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A Role for the Ecdysone Response Gene E93 in Imaginaling Patterning during Metamorphosis

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A ROLE FOR THE ECDYSONE RESPONSE GENE *E93* IN IMAGINAL PATTERNING DURING METAMORPHOSIS

by

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Abstract

Drosophila melanogaster E93 is an early ecdysone response gene that encodes a pipsqueak domain transcription factor. E93 is induced by ecdysone at the end of larval development, and directs the death and elimination of several larval tissues during metamorphosis. Although E93 has been considered a dedicated regulator of larval cell death, I have found that E93 is also widely expressed in imaginal tissues during metamorphosis, where it is required for the proper patterning of many adult structures. Our working hypothesis is that E93 functions in imaginal tissues as a metamorphosis-specific cofactor that determines the pupa-specific action of numerous other transcription factors involved in imaginal patterning. For my thesis work, I focused on a single E93-dependent patterning process, the induction of bracts by bristle cells in the pupal leg. This induction is known to be mediated by EGFR signaling. My studies position E93 downstream of the transcription factor Pointed in the EGFR pathway, and upstream of the bract target gene Distal-less, consistent with the view that E93 functions to control target gene specificity of EGFR signaling during metamorphosis. I also present the results of experiments to dissect functional domains within the E93 protein and to determine whether alternate products of E93 execute the cell death and imaginal patterning functions of E93.

Chapter II-V will present these works in detail and Chapter VI will discuss future directions of this interesting project.

Acknowledgments

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Chapter I : Introduction and background

In this chapter, I first present background information on ecdysone signaling and imaginal patterning during metamorphosis. I then introduce the main character of my research: *E93*, a gene that appears to lie at the intersection of these two processes. I end with our hypothesis that E93 functions as a metamorphosis-specific cofactor for many transcription factors involved in imaginal patterning.

Ecdysone signaling

Ecdysone and the ecdysone receptor

Ecdysone is a steroid hormone that triggers major developmental transitions in *Drosophila* and induces different gene responses in a stage specific and tissue specific manner (Fig. I-1). Ecdysone is synthesized in the prothoracic gland, and released into the haemolymph. The first ecdysone pulse occurs during embryogenesis, and may play some role in cuticle deposition and morphogenesis (Chavez et al. 2000). Ecdysone pulses during the first and second instars occur in combination with a high level of juvenile hormone, and trigger the two larval molts (Dubrovsky et al. 2000; Zhou and Riddiford 2001). As the level of juvenile hormone goes down at the end of the 3rd instar, a sharp increase of ecdysone drives the larva into pupariation and initiates metamorphosis. During metamorphosis, most larval tissues are eliminated, whereas imaginal tissues (future adult tissues) survive and differentiate into appropriate adult structures (Robertson 1936).

The ecdysone receptor is a heterodimer of the ecdysteroid receptor (EcR) and Ultraspiracle (Usp), the *Drosophila* homologue of the retinoid X receptor (Yao et al. 1992; Thomas et al. 1993). Both EcR and Usp are members of the nuclear receptor superfamily (Mangelsdorf et al. 1995). EcR and Usp heterodimers bind ecdysone and the complex then interacts with ecdysone response elements (EcRE) in target genes (Cherbas et al. 1991; Yao et al. 1993). The EcR/Usp/ecdysone complex activates transcription of some targets, but represses others (Tsai et al. 1999; Schubiger et al. 2005). The EcR has three isoforms in Drosophila: EcR-A, EcR-B1, and EcR-B2 (Talbot et al. 1993). These isoforms are expressed with different spatial and temporal patterns, and induce different transcriptional activities (Talbot et al. 1993; Truman et al. 1994; Cherbas et al. 2003). Thus, a variety of cellular responses have already been generated at the top of the ecdysone signaling hierarchy.

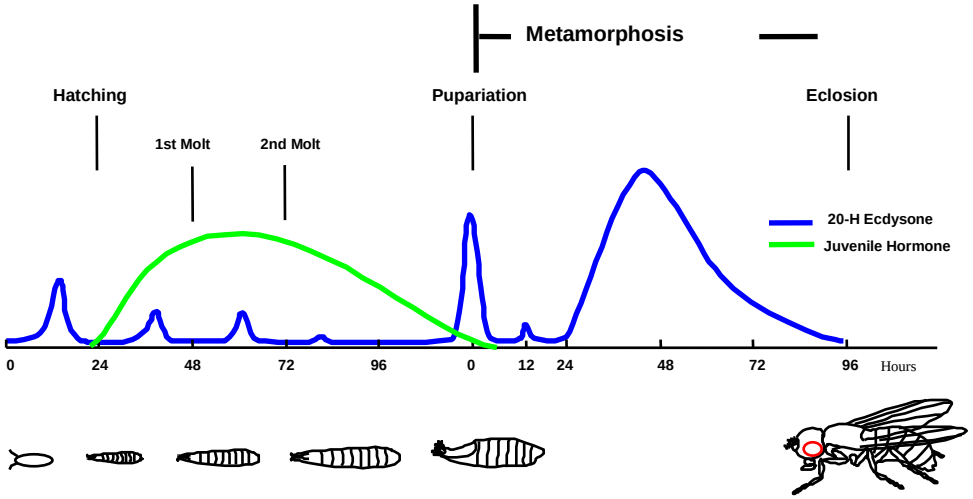


Figure I-1: The hormonal regulation of the life cycle of Drosophila melanogaster

Blue line represents ecdysone. Green line represents juvenile hormone. X axis represents development time in hours. It starts from the embryo and re-zeros at pupariation.

Ecdysone response genes

The next components of the ecdysone signaling hierarchy are ecdysone response genes. At the end of the third instar, the EcR/Usp/ecdysone complex induces a cascade of ecdysone response genes. Many of these genes were identified as puffs in late larval polytene chromosomes (Ashburner et al. 1974; Ashburner and Richards 1976). Among these puff genes, a half dozen are called Early Puff Genes, which include the *Broad-Complex (BR-C), E74, E75, E63-1, E23* and *E93* (Fig. I-2, Ashburner et al. 1974; Ashburner and Richards 1976; Thummel 2002). The protein products of these Early Puff Genes work as transcription factors to induce more than 100 Late Puff Genes and to repress their own expression. The Late Puff Genes are divided into early-late genes (e.g. *DHR3, E78, and E62E*) and late-late genes (e.g. *22C, 63E, 71E, 82F*). The early-late genes require ecdysone/receptor complex for expression, whereas the late-late genes are repressed in the presence of ecdysone/receptor complex and hence are not induced until after the ecdysone pulse ceases. The protein products of the Late Puff Genes function as effectors to regulate specific responses in different tissues. The second pulse of ecdysone around 10hr AP induces the Early Puff Genes and Late Puff Genes again (Ashburner et al. 1974; Ashburner and Richards 1976; Thummel 1996; Thummel 2002).

The best characterized ecdysone response genes are the early genes *BR-C* and *E74*, which have a widespread impact in both larval and imaginal tissues (Kiss et al. 1988; Fletcher et al. 1995). The BR-C is further divided into three complementation groups: *br, rbp* and *2BC* (Belyaeva et al. 1980; Kiss et al. 1988). Each complementation unit encodes specific protein isoforms and its mutant alleles cause specific defects. For example, *br* mutants

fail in imaginal disc eversion, *rbp* mutants fail to undergo salivary gland histolysis, and *2Bc* mutants are defective in imaginal disc fusion (Kiss et al. 1988; Emery et al. 1994).

