraction (Table 2). Additionally, I noticed that loss of maternal but not zygotic *nmo* results in ~60% embryonic lethality, and most embryos that hatch are thought to survive to adulthood.

nemo transcript exhibits dynamic expression in the embryo (Verheyen et al., 2001). It is expressed ubiquitously in blastoderm-stage embryos and expression is refined to a segmentally repeating pattern during germband extension. By late stage embryogenesis, *nmo* transcript is enriched in the central nervous system (CNS) and brain. I wanted to confirm these expression patterns using the *nmo[P]* enhancer trap line, which has been found to express ß-Galactosidase in a pattern that reflects the endogenous expression pattern of *nmo* in larval discs (Choi and Benzer, 1994, Zeng and Verheyen, 2004). I found that *nmo[P]* is expressed throughout the embryo but is enriched in alternating segments in stage 8 and 11 embryos (Figure 10 A,B) and in a segmentally repeating pattern in the CNS in stage 13 (Figure 10 C). *nmo[P]* is also expressed in cells of the dorsal vessel (Figure 10 D) and is refined to a specific subset of neurons in the CNS and PNS by late embryogenesis (Figure 10 E,F).

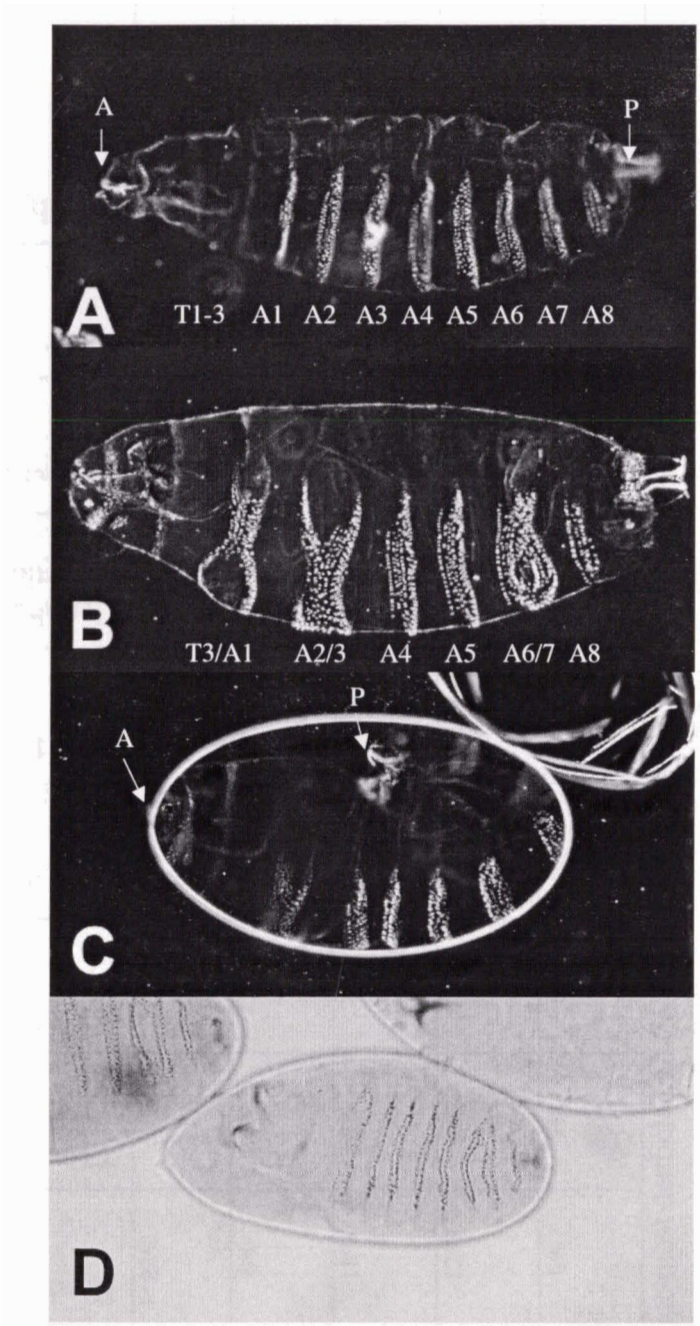


Figure 9: Cuticle preparations of wild-type (A) and *nmo* GLC embryos (B-D). Segments are labeled in (A) and (B) and the anterior and posterior ends of the embryos are indicated by arrows in (A) and (C). In these and all subsequent images, anterior is to the left. Common phenotypes include the fusion of alternating denticle belts (B), failure in germband retraction(C), and head defects (D).

Table 2: Denticle belt defects exhibited by embryos derived from *nmo* GLCs and heterozygous for various mutants.

paternal contibution (all embryos derived from *nmo* GLCs)	Head holes	Head defects	T3/A1 fusion	A2/3 fusion	A4/5 fusion	A6/7 fusion	Germband retraction failure	no phenotype	Other	lethality	N
w^{1118}	15%	23%	59%	20%	10%	74%	n.d.	7%	0%	60%	70
Df(2R)eve	14%	8%	26%*	77%*	62%*	79%*	9%	1%	0%	90%	121
ftz^{11}	12%	18%	9%	7%	2%	20%	18%	32%	6%**	73%	85
Cyo,twiGFP or *TM3,twi-GFP*	20%	12%	22%	10%	11%	23%	22%	22%	1%***	60%	83
fsh^{87}	28%	6%	9%	5%	27%	40%	19%	4%	13***	n.d.	93

*complete loss of the anterior-most denticle belt (ie T3, A2, A4 and A6)
** missing A5
*** missing A4

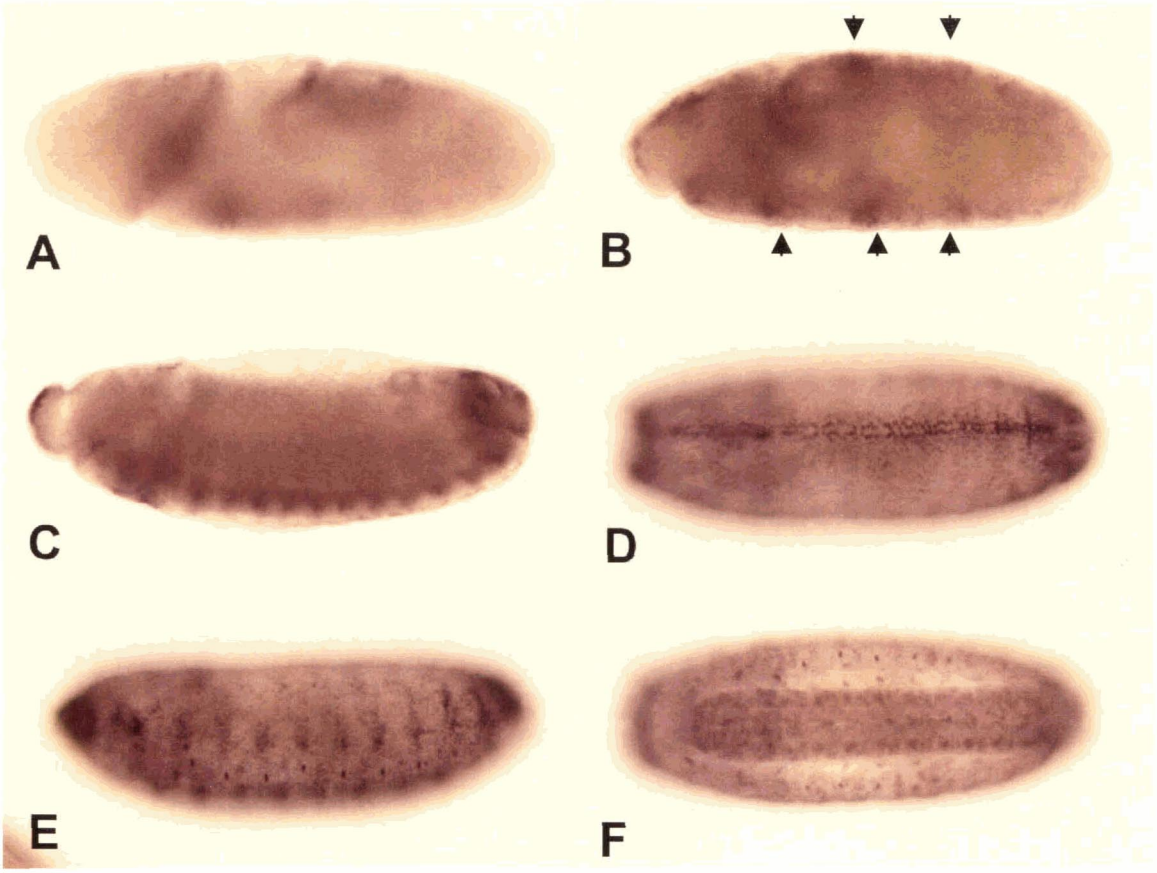


Figure 9: Expression off the *nmo[P]* reporter detected by anti-ß-Gal antibody. Lateral views are shown in (A-C, E), dorsal view in (D) and ventral view in (F). *nmo[P]* exhibits dynamic expression patterns during embryogenesis. Reporter expression appears to be enriched in alternating parasegments in stage 8/9 (A) and stage 11 (arrows in B) embryos. By stage 13 it is enriched in a segmentally repeating pattern in the CNS(C) and is also expressed in the dorsal vessel (D). At the end of embryogenesis (stage 16), the *nmo* reporter is expressed in the PNS (focal plane in E) and CNS (focal plane in F).

This would immediately suggest a role for *nemo* in segmentation and CNS/brain development.

Due to the cuticular phenotypes exhibited by *nmo* GLCs, the expression patterns of *nmo[P]*, and the involvement of *nmo* in Wg signaling, I wanted to explore some of the possible roles for *nemo* in segmentation, denticle patterning and CNS development.

Part 1: Segmentation

Nemo and the Pair-Rule Genes

Segmentation in the early Drosophila embryo depends on a series of highly orchestrated signaling events. Initially, asymmetric distribution of the maternal and gap genes provides positional information along the anterior/posterior (A/P) axis. Differential expression of the gap genes leads, in turn, to the expression of the pair-rule genes in a characteristic seven-stripe pattern. Activities of the pair-rule genes lead to the expression of the segment-polarity genes *engrailed* (*en*) and *wingless* (*wg*), and thus to the formation of the cuticular pattern observed in the larva. Disruption of a single gene within this hierarchy leads to phenotypes characteristic of the particular class to which the gene belongs (see Figure 11). For example, disruption of a gap gene leads to the deletion of a contiguous section of the embryo, disruption of pair-rule patterning leads to the deletion of alternating segments, and disruption of the segment polarity pathway leads to a phenotype, such as loss of naked cuticle, in every segment. Thus, the pattern of denticle belt defects observed in *nmo* GLCs supports a role for *nmo* in modifying expression or activity of the pair-rule genes.

Pair-rule patterning is established through a complex array of expression patterns and interactions among different genes (Figures 12 & 13). The end result is the division

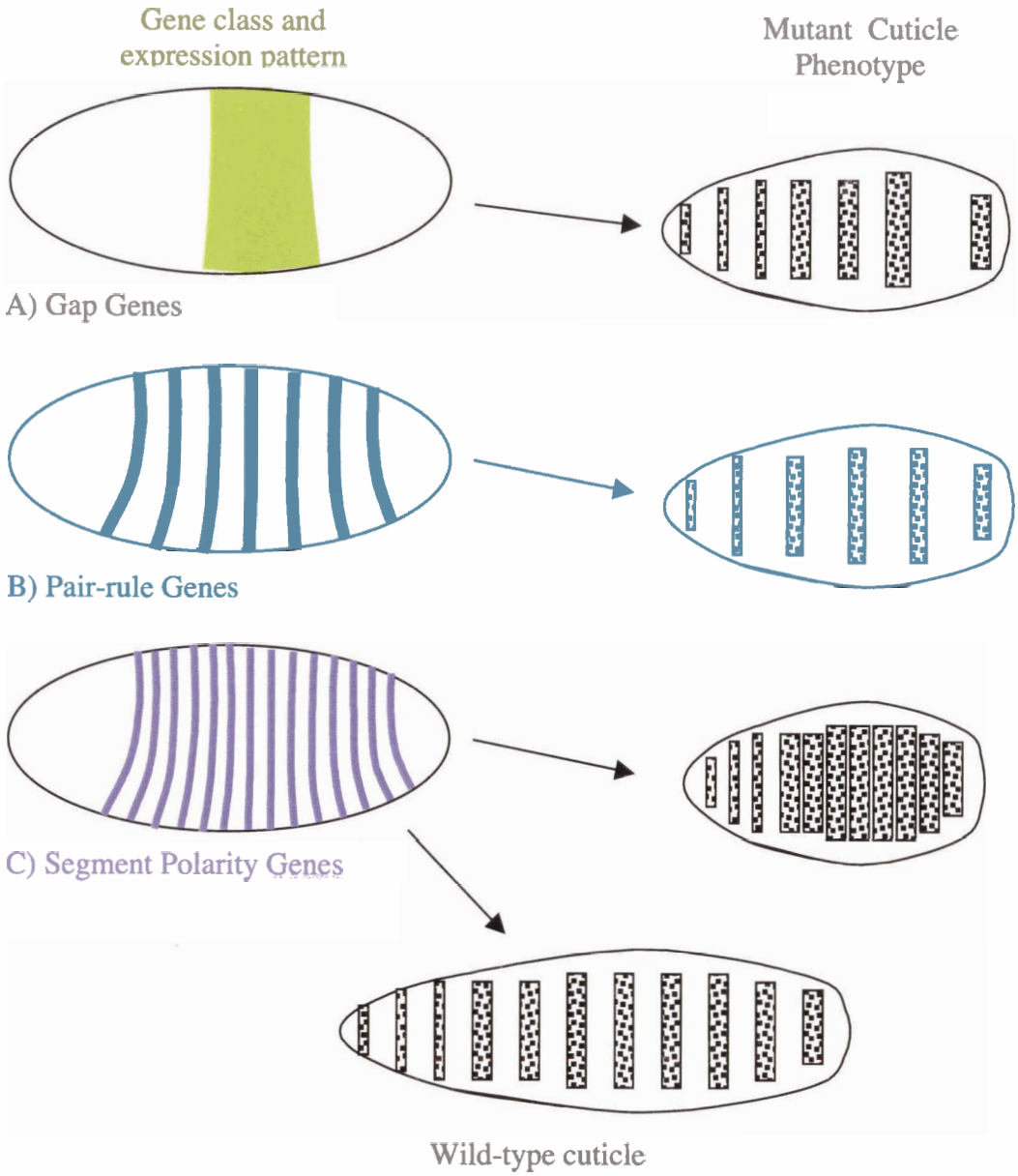


Figure 11: Gene classes involved in embryonic segmentation and their mutant phenotypes. Loss of gap gene function (A) results in the loss of a contiguous section of cuticle, while loss of pair-rule gene function (B) results in the loss of alternate segments. Loss of segment polarity genes (C) leads to a phenotype in every segment.

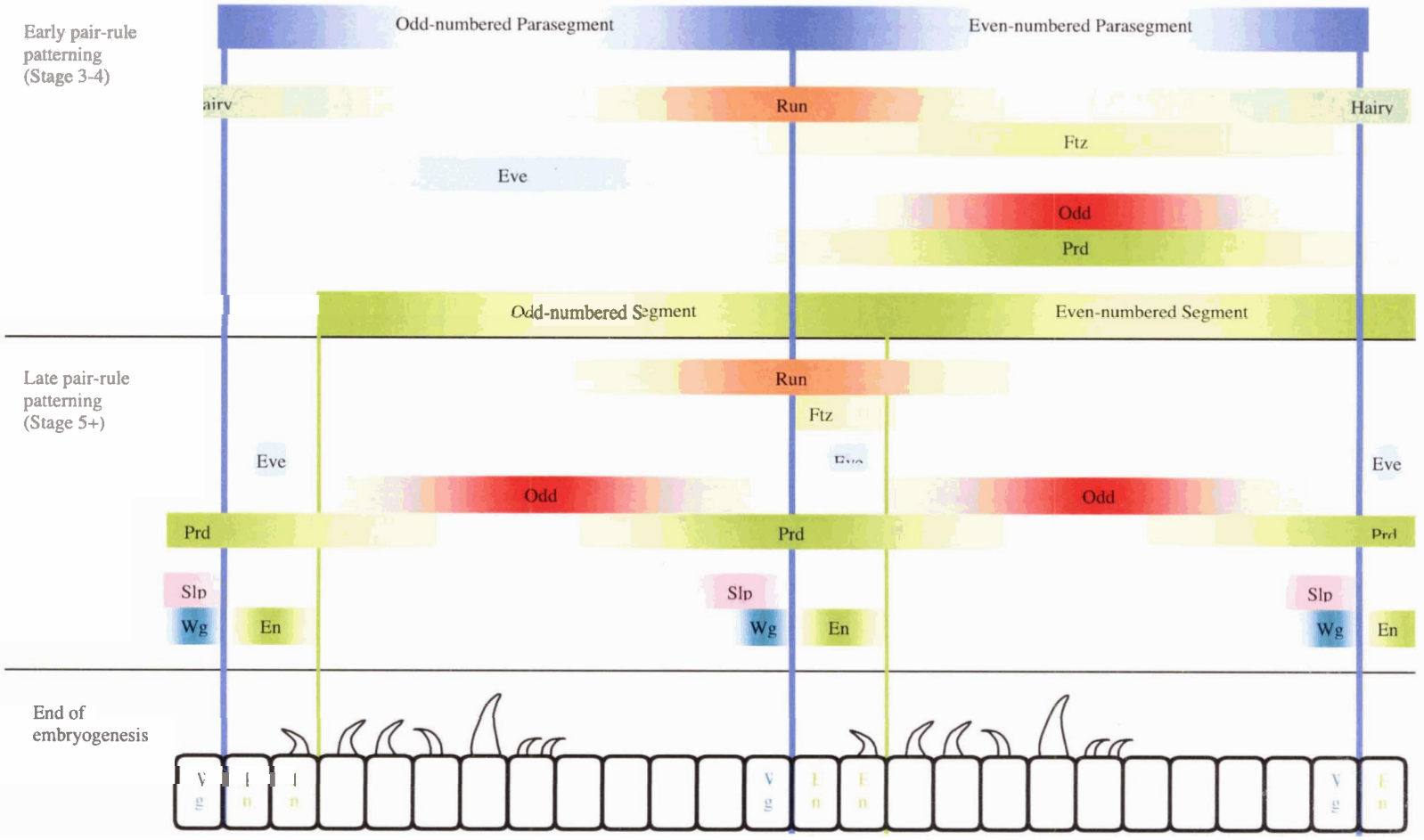


Figure 12: Expression patterns of the pair-rule genes during different stages of embryogenesis and their relationshipb with the final segmentation pattern of the ventral epidermis.