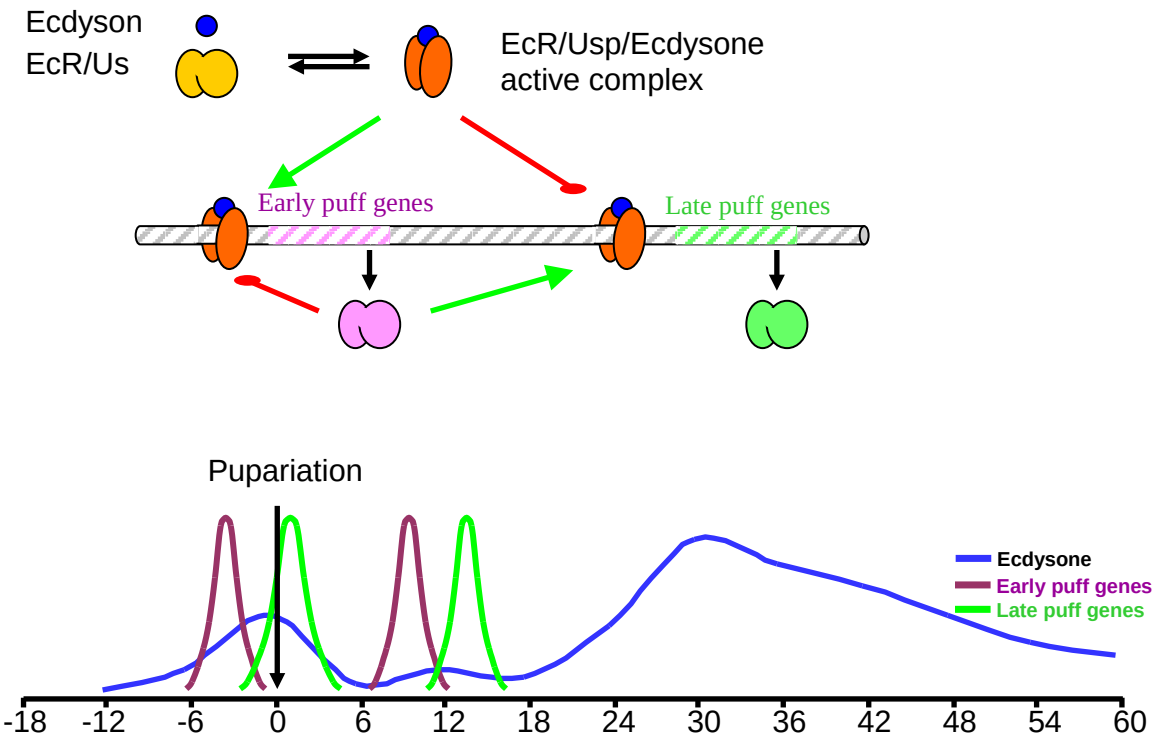


Figure I-2: The Ashburner model for the gene regulation by ecdysone during the metamorphosis

Ecdysone binds to its receptor to form an EcR/Usp/Ecdysone active Complex, which induces Early Puff Genes and inhibits Late Puff Genes. The protein products of Early Puff genes induce Late-Puff genes and inhibit their own expression. The second pulse of ecdysone around 10hr AP induces Early Puff Genes and Late Puff Genes again.

Imaginal patterning

Imaginal patterning and larval remodeling during metamorphosis

Most work on the role of ecdysone signaling during metamorphosis has focused on early events after pupariation, including the death of larval tissues (such as the larval midgut

and salivary gland), and the evagination of the imaginal discs. These events are controlled by the ecdysone peaks that initiate pupariation and that drive the prepupal to pupal molt. Relatively little attention has been devoted to the subsequent strong and very broad ecdysone peak that occurs during mid-pupal development. Many different imaginal patterning events take place at this time, and some of these events have been shown to require ecdysone signaling. However, the ecdysone response genes responsible for executing these effects have not been identified. The work presented in this thesis indicates that the early response gene E93 is an important regulator of many of these pupal patterning processes. Here I review the imaginal patterning processes known to take place in mid-pupal development. These processes are of two types: the development of structures derived from imaginal discs and histoblasts, and the generation of adult structures by the remodeling of pre-existing larval structures.

Patterning of imaginal discs and histoblasts during metamorphosis.

A common misconception is that the patterning of imaginal discs is essentially complete by the end of larval life, and that the role of ecdysone is simply to induce the morphogenesis and final differentiation of adult structures. In fact, cell fate decisions involved in the patterning of imaginal structures continue throughout most of pupal development. For example, in eye development the patterning of 2˚ and 3˚ pigment cells and the programmed cell death of excess lattice cells occurs between 24-48hr After Pupariation (AP) (Freeman 1996; Brachmann and Cagan 2003). In the antenna, chemosensory sensilla are patterned in the mid pupal period (Sen et al. 2003) and outgrowth of the aristal plume and apoptosis of central core cells begins at about 30hr AP

and lasts for 18-20hr (He and Adler 2001). Specification of the maxillary palps takes place entirely after pupariation (Lebreton et al. 2008). In the wing, final patterning of the veins by *decapentaplegic (dpp)* takes place during the pupal stage (for review see deCelis 2003). In the leg, induction of bracts by bristle cells takes place between 8 and 28 hrs after pupariation (del Alamo et al. 2002; Held 2002). Finally, the abdominal cuticle is patterned almost entirely after pupariation, as the imaginal cells that give rise to the abdomen (the abdominal histoblasts) do not proliferate and replace the larval epidermal cells until after this time (Kopp and Duncan 1997; Kopp and Duncan 2002; Lawrence et al. 2002).

Remodeling of pre-existing larval structures during metamorphosis

Several adult structures are produced by remodeling of larval structures during the pupal stage. Such remodeling has been studied extensively in neuronal cells and in the heart. Neuronal remodeling occurs in both the central nervous system (CNS) and in the peripheral system. In the CNS, the early-born γ neurons (these are born between the embryonic and mid 3rd larval stages, and project into the γ lobe) of the mushroom bodies lose their larval axonal projections at 0-18hr AP, and then elaborate new adult-specific axonal projections during the early pupal stage (24-36hr AP) (Lee et al. 1999). Also in the CNS, the embryo-born olfactory Projection Neurons (PNs) (these are born in the embryo, receive input from olfactory receptor neurons in the antennal lobe, and project into the MB calyx and lateral horn) function in both the larval and adult olfactory system. These neurons prune their dendrites and axon branches during the first few hours of metamorphosis and later reinervate the adult mushroom body and lateral horn (Marin et

al. 2005). The pruning process of both early-born (γ) neurons and embryo-born PNs requires cell-antonomous reception of ecdysone signaling through the heterodimeric EcR-B1/Usp ecdysone receptor (Lee et al. 2000; Marin et al. 2005). Another example of neuronal remodeling in the CNS is the Thoracic ventral (Tv) neurons. The Tv neuron dendrites are pruned back in the early pupal stage (5-18hr AP), and then establish adult-specific branches in later pupae (18-48hr AP) (Schubiger et al. 1998; Brown et al. 2006). The pruning of Tv neurons requires the EcR-B1 or EcR-B2 isoforms (Schubiger et al. 1998). The pruning of larval dendrites in Tv neurons seems to result from transcriptional activation by the EcR complex, but the outgrowth of adult dendrites is more complicated. The outgrowth could either be a result of EcR-dependent transcriptional activation or a transcriptional derepression (Schubiger et al. 1998; Brown et al. 2006).