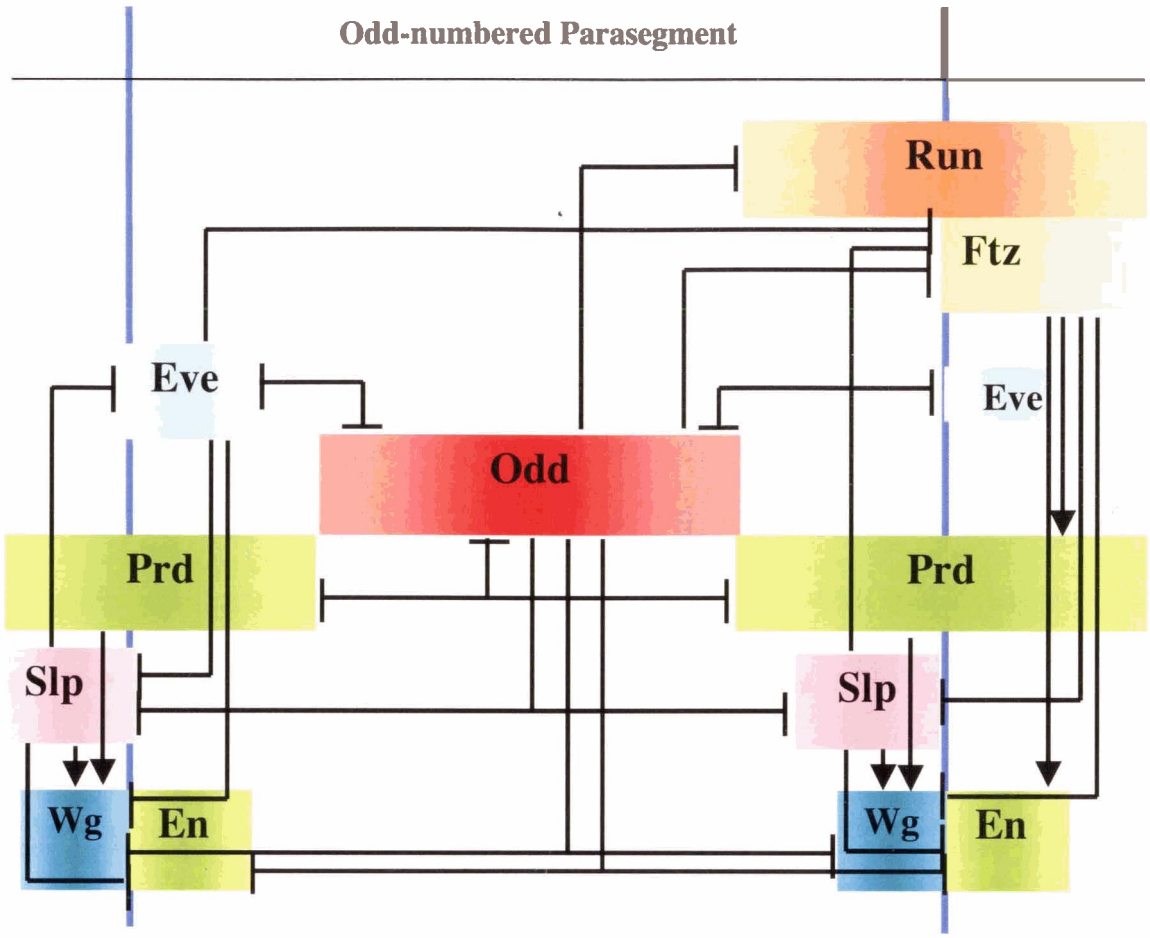


Figure 13: Schematic diagram of the regulatory interactions among the pair-rule genes following the refinement process. Solid arrows indicate positive interactions and blunt lines indicate negative interactions.

of the embryo into parasegments, the embryonic counterpart of the adult segment, through establishing the expression of the segment-polarity genes, *en* and *wg*, in 14 stripes along the anterior/posterior axis. Pair-rule patterning is initiated by the primary pair-rule genes, *even-skipped* (*eve*), *fushi-tarazu* (*ftz*), *hairy* (*h*) and *runt* (*run*). The promoter regions of these genes contain multiple stripe-specific enhancers that respond directly to maternal and gap gene products, and thus their expression tends to develop on a stripe-by stripe basis (see Sánchez and Thieffry, 2003). The initial 7 stripes of the primary pair-rule genes are off-set from one another such that *eve* and *ftz* expression is largely complementary, but each overlaps with the expression domains of *run* and *h*, which are also complementary to one another (see Figure 12). At the time of initiation, the primary pair-rule genes are expressed in relatively broad swaths that are later refined to narrow stripes as activation of the secondary pair-rule genes is initiated.

The 'classical' pair-rule genes identified by Nüsslein-Volhard and Weischaus (1980) all encode transcription factors, which, for the most part, act as transcriptional repressors. As a result, refinement of pair-rule expression patterns occurs as much from mutual repression as from direct activation. A combinatorial model has largely been used to explain how activation and refinement occurs, taking into account the presence and/or absence of activators and repressors in a specific domain (See Figure 13 and Sánchez and Thieffry, 2003). However, these models are limited in their ability to account for the complexity of interactions displayed in the developing embryo for several reasons. Expression patterns of the pair-rule genes are dynamic and therefore their potential interactions are also dynamic. A classic example of this was demonstrated using ectopic expression of *odd-skipped* (*odd*) at different times during embryogenesis and assessing its

effects on the expression of other genes (Saulier-Le Dréan et al., 1998). Specifically, ectopic *odd* resulted in expansion of *ftz* expression when induced in stage 4/5 embryos. However, it also repressed *ftz* in stage 7 embryos, but was able to do so only in those regions in which it is normally expressed, indicating that its activities are very context dependent. Additionally, a gene product can often affect only a subset of interactions of another. For example, *gro* has been shown to be required for only a subset of the repressive activities mediated by *eve* (Kobayashi et al., 2001). Teasing apart these regulatory loops therefore can be a challenging task.

In an attempt to shed light on possible molecular mechanisms for the cuticular phenotypes observed in *nmo* GLCs I examined En and Wg expression. In *nmo* mutant embryos En (Figure 14) and Wg (Figure 15) expression are disrupted in alternating stripes, although the complete loss of any stripe was rarely observed. In addition to the partial loss of En stripes, I also note that overall background En expression is consistently much higher in *nmo* GLCs. To determine which stripes of En and Wg were being lost, embryos were double stained for En or Wg and a *ftz-lacZ* reporter, as *ftz* is expressed in the even parasegments. In both cases the disruption occurs in those stripes just anterior to *ftz* expression (arrows in Figure 14G and 15E), which corresponds to even En stripes and odd Wg stripes. The observed loss of En and Wg expression in *nmo* GLCs likely directly results in the observed denticle defects. It is interesting to note that those regions in which *nmo[P]* is most highly expressed (arrows in Figure 10B) also appear to be those regions in which En (arrows in Figure 14D) and Wg are most commonly disrupted. Additionally, the anterior-most segments (where En and Wg are commonly disrupted in

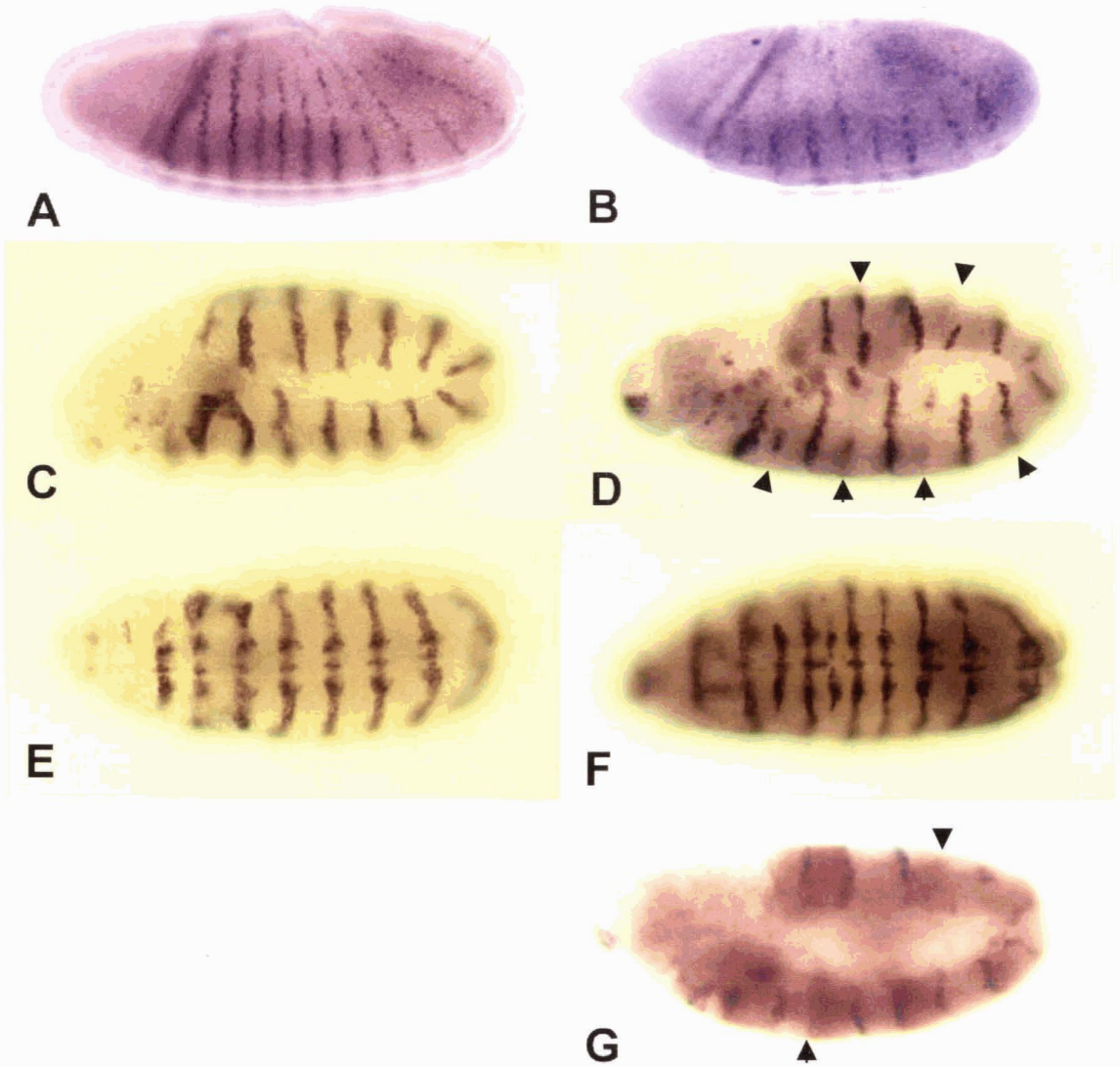


Figure 14: Anti-En antibody stain (black) in wild type (A,C,E) and *nmo* GLC (B,D,F,G) embryos. The embryo in (G) is from a *nmo* GLC mother crossed to a *ftz-lacZ* balancer and is also stained with anti-ß-Gal antibody (brown). (A-D,G) lateral view, (E,F) ventral view. Even En stripes are disrupted in *nmo*GLCs. En expression appears to be initiated normally in *nmo* GLCs (compare A to B), although by stage 11 En expression is disrupted in alternating stripes in(compare D,F to C,E; disrupted En stripes are marked by arrows in D). The stripes that are affected are those just anterior to the expression of a *ftz-lacZ* (brown) reporter construct (arrows in G), which reflects the early expression of *ftz* in even parasegments.

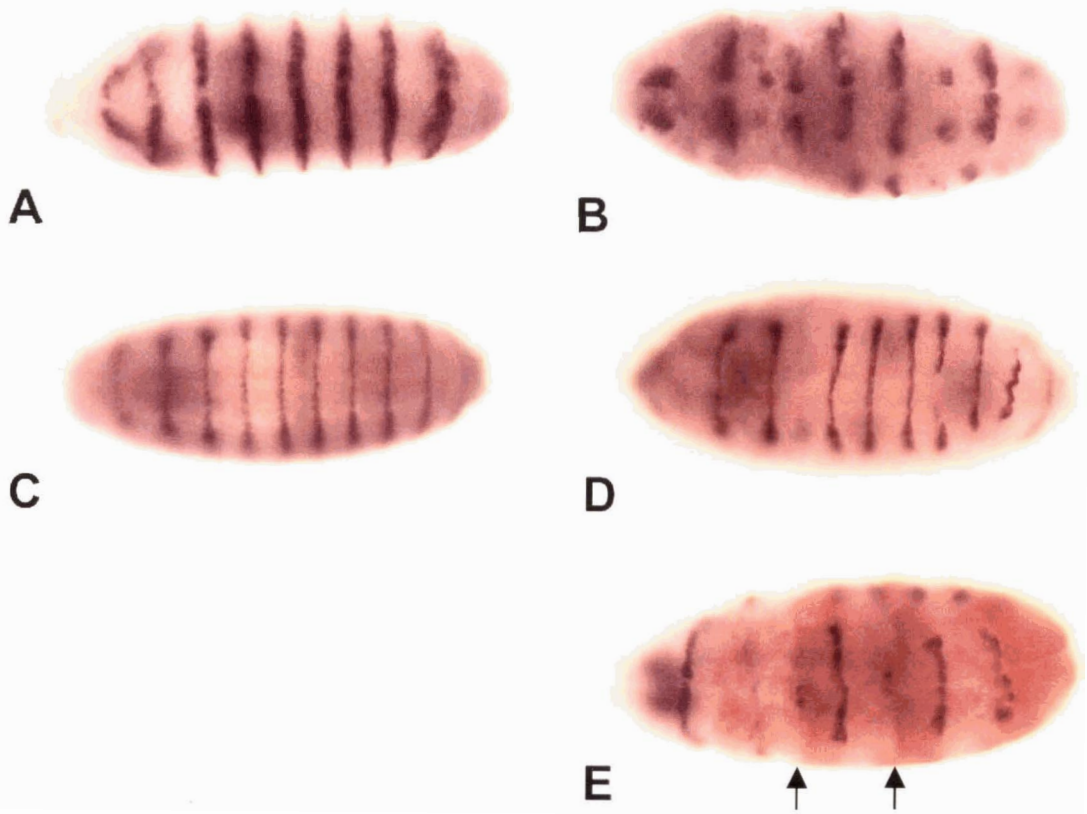


Figure 15: Anti-Wg antibody staining in wild type (A,C) and *nmo* GLC (B,D,E) embryos. Embryo in (E) is from a *nmo* GLC mother crossed to a ftz-lacZ balancer and is also stained with anti-ß-Gal antibody (brown). Odd Wg stripes are disrupted in *nmo*GLCs. Wg expression is disrupted in alternating stripes in stage 11 and 12 embryos (compare B,D to A,C). The stripes that are affected are those just anterior to the expression of a *ftz-lacZ* reporter construct (brown in E), which corresponds to the odd Wg stripes.

nmo GLC embryos) are required for the development of head structures, which are also commonly aberrant in *nmo* GLC embryos.

It appears from cuticle preparations that *nmo* GLCs lose naked cuticle from *eve*-dependent segments (eg. the posterior half of T3 or A2). On the other hand, the En and Wg stripes that are disrupted are those that are dependent on the later activity of *ftz*. Additionally, *run* is expressed in all tissues that are lost/disrupted in *nmo* GLCs. Therefore these seem to be reasonable candidates for targets of *nmo*. I examined the expression of *eve* and *run* in stage 5 embryos, just after expression is initiated, and found them to be unaltered (Figure 16). However this does not preclude the possibility that Nemo may alter the activity of these gene products rather than their expression.

I also examined the expression of a *ftz-lacZ* reporter gene in *nmo* GLCs and found that stripes 3 and 6 are initially expressed at lower levels than other stripes, although expression appears to be comparable in all stripes at later stages (Figure 17). However, since ß-galactosidase is relatively stable when expressed *in vivo*, the expression of ß-gal apparent at later stages could be due to the gradual accumulation of lower levels of expression. Additionally, the differences in expression could reflect decreased expression of stripes 3 and 6, or increased expression of the remaining stripes. Because denticle phenotypes commonly manifest in those segments corresponding to *ftz* stripes 3 and 6, I initially interpreted these findings as a delay in the initiation of *ftz* stripes 3 and 6. Unfortunately due to time constraints, I was unable to confirm by RNA *in situ* whether the initiation of *ftz* stripes 3 and 6 is delayed or to determine if expression of these stripes does indeed occur at normal levels once initiated.

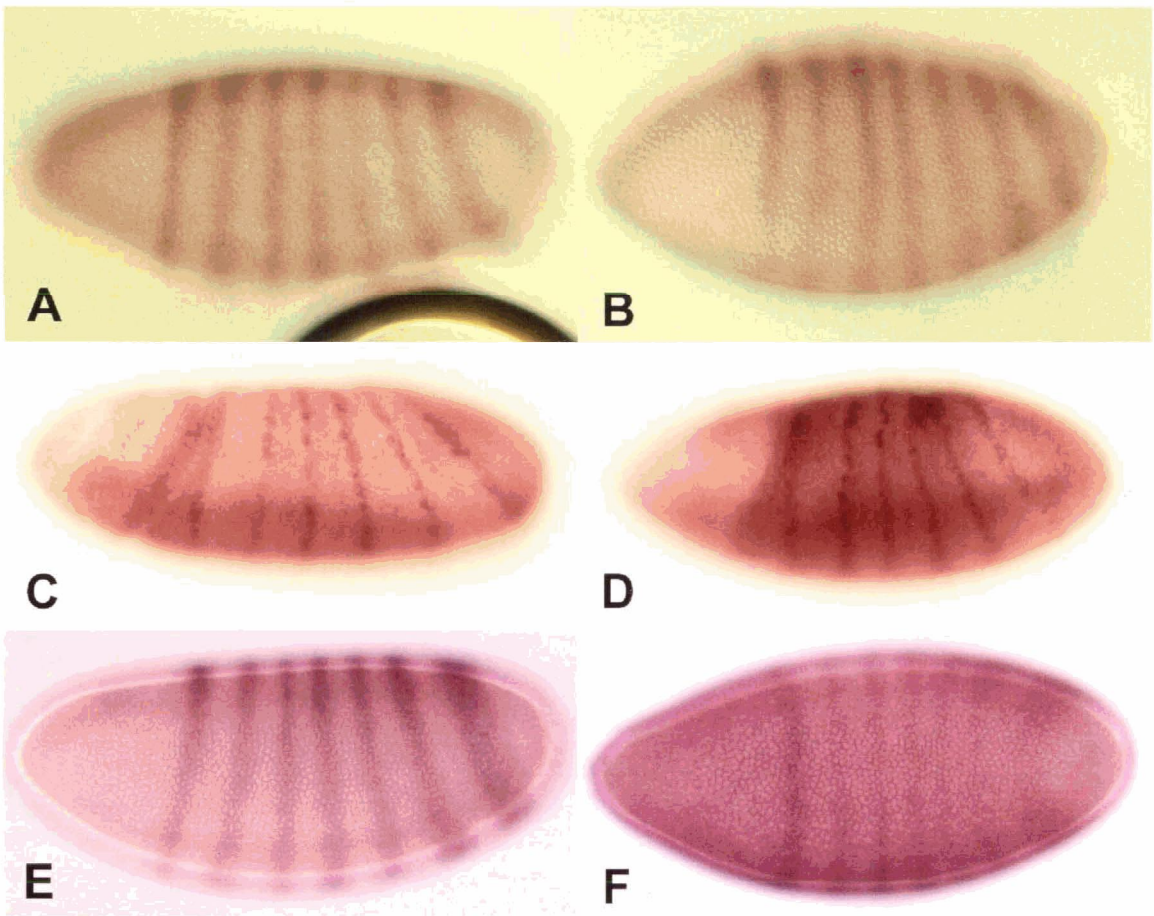


Figure 16: Wild-type (A,C,E) and *nmo* GLC (B,D,F) embryos are stained using anti-sense RNA probes against *eve*(A-D) or *run* (E,F). Expression of the primary pair-rule genes *eve* and *run* appears to be initiated normally in *nmo* GLCs. s.