In the peripheral nervous system, some of the dendritic arborization (da) neurons are remodeled during metamorphosis and function in the adult nervous system. For example, the larval dendritic arbor of the dorsal da sensory neuron E (ddaE) is pruned back in the early pupal stage (10-25hr AP), and then regrows to produce the adult arbor at 40-54hr AP (Williams and Truman 2005). The pruning of dendritic and axonal arbors requires the EcR-B and EcR-B2 isoforms, but not the EcR-A isoform of the ecdysone receptor (Schubiger et al. 1998; Lee et al. 2000).

The heart is also remodeled during metamorphosis. The morphology of the heart has been well characterized: in the embryo and larva, the cardiac tube is derived primarily from thoracic segment T3 and abdominal segments A1-A7, with segments T3-A4 forming the

larval posterior aorta, and segments A5-A7 forming the larval heart. Each segment is composed of two Seven Up (Svp) expressing anterior myocytes and four Tinman (Tin) expressing posterior myocytes (Monier et al. 2005). During the remodeling process, segments T3-A4 differentiate as the adult heart, and segment A5 becomes the terminal chamber of the adult heart by transdifferentiation. In this process, A5 loses contractile activity, gains specific innervation, and undergoes changes in cell shape and polarity. Segments A6-A7 are eliminated by apoptosis (Monier et al. 2005).

Remodeling of the larval heart is accomplished at least in part by the ecdysone-dependent transcriptional reprogramming of the Hox genes *Ultrabithorax (Ubx)* and *abdominal-A (abd-A)* (Monier et al. 2005). *Ubx* and *abd-A* are expressed within the larval aorta and larval heart, respectively, and are known to specify the identities of these regions (Lo et al. 2002; Lovato et al. 2002; Ponzielli et al. 2002). During metamorphosis, ecdysone causes *Ubx* to change its expression pattern: *Ubx* becomes repressed in Tin-expressing myocytes and restricted in expression to Svp-expressing myocytes. Ecdysone has a very different effect on *abd-A*: in A5, it causes Abd-A to transition from specifying larval heart to specifying terminal chamber. This transition involves a shift in the target gene specificity of Abd-A (see below) (Monier et al. 2005).

Key transcription factors and signaling systems change their target gene specificity in the transition from larval development to metamorphosis

Several of the metamorphosis-specific patterning events described above are known to involve dramatic changes in the effects of key transcription factors or developmental

signaling systems. A good example occurs during wing vein patterning. During larval life and in the first 8 hr APF, *dpp* is expressed in wing discs in anterior compartment cells located along the compartment boundary. At this time, the dpp morphogen shapes the wing by controlling *spalt*, *optomotor-blind* (*omb)*, and other downstream targets (de Celis et al. 1996; Grimm and Pflugfelder 1996; Nellen et al. 1996). The expression of dpp along the compartment boundary is activated by Hh, which is secreted by posterior compartment cells and diffuses across the compartment boundary. Although *hh* continues to be expressed in the posterior compartment of the wing throughout pupal development, at about 14 hr APF *dpp* becomes insensitive to Hh signaling, and expression of *dpp* along the compartment boundary is lost. At about this same time, *dpp* becomes competent to respond to *EGFR* signaling, which drives *dpp* expression in the wing-vein primordia, where it directs the final differentiation of the veins (reviewed in De Celis 1998; Martin-Blanco et al. 1999; De Celis 2003). Strikingly, expression of rhomboid, which is required for secretion of the EGFR ligand Spitz, is expressed in preveins in third instar larval discs as well as in pupal wings. Thus, although both Hh and EGFR signals are present in both larval and pupal stages, dpp responds only to Hh in the larva, and only to EGFR signaling in the pupa. The factors that drive this transition in the competence of dpp have not been identified. The targets of Dpp signaling must undergo a similar transition during metamorphosis, as Dpp controls genes involved in patterning the wing blade in the larva, and genes involved in elaborating veins in the pupa.

Many other examples indicate that a distinct regulatory environment exists during metamorphosis. In the larval heart, Abd-A activates *Ih* (which encodes a protein for

intracellular cyclic nucleotide activated cation channel activity) and *Ndae1* (Na^+-driven anion exchanger 1), whereas in the pupa during remodeling, Abd-A transitions to repressing Ih and Ndae1 in the same tissue (Perrin et al. 2004; Monier et al. 2005). In abdominal development, *omb* is regulated by Wg in early pupal development, but then loses its competence to respond to Wg and acquires competence to be activated by Hh signaling (Kopp and Duncan 1997; Kopp and Duncan 2002; Lawrence et al. 2002). Maxillary palp development is initiated by expression of Wg in the ventral region of the antennal disc after pupariation (Lebreton et al. 2008). However, when Wg expression is driven here in the larva, antennal development is induced (Lebreton et al. 2008). In the eye, ectopic expression of EGFR signaling has very different effects after pupariation than before (Freeman 1997). In the wing, ectopic EGFR signaling induces vein development early in metamorphosis, but represses it at later times (Martin-Blanco et al. 1999). Additional evidence that a distinct regulatory environment is present during metamorphosis is provided by the identification of many enhancers that are specific for the larval or pupal stages. Examples include pupal-specific antennal enhancers from the *spineless* (Emmons et al. 2007) and *distal antenna related* genes (D. Duncan, unpublished).

A new connection between ecdysone signaling and imaginal patterning during metamorphosis

It seems very likely that the metamorphosis-specific patterning events described above and their associated transitions in target gene specificity are controlled by ecdysone signaling. However, this has been demonstrated to be true only for neuronal remodeling

and remodeling of the heart. For my thesis work, I have shown that the ecdysone response gene E93 is required for many imaginal patterning events during metamorphosis, a finding that establishes a new connection between ecdysone signaling and these events. Below, I review what is known about E93.

Basic knowledge about E93

E93 was identified as an early puff gene induced by ecdysone (Baehrecke and Thummel 1995). E93 shows tissue-specific regulation not seen for other early ecdysone response genes. In the larval midgut, E93 is induced by both the larval-prepupal ecdysone pulse at 0 hr APF and by the prepupal-pupal pulse at about 10 hr APF. However, in salivary glands, E93 is induced only by the second of these pulses (Baehrecke and Thummel 1995). The explanation for this difference is that E93 expression requires bftz-F1, which is not expressed in the salivary gland until mid-prepupa (Lavorgna et al. 1993; Baehrecke and Thummel 1995; Broadus et al. 1999). *BR-C*, *E74* and *E93* trigger salivary gland apoptosis by inducing at least two cell death genes, *rpr* and *hid* (Lee et al. 2000; Thummel 2001). Northern blotting of RNA from whole pupae indicates that E93 is induced until about 48hr AP (Fig. I-3, Fig. V-1, Baehrecke and Thummel 1995).