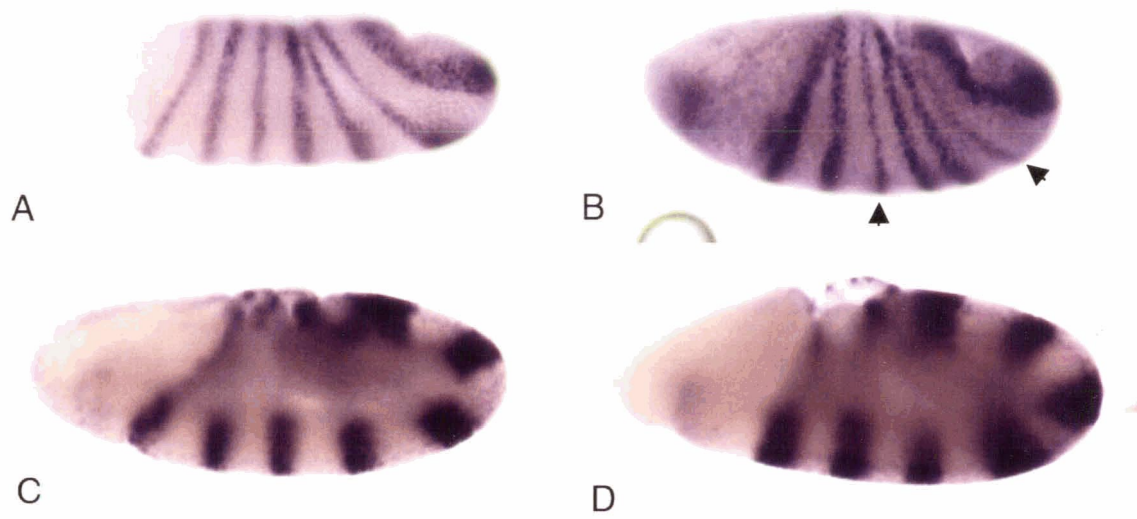


Figure 17: Anti-ß-Gal antibody staining of wild type (A,C) and *nmo* GLC (B,D) embryos carrying a *ftz-lacZ* reporter. In *nmo* GLC embryos the *ftz-lacZ* reporter is expressed at different levels in stripes 3 and 6 at stage 6 (arrows in B) but appears to be expressed at levels comparable to other stripes by stage 10 (D).

To address the potential functional significance of the observed loss of ftz-lacZ expression, I crossed *nmo* GLC mothers to *ftz*^{11}*/TM3, twi-GFP* males. Heterozygosity for *ftz*^{11} had no observable effect on the cuticle defects observed in *nmo* GLCs (Figure 18). In addition, the survival rate of *nmo* GLCs heterozygous for *ftz* was not reduced compared with balanced sibs (Table 2). This indicates that *nmo* does not interact genetically with *ftz*.

I also crossed *nmo* GLC mothers to *Df(2R)eve/Cyo,twi-GFP* males to see if *nmo* GLCs were sensitive to levels of *eve*. Surprisingly, *nmo* GLCs that were also heterozygous for the *eve* deficiency exhibited increased lethality (Table 2) and most hatched larvae died at the first instar. Significantly none survived to adulthood. Additionally, these embryos also exhibited a severe pair-rule cuticle phenotype resembling the *eve* mutants originally described in (Nüsslein-Volhard and Wieschaus, 1980)(Figure 18). I therefore suspect that *nmo* is required either for a subset of *eve* activities, or for expression (or more likely repression) of specific targets of *eve*. *In vitro* binding assays are currently in progress to determine if Nmo might bind Eve. It is possible that Nemo is required for Eve activation, and thus the absence of Nemo would prevent Eve activity, mimicking loss of Eve function.

eve represses a variety of pair-rule genes including *odd-skipped* (*odd*) and *sloppy-paired* (*slp*). I examined the expression of ß-Galactosidase from *odd* and *slp* enhancer trap lines in *nmo* GLCs. The specific expression pattern of *odd* did not appear to be affected in *nmo* GLCs, however, the overall levels at which it was expressed appeared to be considerably higher (Figure 19). Like Wg and En, *slp* reporter expression appeared to be initiated normally, but decayed at later stages in specific stripes (Figure 20).

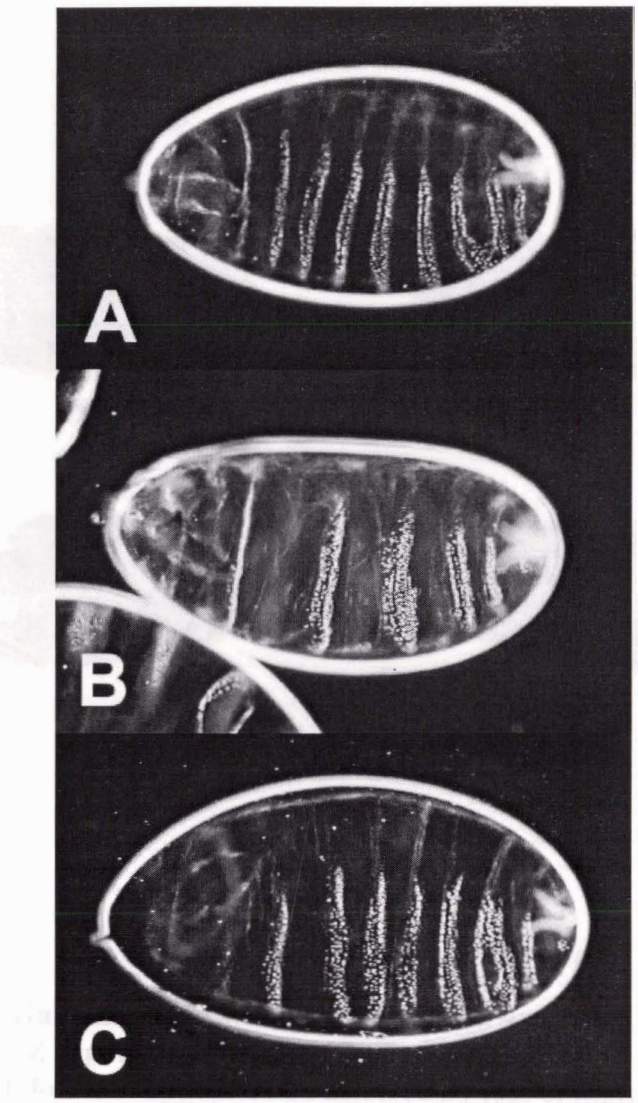


Figure 18: (A) ***ftz11/+; nmo/+*** **(B)** ***Df(2r)eve/+; nmo/+*** **(C)** ***+/Cyo,twi-Gal4, UAS-GFP; nmo/+*** **sibling from (B). All embryos are derived from** ***nmo*** **GLCs.**

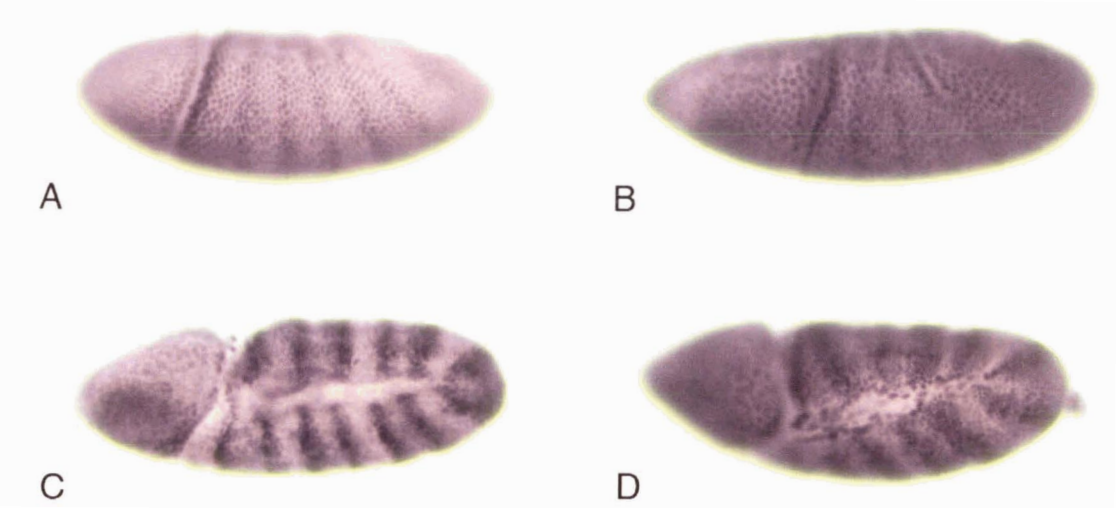


Figure 19: Anti-ß-Gal staining of wild type (A,C) and *nmo* GLC (B,D) embryos carrying an *odd-lacZ* reporter construct. Expression pattern of the *odd-lacZ* reporter construct does not appear to be affected by loss of maternal *nmo*, but does appear to result in higher levels of reporter expression in both stage 6 (A,B) and stage 9 (C,D) embryos.

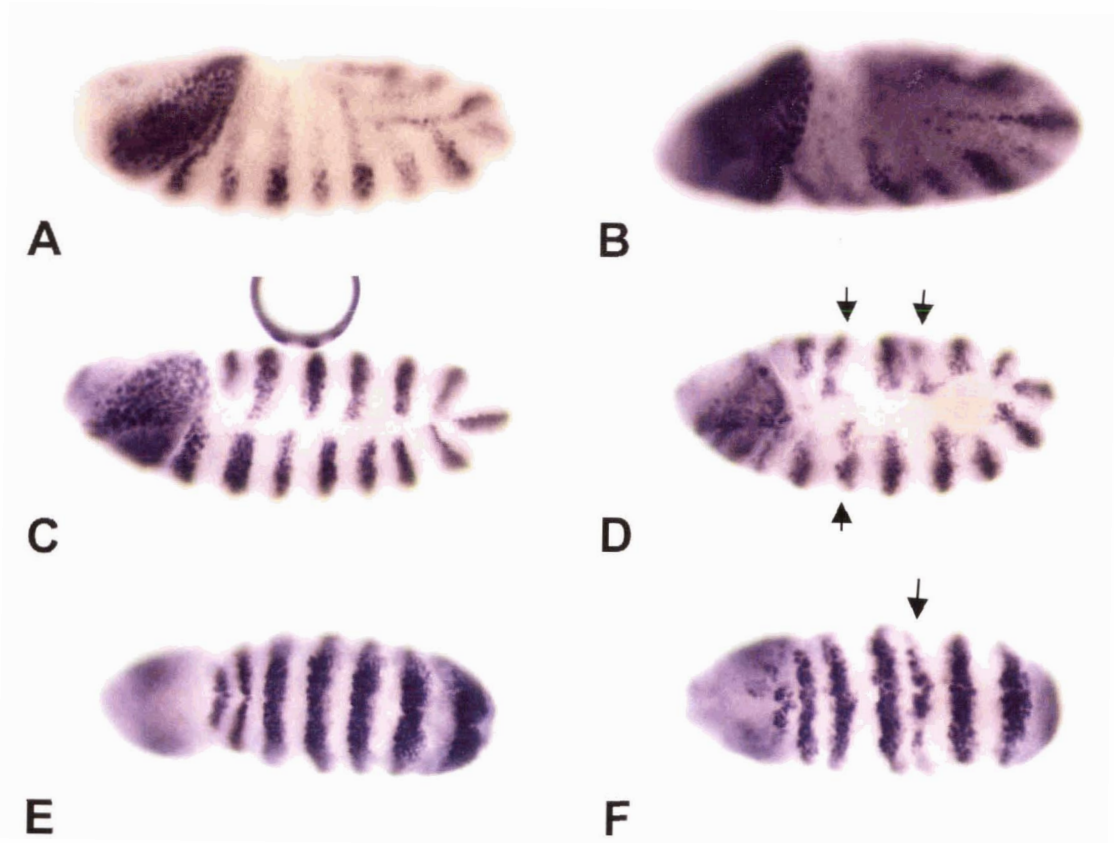


Figure 20: Anti-ß-gal staining of wild type (A,C,E) and *nmo* GLC (B,D,F) embryos, (A-D) lateral view, (E,F) dorsal view. Expression pattern of the slp-lacZ reporter construct mimics expression of En and Wg, being disrupted in alternating segments. Expression appears to be relatively normal in stage 6 embryos (A,B) but by stage 9 (C-F) alternating stripes are disrupted (arrows in D,F).

Unfortunately, due to time constraints, the effects of *nmo* on the expression of other pair-rule genes could not be addressed; this may provide more insight into the functions of *nmo* in pair-rule patterning in the future.

Nemo and SOX

Interestingly, the fly homologue of the NLK binding partner xSOX11, SOX70D (also called *Dichaete* or *fish hook*, henceforth referred to as *fsh*) has been shown to be required for wild-type expression of *eve*, *h*, *run*, *ftz* and Wg (Nambu and Nambu, 1996, Russell et al., 1996). *fsh* mutants also exhibit loss or fusion of denticle belts. I generated several nmo^{adk}, *fsh* recombinant lines. Homozygotes from these recombinants exhibit cuticular phenotypes indistinguishable from *fsh* mutants alone (Figure 21). This is not particularly surprising, as *nmo* is maternally loaded and should be able to compensate for the loss of the zygotic *nmo* gene product.

To address if *fsh* might be required for *nmo* function, I crossed *nmo* GLC mothers to fsh^{87}/*TM3,twi-GFP* males to look for an interaction. Heterozygosity for *fsh* had little effect on the cuticle phenotype exhibited by *nmo* GLCs, except perhaps a subtle shift in which denticle belts were predominantly affected (see Table 2). These results give no indication that *nmo* and *fsh* interact genetically but do not exclude the possibility. If *nmo* and *fsh* were to interact, it seems likely that *nmo* would affect only a subset of *fsh* activities, as *fsh* has been found to be required for the expression of multiple primary pair-rule genes, and this does not appear to be the case for *nmo*. *In vitro* binding studies are currently underway to assess the potential for *nmo-fsh* interactions.

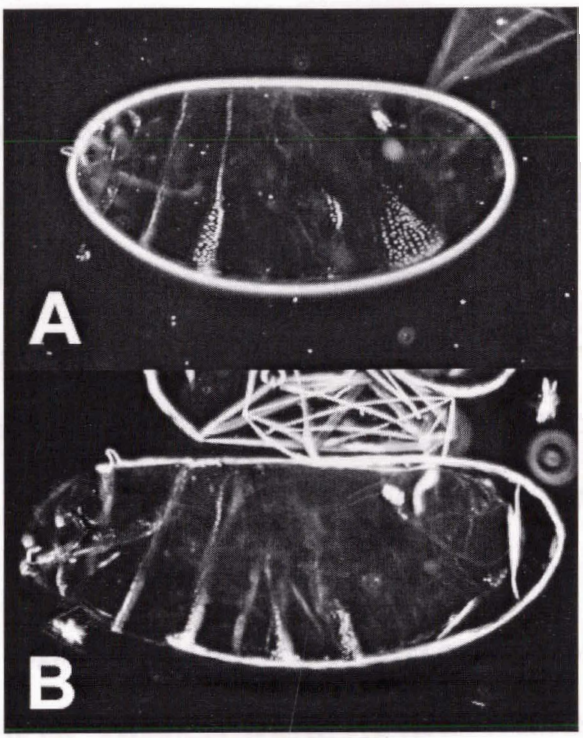


Figure 21: (A) *nmo*^{adk2}, *fsh*^{96} recombinant, (B) *fsh*^{96}. Loss of zygotic *nmo* has no effect on the *fsh96* homozygous cuticle phenotype.

Part 2: Nemo and denticle formation

Patterning of the embryonic cuticle depends on the co-ordination of inputs from several different signaling pathways including Wingless, EGFR, and Hedgehog. Contributions from each of these pathways specify not only the presence or absence of denticles, but the diversity of denticle types found in the cuticle. For example, one of the early functions of wingless is to specify naked cuticle by preventing the expression of the *shavenbaby* (*svb*) transcription factor, while EGFR signaling has the opposite effect, inducing *svb* expression and leading to the formation of denticles (Payre et al., 1999). I wanted to confirm that *nmo* cuticle phenotypes were a direct result of altered expression of the pair-rule or segmentation genes and not due to a secondary role for *nmo* in specifying denticle fate through modulation of cross-talk between wingless and EGFR signaling. I looked at denticle formation in embryos in which *nmo* and/or components of the EGFR pathways were mis-expressed. Ectopic expression of a secreted form of spitz, an EGFR ligand, (*UAS-sSpi*) using the engrailed-Gal4 driver leads to an ectopic row of denticles immediately anterior to the first row of denticles (O'Keefe et al., 1997). In contrast, ectopic expression of either a dominant-negative form of EGFR (UAS-DERDN) or an activated form of Armadillo (UAS-Arm^{Act}) using the same driver leads to the loss of the first denticle row. If Nemo were to play a role in cross-talk between wingless and EGFR in this context (or if Nemo were to potentiate or inhibit signaling of one pathway at the expense of the other) I would expect that ectopic expression of *nemo* using the same driver would result in either loss of the first row of denticles (as when EGFR signaling is compromised), or ectopic denticles anterior to the first row (as when EGFR signaling is enhanced or Wg signaling is compromised). However, ectopic expression of a single

copy of *UAS-nmo* under the control of the *en-Gal4* driver at 25°C gave no phenotype (not shown). In addition, ectopic expression of a single copy of *UAS-nmo* in combination with *UAS-sSpi* was unable to rescue the ectopic row of denticles exhibited with *UAS-sSpi* alone (not shown). Similarly, co-expression of *UAS-nmo* with *UAS-sSpi* in the epidermis using the 69B-Gal4 driver had no effect on the lawn of denticles exhibited by *69B-Gal4>UAS-sSpi* at 29°C (not shown). These results are not surprising, as both *en-Gal4>UAS-nmo* and *69B-Gal4>UAS-nmo* are known to be viable and lead to very mild defects in adult flies.

Part 3: Nemo and CNS development

Due to the enrichment of *nmo* reporter construct in the embryonic CNS and PNS, I decided to investigate a possible role for *nmo* in nervous system development. I stained *nmo* GLCs with antibodies to elav, BP102 and FasII, which stain the nuclei of all neurons, all CNS axons by stage 13, and a specific subset of neurons, respectively. Each of these revealed defects in CNS and PNS development in *nmo* GLCs (Figures 22-24). Specifically, the CNS appears disorganized, and neuromeres, the CNS equivalent of segments, are often fused (arrows in Figure 23D), or fail to form properly (Figures 22 & 24).