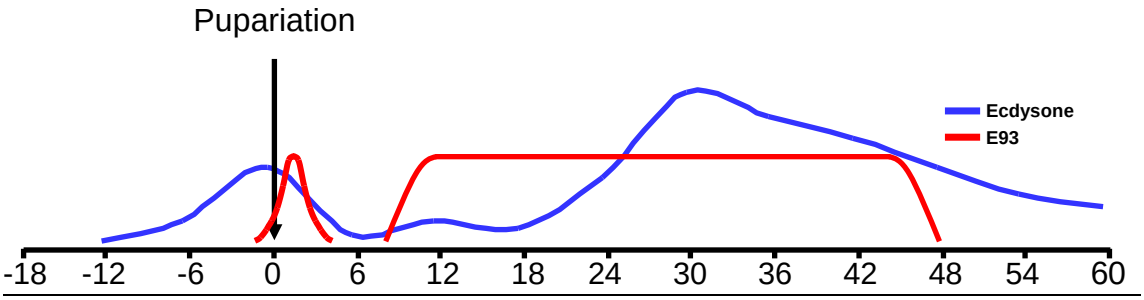


Figure I-3: The stage specificity of the expression of *E93*

E93 is induced by the first and second ecdysone pulses (0hr AP, 10hr AP) in the midgut and fat body. It is induced by the second ecdysone pulse (10hr AP) in the salivary gland. Northern blot from the whole animal indicates its expression is sustained until about 48 hr AP. The blue line represents ecdysone pulses; the red line represents the induction of E93 expression.

In the central nervous system, *E93* is activated at 12hr AP, but this activation seems to be independent of ecdysone in organ culture experiments; in imaginal discs, *E93* is induced mildly at 12hr AP, and input of ecdysone to this induction is unknown; in the fat body and midgut, *E93* responds to both ecdysone pulses at 0hr AP and 10hr AP (Fig. I-4, Baehrecke and Thummel 1995).

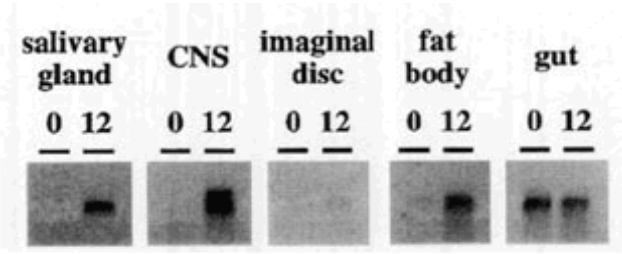


Figure I-4: The tissue specificity of the expression of *E93*

Northern blot of E93 total RNA from salivary glands, CNS, imaginal disc, fat body and gut at 0hr and 12hr AP (Baehrecke and Thummel 1995).

The transcriptional products of E93

In their molecular characterization of *E93*, Baehrecke and Thummel pieced together several partial cDNAs from the locus to infer a transcript of 9.5 kb that covers some 37kb of genomic sequence (the red transcript in Fig. I-5, Baehrecke and Thummel 1995).

Consistent with this transcript size, a transcript of 9.5 kb is detected on Northern blots using a probe from the 3' UTR (Baehrecke and Thummel 1995). The predicted ORF of this transcript is 1165 amino acids long (the red shaded region in Fig. I-5). Recently, an alternate *E93* cDNA of 4.5kb was described on FlyBase (*LP08695*; designated here as *LP0*, the green transcript in Fig. I-6). The *LP0* cDNA has 3 novel 5' exons and a shortened 3' exon, and its predicted ORF (the green shaded region in Fig. I-5) encodes a protein of 1188 amino acids in which the N-terminal 9 amino acids of the E93 ORF are replaced by an alternate sequence of 32 amino acids. In my own cDNA screening, another cDNA of 4.6kb (designated *Clone 2*) was identified (the yellow transcript in Fig. I-5). *Clone 2* has novel 5' exons and a shortened 3' exon, but its predicted ORF (yellow shaded region) is the same as *E93*. Since the 5' end of Baehrecke's *E93* transcript was never determined, it is possible that its 5' end is the same as *Clone 2*.

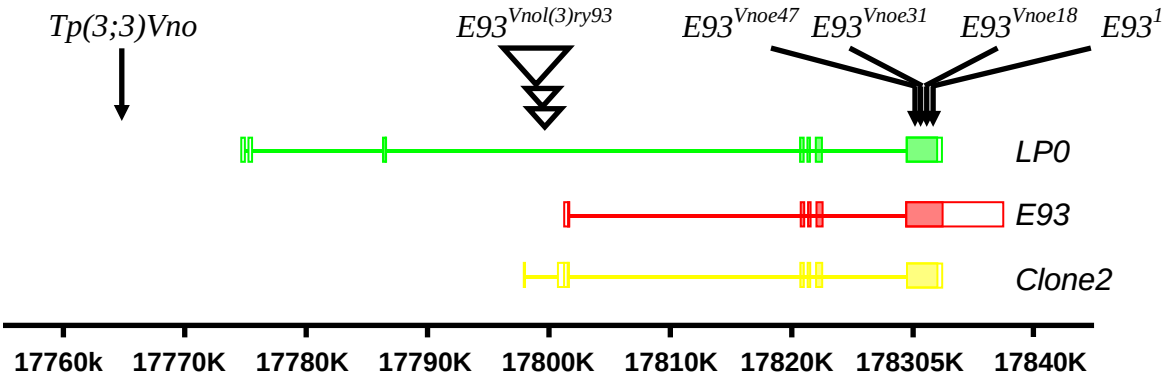


Figure I-5: Molecular map of three alternate transcripts of *E93*

The green transcript represents *LP0*. The red transcript represents *E93*. The yellow transcript represents *Clone2*. Shaded areas represent the predicted ORF. The black arrows indicate the breakpoint of the dominant *Tp(3;3)Vno* mutant and the stop codon positions

of *E93*^{Vnoe31}, *E93*^{Vnoe47}, *E93*^{Vnoe18} and *E93*^{1} mutants respectively. The black triangles are insertions of two Doc elements and a P-element in the *E93*$^{Vnol(3)ry93}$ allele.

The protein homology of E93

E93 is likely a transcription factor, as it contains a pipsqueak (pipsq) domain, which is a DNA-binding domain (Fig. I-6, pink region, Lehmann et al. 1998; Siegmund and Lehmann 2002). A second cryptic pipsq domain, which contains amino acid substitutions within the conserved pipsq domain helix-turn-helix region, is located 385 amino acids upstream of the canonical pipsq domain (Siegmund and Lehmann 2002). Both domains have high homology with honey bee Mushroom body large-type Kenyon cell specific protein-1 (Mblk-1) in two regions, designated as RHF1 (region conserved between honeybee and fruit fly 1) and RHF2 (Takeuchi et al. 2001). RHF2 is also conserved in the mammalian proteins Mblk1-related protein-1(Mlr1) and Mlr2 (Kunieda et al. 2003). Mlr1 is expressed in mouse testis, implying a potential role in spermatogenesis (Kunieda et al. 2003). Mblk-1 is expressed in the honeybee mushroom body, where it may be involved in synaptic plasticity (Takeuchi et al. 2001).

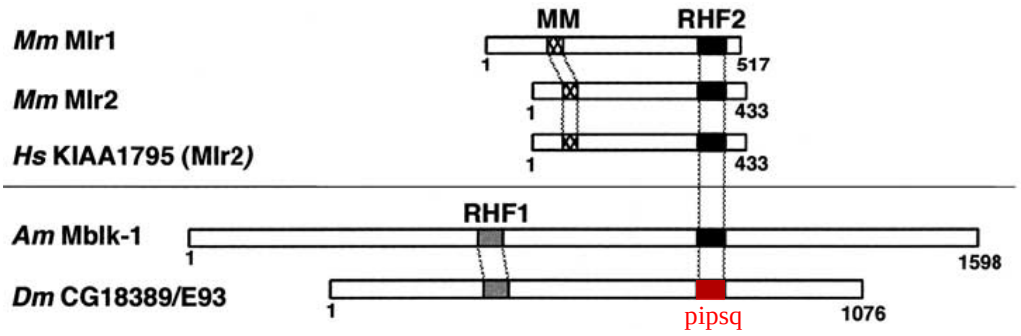


Figure I-6: *E93* homologues from other species

Mm (Mus musculus); Hs (Homo sapiens); Am (Apis mellifera); Dm (Drosophila melanogaster). MM, RHF1 and RHF2 are conserved motifs across species. Red pipsq is the canonical pipsqueak domain in fruit fly (modified from Kunieda et al. 2003).

The phenotype of E93 alleles

Lee et al. (2000) have described three mutant alleles of E93. These are all defective in the death of larval midgut and salivary gland cells during metamorphosis (Fig. 1-7 A'). The $E93^{1}$ allele is associated with a nonsense change at codon 995, near the C terminus of the coding sequence (Fig. I-5). Sequence changes have not been identified for the other two alleles ($E93^{2}$ and $E93^{3}$), which are unaffected in their coding sequences. $E93^{2}$ and $E93^{3}$ mutants show reduced accumulation of 9.5 kb *E93* transcripts, suggesting that these alleles affect regulatory sequences of the gene (Lee et al. 2000). Because of the nature of the E93 product and the phenotypes of these mutant alleles, *E93* has been viewed in the literature as a dedicated regulator of programmed cell death (Lee et al. 2000; Siegmund and Lehmann 2002).

My thesis work grew out of the discovery of three new alleles of *E93* that have little effect on larval cell death, but instead cause numerous defects in pattern formation during

the pupal stage. These alleles were isolated by Eric Baehrecke in a screen for EMS-induced lethals that fail to complement a deficiency that includes the *E93* gene. This was the same screen that led to the recovery of the $E93^{1}$, $E93^{2}$, and $E93^{3}$ alleles. When Baehrecke sorted the lethals he recovered into complementation groups, the three new alleles affecting imaginal patterning were placed into a separate complementation group, as they fully complement the $E93^{1}$, $E93^{2}$, and $E93^{3}$ alleles. Baehrecke went on to show that the three mutations fail to complement the dominant wing-vein mutation *Tp(3;3)Vein-off (Tp(3;3)Vno)*, and so he designated them as *Vno* alleles (Vno^{e18}, Vno^{e31}, and Vno^{e47}). An additional allele [$Vno^{l(3)ry93}$] was identified in the Bloomington P-element insertion collection. Because our laboratory was interested in the effects of the *Tp(3;3)Vno* mutation on the development of the abdomen, Dr. Baehrecke sent his *Vno* alleles to us for analysis. Our lab mapped the P-element insertion in $Vno^{l(3)ry93}$ to be 1.5kb upstream of the 5' end of the *E93* transcripts (see Fig. I-5) and then identified the Vno^{e47}, Vno^{e31} and Vno^{e18} mutants as nonsense mutations in codons 360, 545, and 783, respectively of the 1165 codon *E93* sequence (Fig. I-5). We also showed that *Tp(3;3)Vno* has a breakpoint located 36-37kb upstream of *E93* (Fig. 1-5). In light of these findings, the *Vno* mutations were renamed $E93^{Vnoe18}$, $E93^{Vnoe31}$, $E93^{Vnoe47}$, and $E93^{Vnol(3)ry93}$.

$E93^{Vno}$ alleles fully complement the $E93^{1}$, $E93^{2}$, and $E93^{3}$ alleles, and hemizygotes show almost normal midgut and salivary gland cell death after pupariation. However, $E93^{Vno}$ mutants do show numerous defects in imaginal patterning. Wing veins are completely absent, the abdominal tergites are only partially formed, most chemosensory sensilla are absent in the antenna and palp, the arista fails to branch, 2° and 3° pigment cells fail to be

specified properly in the eye, and bracts fail to form in the leg (Fig I-7). Strikingly, all of these defects are in structures that are patterned during metamorphosis. Our lab has failed to find any defects in $E93^{Vno}$ mutants prior to pupariation; even hemizygous mutant larvae from homozygous mutant germ line mothers develop normally until this time. This stage-specificity suggests that E93 encodes a metamorphosis-specific cofactor required in many imaginal patterning processes. My working hypothesis is that E93 directs many of the pupal-specific target gene transitions described above. My studies help establish a connection between ecdysone signaling and imaginal patterning during metamorphosis and are likely to provide important new insights into the temporal control of target gene specificity for key signaling systems used in development. Why the $E93^{Vno}$ alleles complement the $E93^{1}$, $E93^{2}$, and $E93^{3}$ alleles has been difficult to understand, and is addressed in Chapter IV of my thesis.