However, it is not clear whether these observed defects are bona fide defects in nervous system development, or occur as a result of defects in segmentation. To address this, I double-labeled *nmo* GLCs with the CNS marker BP102 and En. I reasoned that defects in segmentation would be marked by defects in En. Thus, if CNS/PNS defects were restricted to segments in which En was disrupted, the observed nervous system defects would likely reflect *nmo*'s role in segmentation, rather than indicate an

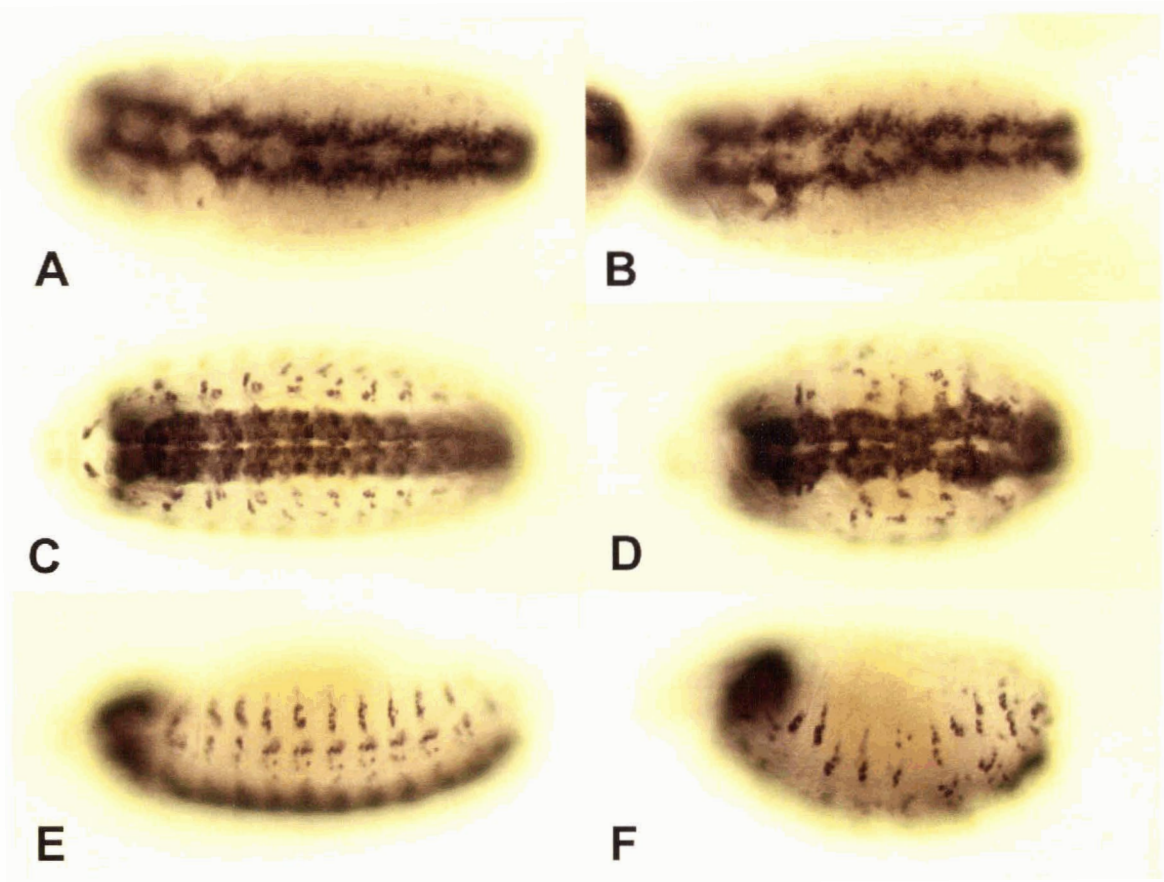


Figure 22: Anti-elav staining in wild-type (A,C,E) and *nmo* GLC (B,D,F) embryos, (A-D) ventral view, (E,F) lateral view. Loss of maternal *nmo* results in disorganization of the CNS and PNS. Staining in stage 9 embryos (A,B) indicates that some neuromeres fail to form properly, while a general disorganization of the CNS (C,D) and PNS (E,F) is evident in stage 13 embryos.

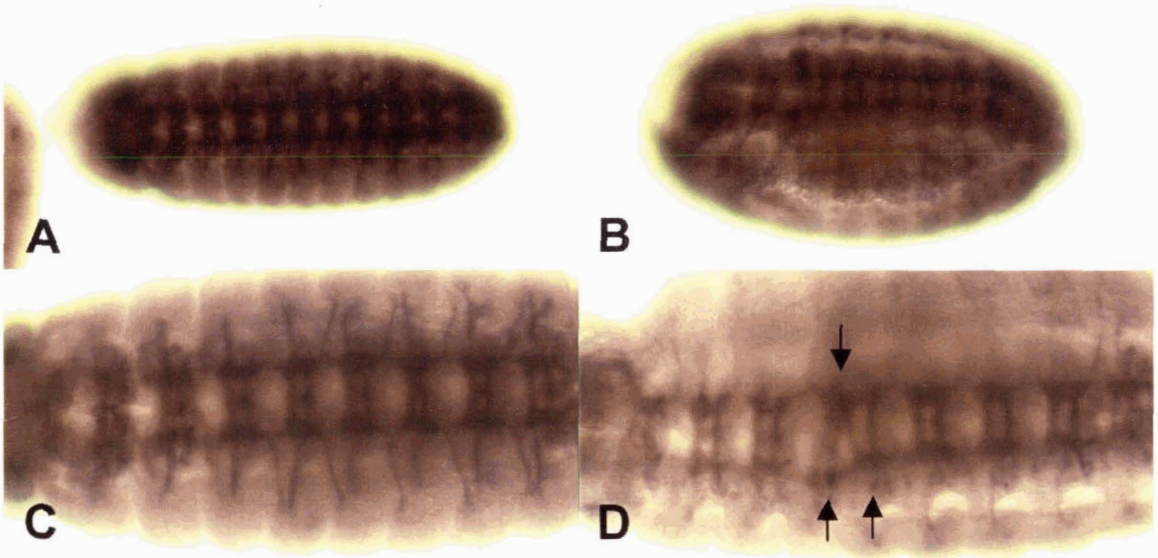


Figure 23: Ventral views of wild-type (A,C) and *nmo*GLC (B,D) embryos stained with anti-BP102 are shown. Loss of maternal *nmo* results in fusion of neuromeres in *nmo* GLC embryos (arrows in D).

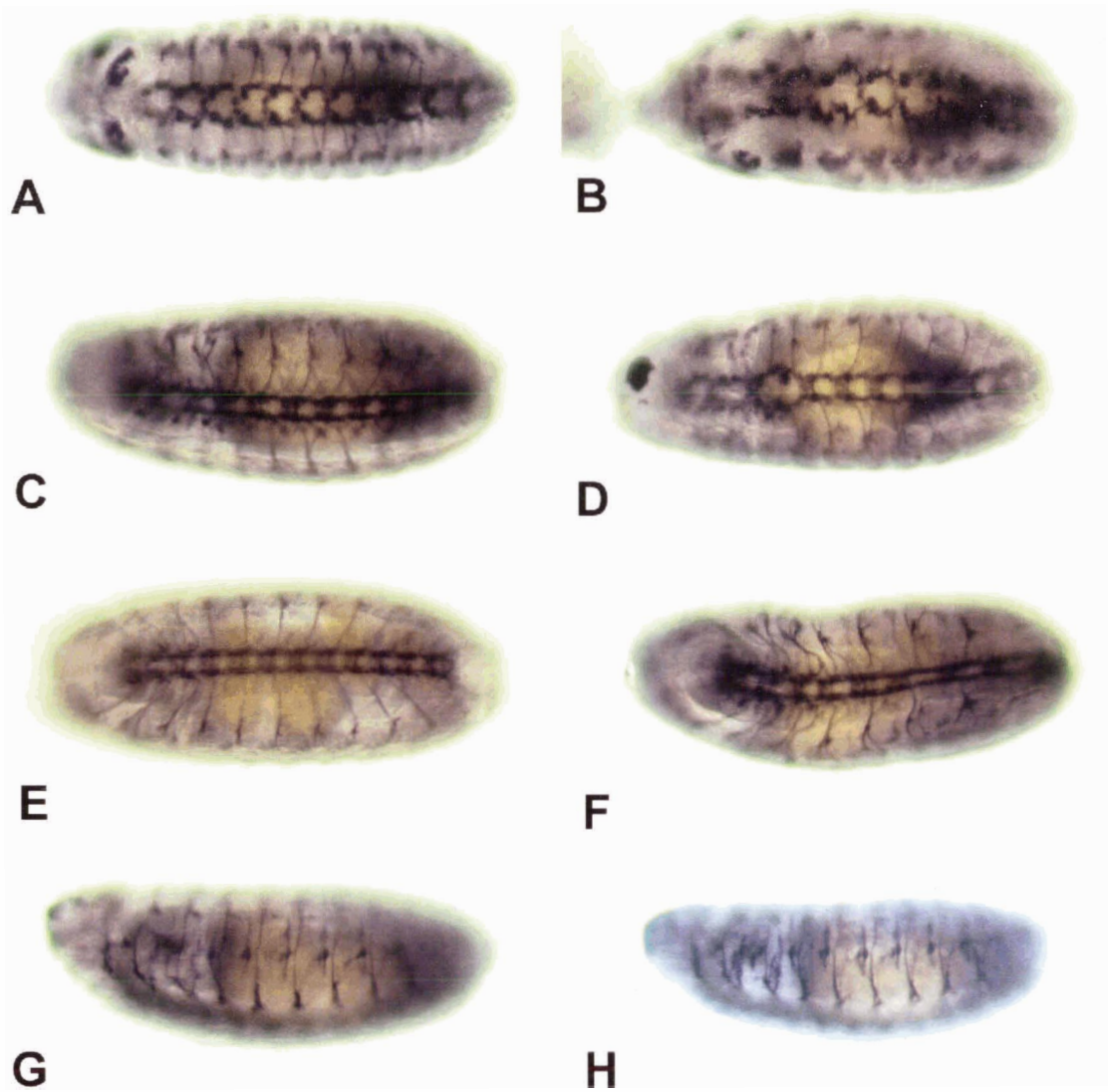


Figure 24: Ventral views (A-F) and lateral views (G,H) of wild-type (A,C,E,G) and *nmo* GLC (B,D,F,H) embryos stained with anti-FasII are shown. Loss of maternal *nmo* results in disorganization of the CNS . Staining in stage 12 embryos (A-D) indicates that some neuromeres fail to form properly, while a general disorganization of the CNS (C,D) and PNS (E,F) is evident in stage 14 embryos.