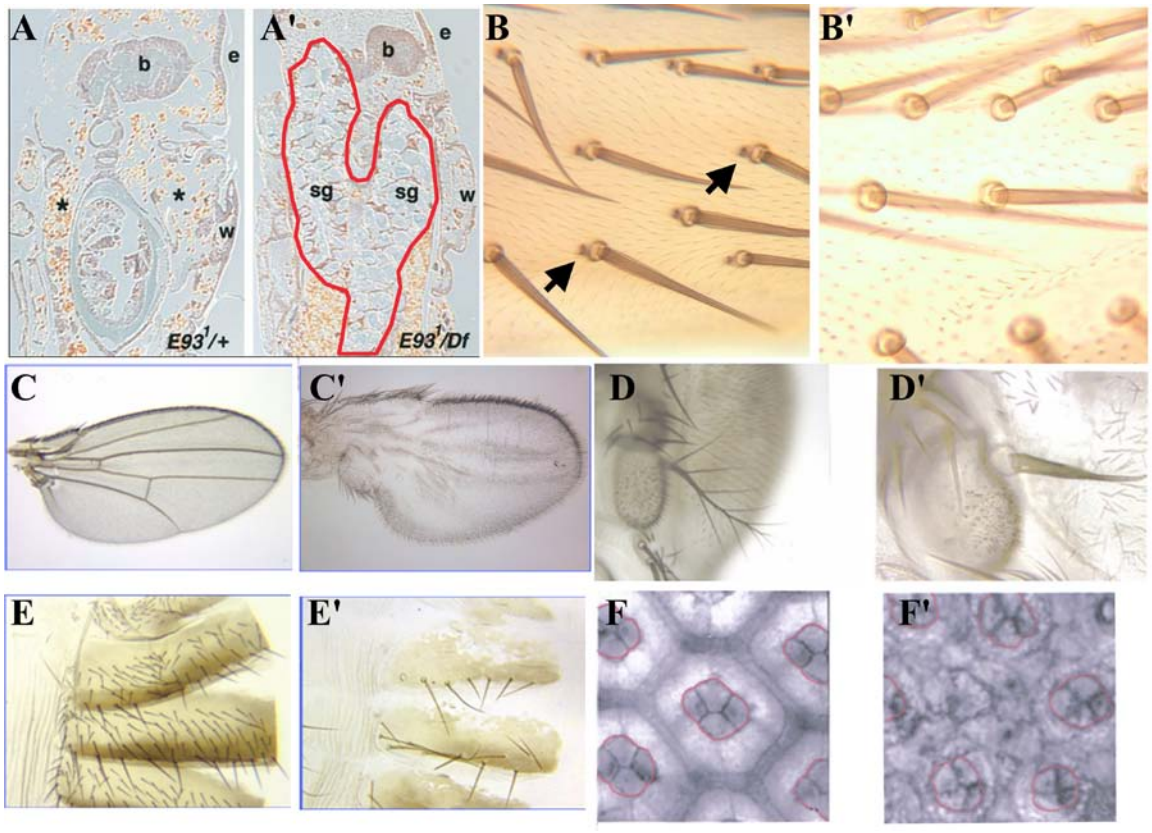


Figure I-7: The phenotype of *E93*^{1} alleles and *E93*^{Vno} alleles

A: Wild-type *(E93*^{1}*/+)* pupa at 24hr AP; salivary glands have been eliminated by this time (asterisk); but other tissues formed normally: adult eyes (e), brain (b), and adult wings (w). A': *E93*^{1}*/Df* pupa at 24hr AP; redline circle the persistent salivary glands (sg) (Lee et al. 2000). B-F are wild type; B'-F' are *E93*^{Vno} mutants. B and B' are legs (bracts are marked by arrows). C and C' are wings. D and D' are abdomen. E and E' are antenna. F and F' are eyes. The red lines outline the shape of cone cells in ommatidia.

Summary of my work

Through my four and half years work in Dr. Ian Duncan's lab, I have made progress in the following areas (detailed information presented in Chapter II-V separately).

1) Determination of the protein expression profile of E93.
2) Determination of the position of *E93* in the signaling pathway leading to the formation of bracts.
3) Dissection of E93 protein domains required for its cell death and patterning functions.
4) Identification of *E93* transcripts responsible for the cell death and patterning functions of *E93*.

Chapter II : Determination of the protein expression profile of E93

Abstract

I designed antigens from three regions of the E93 coding sequence, produced these proteins in *E. coli*, and used them to immunize rabbits and mice either as single or mixed E93 antigens. The E93 antisera obtained allowed the immunostaining of E93 in pupal tissues. E93 is detected in the antenna, arista, eye, labellum, wing and leg in the pupal period, consistent with the multiple imaginal defects seen in $E93^{Vno}$ mutants. The $E93^{Vnoe47}$ mutant shows dramatically reduced staining, demonstrating specificity of the antisera.

Rationale

Although expression of E93 has been characterized early in metamorphosis in the larval midgut and salivary gland (Lee et al. 2000), later expression in imaginal tissues has not been examined. An E93 antiserum (against codon 279-837) was generated by Dr. Eric Baehrecke (Lee et al. 2000). However, this antiserum did not work well for Western blotting or tissue staining (personal communication), and is no longer available. In order to gain a more complete picture of the expression of E93 during metamorphosis, it became necessary to generate a new E93 antiserum.

Methods

DNASTAR Lasergene 7 software was used to predict three regions with high antigenicity (Antigen I, II, and III, which include codons 47-126, 285-400, 999-1125 respectively (Fig.

IV-1); detailed sequence information can be found in the Appendix). These three regions were amplified and cloned into the pET28a vector by EcoRI and HindIII double digestion. Expression was induced in BL21 (DE3) cells, and proteins purified by His-Bind nickel chromatography, followed by dialysis in PBS overnight. Preimmune serum was collected from animals prior to immunization, and stored in 50% glycerol at -70˚C. Mixtures of the 3 antigens were injected into 2 rabbits (Rabbit A and Rabbit B) and 3 mice (Mice 1-3). Individual antigens (antigens I-III) were injected into 3 mice separately (Mice 4-6). Rabbits were immunized 6 times with 110ug of protein at 1 month intervals. Mice were immunized 6 times with 10ug of protein at 1 month intervals. All immunization and bleeding procedures followed standard protocols. Sera were stored in 50% glycerol at -70˚C. Rabbit B generated strong immune response in the 1^{st} bleeding, and then the strength of the immune response went down (tested bleedings include preimmune, 1^{st}, 2^{nd}, 3^{rd} and 6^{th} bleeding). Rabbit A didn't generate good immune response in the 1^{st} bleeding, and the strength of the immune response didn't get better until the 6^{th} bleeding (tested bleedings include preimmune, 1^{st}, 2^{nd}, 3^{rd}, and 6^{th}). And even the strongest response from Rabbit A (6^{th} bleeding Rabbit A) is weaker that the weakest bleeding from Rabbit B (1^{st} bleeding from Rabbit B). All mice sera, except Mouse 4, generated good immune response, and the strength of the immune response got better and better in each bleeding (tested bleedings include Mouse 1 preimmune, 1^{st}, 2^{nd}, Mouse 2 preimmune, 2^{nd}, Mice 3-6 preimmune, 1^{st}, 2^{nd}, 3^{rd}). More details of the bleeding tests are available in the Appendix. The specificity and authenticity of tests of E93 antisera are shown in the Result section.

Antibody staining was by standard methods. Embryos were dechorionated in 50% freshly diluted Chlorox for 3 minutes, and fixed in 8ml PMG buffer (0.1M PIPES, 2 mM EGTA, 1 mM MgSO4, pH 6.9), 10ml heptane, and 2ml 37% formaldehyde for 20 minutes, and devitellinized in 100% MeOH. Larvae and pupae were staged and fixed in 4% paraformaldehyde. For 1˚ antibodies, chicken anti β -gal was used at a dilution of 1:200 and all others (rabbit anti E93, mouse anti E93, mouse anti Distal-less (Dll), rabbit anti Pointed.P1) were used at 1:400. 2˚ antibodies (anti chick, anti rabbit, or anti mouse) were all used at 1:200.

Images were taken using a Leica TCS SP 2 confocal system, or a Zeiss Photomicroscope III.

Results

The specificity and authenticity of E93 antisera

Previous experiments indicated that E93 protein is expressed in midgut cells beginning at around 2hr AP and disappearing by about 13hr AP, whereas in the salivary gland, E93 protein is not detected until around 10 hrs AP (Lee et al. 2000). With one exception, all rabbit and mouse antisera against E93 gave the expected result for staining of the midgut: positive nuclear staining was seen at 2hr AP (Fig. II-1 A), whereas staining was lost by 13hr AP (data not shown). The antiserum from mouse 4, which was immunized with antigen I alone, did not show positive staining in midgut. Staining of salivary glands was not as expected: antiserum from mouse 1 showed weak staining of salivary nuclei in both

13hr (Fig. IV-5 E) and 2hr AP pupae, but not in larvae. Mouse 2 showed weak staining of salivary nuclei in 2hr AP pupae, but not in larvae. Mice 3, 5 and 6 showed weak staining of salivary nuclei in both 2hr AP pupae and larvae. Rabbits A and B showed weak staining of salivary nuclei in larvae, but not in 2hr pupae. The reason for these complicated staining results for salivary glands has not been explored further.

Two lines of evidence demonstrate the specificity of the mouse and rabbit antisera generated. First, *E93*^{Vnoe47} hemizygotes show a dramatic reduction in staining in both imaginal (bract - see below) and midgut cells (Fig. II-1 C, D). Second, both mouse and rabbit antisera detect full length E93 protein expressed in en-Gal4; UAS-E93 embryos (Fig. II-1 B). The rabbit antisera also show some staining in the anterior gut of the embryo. This staining is likely due to cross reaction, as it is not reduced in the *E93*^{Vnoe47} mutant, and is not seen with the mouse antisera. Antisera from mice injected with single antigens show the expected staining specificity when used to stain embryos in which en-Gal4 is used to drive expression of fragments of E93 (UAS-E93^{1}Nterm and UAS-E93^{Vnoe47}Cterm, see their molecular map in Fig. IV-1) (Fig. II-1 E-L). Rabbit antisera were used for most of my experiments, as they are abundant. Mouse antibodies were used only when required for double or triple antibody staining.

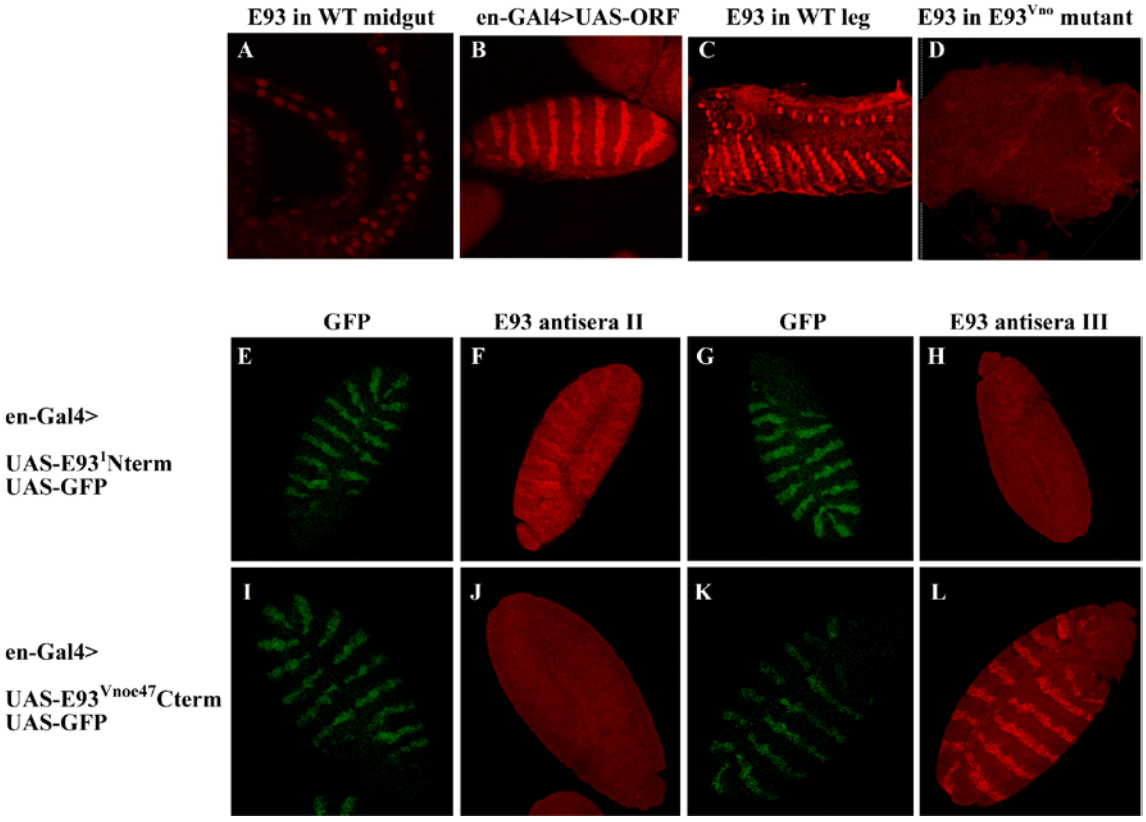


Figure II-1: E93 antigen authenticity tests

A: Wild-type 2hr midgut stained by E93 antiserum from Mouse 3; B: Embryo carrying en-Gal4>UAS-ORF-D1 stained by E93 antiserum from Rabbit B; C: Wild-type pupal leg at about 35hr AP stained by E93 antiserum from Rabbit B; D: *E93*^{Vno} mutant pupal leg stained by E93 antiserum from Rabbit B; E-H are embryos carrying en-Gal4>UAS-E93^{1}Nterm and UAS-GFP; I-L are embryos carrying en-Gal4>UAS-E93^{Vnoe47}Cterm and UAS-GFP; E, G, I and K are GFP positive controls to show that en-Gal4 drives UAS constructs in the expected pattern; F and J are stained by E93 antiserum II (Mouse 5); H and L are stained by E93 antiserum III (Mouse 6); F and L suggest antisera from Mouse 5 and Mouse 6 are specific to antigens II and III, respectively

The expression profile of E93 in WT

When I stained wild-type pupae between 20-60hr AP, I detected E93 in multiple tissues. In general, E93 is expressed strongly between 30-35hrAP, and then gradually declines. At 30-35hr AP, E93 is expressed in every cell of antennal segments II and III (Fig. II-2 A), throughout the wing (Fig. II-2 B, E), and in future pseudotracheal cells (pseudotracheae are hollow tubes on the surface of the labellum used for feeding) in the labellum (Fig. II-2 D). In the eye, E93 is expressed in a complex pattern in each ommatidium (Fig. II-2 C, F), and in the leg, E93 is expressed in single cells located just proximally to each bristle (Fig. II-2 G, H). In chapter III, these cells are identified as producing the leg bracts. The expression of E93 detected by immunostaining corresponds well to the structures that are affected in the $E93^{Vno}$ mutants. As described above, E93 is also expressed in the nuclei of midgut caeca at 2hr AP (Fig. II-1 A), but disappears quickly and is not detected at 13hr AP (data not shown). E93 expression in the salivary gland is weak and variably detected by the different antisera.