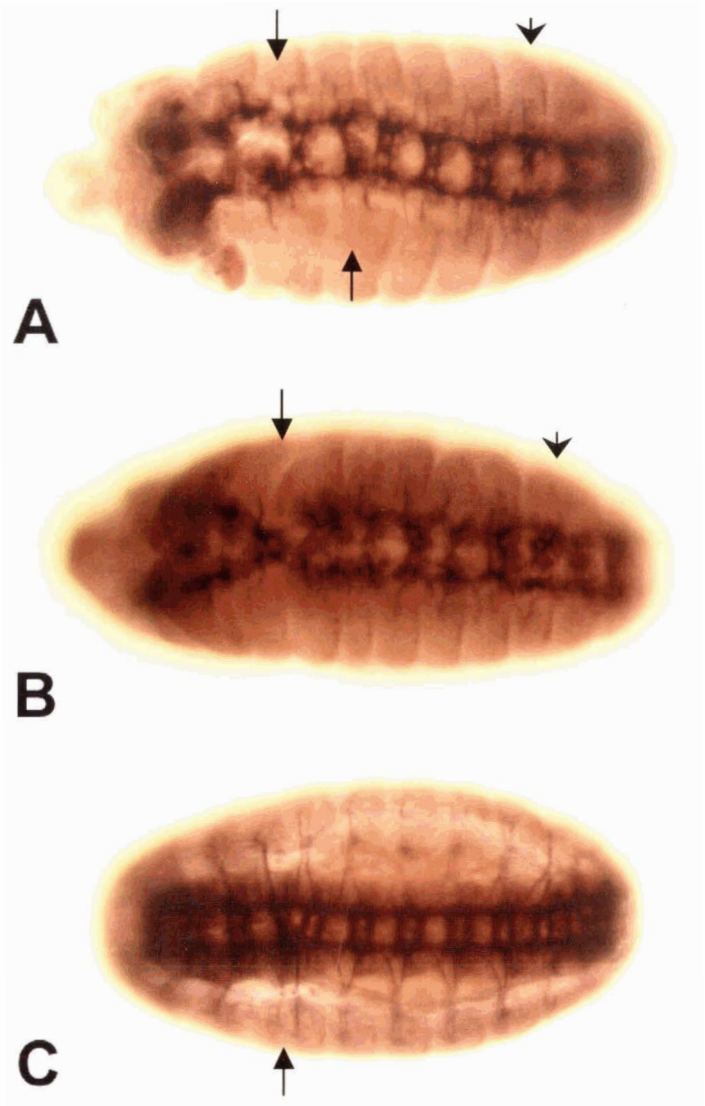


Figure 25: Anti-BP102 (black) and anti-En (brown) staining in stage 13 (A,B) and stage 15 (C) embryos indicates that although most CNS defects occur where segments have fused (arrows in A-C), some defects occur where En patterning and segmentation appears normal (arrowheads in A,B).

involvement in nervous system development. While most observed CNS defects occurred concomitantly with disruptions in En, some CNS defects were observed in neuromeres where En was clearly not disrupted (see Figure 25). This could indicate a role for *nmo* in nervous system development. Unfortunately, due to time constraints, this was not further characterized.

Discussion

It is not clear precisely what role *nmo* plays in segmentation. I believe the cuticular phenotypes I observe in *nmo* GLCs are a direct result of the disruption of even En and odd Wg stripes, particularly because I usually observe either medial or lateral disruption of En and Wg expression, and either medial or lateral fusion of denticle belts. There are several lines of reasoning that would support a role for *nmo* in the maintenance of En and Wg expression, particularly because En and Wg appear to be initiated normally in *nmo* GLCs. One possibility is that *nmo* is required for En function, and through paracrine signaling also its maintenance. Thus in the absence of *nmo*, En would be initiated normally but not maintained. As a result, Wg would decay where En was not maintained, resulting in the loss of Wg-dependent naked cuticle. An interesting consequence of this model is that *nmo* would act to promote wingless signaling (albeit indirectly) rather than inhibiting it as it does in other contexts. Additionally, *en* mutant embryos exhibit cuticle phenotypes reminiscent of *nmo* GLCs, with fusions of the same segments. The significance of increased overall levels of En in *nmo* GLCs is unclear and cannot, for the present, be explained.

It is also unclear why alternating segments are affected. The loss of *ftz* stripes 3 and 6 cannot easily account for this. While disruptions in En expression occur in *ftz*-

dependent stripes in *nmo* GLCs, only the expression of *ftz* stripes 3 and 6 appear to be affected. These stripes normally give rise to abdominal segments A1 and A7, which just overlap the posterior edge of the regions most commonly affected in *nmo* GLCs. However, based on the other cuticle phenotypes I observe, I would expect that additional *ftz* stripes (with the exception of stripe 5, which gives rise to A5 and is not commonly affected) would be affected in many embryos, and this is not observed. It is possible, however, that *ftz* expression is subtly altered such that it was not detected.

There is good evidence to support a role for *nmo* in modulating *eve* activity, as heterozygosity for *eve* in a *nmo* GLC background results in increased embryonic lethality and cuticle phenotypes mimicking *Df(2R)eve* homozygous embryos. However this cannot directly account for the loss of even En stripes or odd Wg stripes – loss of *eve* results in the loss of all En and Wg stripes. It is possible that *nmo* is required for only a subset of *eve* functions, particularly in the posterior of *eve*-dependent parasegments. That *nmo* would affect only a subset of *eve* activities is not unprecedented, as Eve contains repression domains that exhibit both Gro-dependent and Gro-independent functions (Kobayashi et al., 2001). Eve is required for repression of *slp*, *prd*, and *odd*. Loss of the Gro-Eve interaction (either through loss of gene product, or loss of the Gro binding motif in Eve) results in the posterior expansion of both *slp* and *prd* expression in odd parasegments. This results in a posterior-ward shift in odd En stripes and expansion of even Wg stripes. However, loss of Gro has no effect on *odd* expression. Although *slp* expression is abnormal in *nmo* GLCs, it does not appear that expression is expanded posteriorly into the odd parasegments.

One possibility is that *nmo* mediates *gro*-independent repression by *eve*, particularly in the repression of *odd*. Ectopic expression of *odd* leads to a variety of phenotypes (Saulier-Le Dréan et al., 1998). Most notably, ectopic odd induced at 2:40-2:50 AEL leads to the loss of both alternating *slp* stripes, odd *wg* stripes and even *en* stripes, and denticle belt fusions between T1/2, T3/A1, A2/3, A4/5 and A6/7. These phenotypes are somewhat more severe but are otherwise similar to those observed in *nmo* GLCs. In support of this, I notice significantly higher expression of *odd* in *nmo* GLCs. However, it is interesting to note that segments A4/5 are most commonly affected by ectopic *odd*, while this region is the least often affected in *nmo* GLCs.

Another appealing possibility is that *nmo* is required for a subset of *run* function. Early *run* expression overlaps regions deleted in *nmo* GLCs. Loss of *run* results in the complete fusion of T3/A1, A2/3, A4/5 and A6/7, and loss of alternating En stripes (Kaminker et al., 2001). *run* encodes the DNA binding a-subunit of the heterodimeric Core Binding Factor (CBF). Two genes encoding the ß subunits, *brother* (*bro*) and *big brother* (*bgb*) have been identified in flies (Kaminker et al., 2001). Although their functions appear to be largely redundant, DS RNAi for *bro* results in incomplete denticle fusions, reminiscent of *nmo* GLCs. However, because *odd* represses *run* (Saulier-Le Dréan et al., 1998), these similar phenotypes could be the result of decreased *run* due to derepression of *odd* in *nmo* GLCs. While loss of *nmo* was not seen to have an effect on the initial expression of *run*, later expression was not examined, and therefore this possibility cannot be ruled out at the moment.

Conclusions

Previous studies in Drosophila and other organisms have identified roles for *nemo* and *Nlks* in the inhibition of Wingless signaling. Additionally, Drosophila *nmo* has been implicated in the regulation of Notch, EGFR and apoptotic signaling pathways. The results of this study have identified a novel role for *nmo* in embryonic segmentation and nervous system development in Drosophila. Studies are currently underway to determine the molecular mechanism(s) for these roles. It will be interesting to see if these novel roles for *nmo* are conserved in other organisms.

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