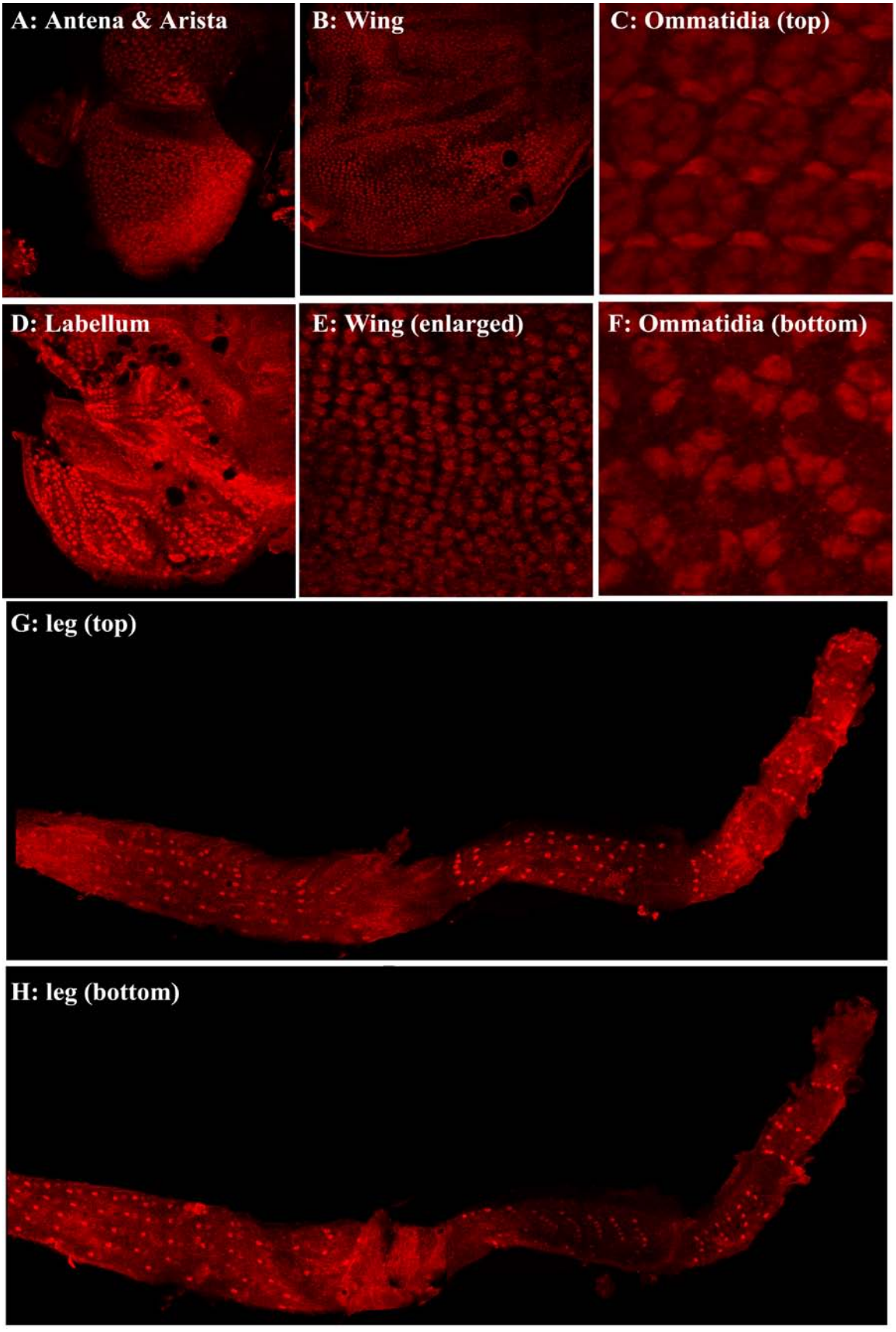

A: Antena & Arista
B: Wing
C: Ommatidia (top)
D: Labellum
E: Wing (enlarged)
F: Ommatidia (bottom)
G: leg (top)
H: leg (bottom)

Figure II-2: The expression profile of E93 in multiple tissues during metamorphosis

Wild-type tissues at 30-35hr AP stained by E93 antiserum from Rabbit B. A: Antenna and arista; B: Wing; C: Ommatidia (top scanning); D: Labellum; E: Wing (enlarged scanning of B); F: Ommatidia (bottom scanning); G: Leg (top scanning); H: Leg (bottom scanning).

E93 *expression in E93 mutant hemizygotes*

Both the $E93^{Vno}$ alleles (including $E93^{Vnoe47}$, $E93^{Vnoe31}$, and $E93^{Vnoe18}$) and the alleles affecting larval cell death ($E93^{1}$, $E93^{2}$, and $E93^{3}$) cause a strong reduction in E93 staining in 1-5hr AP midgut cells in hemizygotes (see Figure IV-3). Hemizygotes for the $E93^{Vno}$ alleles also show strongly reduced E93 staining in prebract cells in 30-50 hr AP legs (Fig IV-3). Hemizygotes for $E93^{1}$, $E93^{2}$, or $E93^{3}$ die early in pupal development, precluding examination of expression in bract cells in these mutants.

Discussion

The detection of E93 in the antenna, arista, ommatidia, wing and leg is consistent with the observation that $E93^{Vno}$ mutants cause patterning defects in these tissues. The finding of E93 expression in the pseudotracheal cells of the labellum was a surprise, and suggests the involvement of E93 in the development of these structures although we haven't checked whether $E93^{Vno}$ mutants affect their development. Based on our hypothesis that E93 functions as a metamorphosis-specific cofactor, our expectation was that E93 would prove to be expressed very broadly in imaginal tissues in the pupal stage. This expectation was borne out in the wing and antenna, where E93 is expressed in all or

almost all cells. However, in the eye and leg, E93 shows highly restricted expression patterns. The identities of E93-expressing cells in the eye have not been determined, but in the leg, expressing cells have been identified as future bract cells (see Chapter III). As described above, bracts fail to develop in the $E93^{Vno}$ mutants. The picture that emerges for E93 is similar to that seen for the Hox genes. Initial descriptions of the expression patterns for Hox genes were a great surprise, as genetic studies had suggested these genes would prove to be expressed uniformly within each developmental compartment. Such uniformity was often found, but in some cases expression was limited to only specific structures within compartments. One example from the recent literature is the finding that the Hox gene Scr, which controls first thoracic segment identity, is strongly upregulated in the sex comb region of the male first leg (Randsholt and Santamaria 2008). A likely possibility is that in the ancestral state Scr was expressed uniformly in the male T1 leg, where it came to specify sex comb development in the male among other T1-specific structures. Selection for increased development of the sex comb then led to selection for increased expression of its positive regulators, including Scr. In response, Scr became incorporated into the patterning system that positions the sex comb, leading to high level expression within this specific structure. If our working hypothesis (that E93 functions as a metamorphosis-specific cofactor) is correct, a similar series of events could have occurred in the evolution of high level E93 expression in bract cells.

The $E93^{1}$, $E93^{2}$, and $E93^{3}$ mutants all cause strong reductions in E93 staining in the 2 hr AP midgut, consistent with the failure in larval cell death seen in these mutants. The $E93^{Vno}$ mutants also cause a strong reduction of E93 staining in midgut cells, as well as in

the pre-bract cells of the pupal leg. Why, then, do the $E93^{Vno}$ alleles almost fully complement the $E93^{1}$, $E93^{2}$, and $E93^{3}$ alleles? One possibility is that the cell death function of *E93* is executed by an alternate product of the locus, a possibility that is explored further in Chapter V. The problem of the complementation of the $E93^{Vno}$ alleles and the alleles affecting larval cell death is addressed more fully in Chapter IV.

Chapter III : Determination of the position of E93 in the signaling pathway leading to the formation of bracts

Abstract

In order to understand the role of *E93* in imaginal patterning, I focused on a single *E93*-dependent process, the induction of bracts in the legs. Bracts are small pigmented outgrowths that lie just proximal to most bristles in the legs. They are patterned in the mid pupal stage by a relatively simple process: bristle or bristle-associated cells secrete the EGFR ligand Spitz, which induces bract formation in an adjacent epidermal cell (del Alamo et al. 2002; Held 2002). In mosaic studies, I demonstrated that *E93* is required in the epidermal (signal receiving) cell for bract formation. I showed that *argos*, a common target of EGFR signaling, is induced in the pre-bract cell in normal development. This induction is unaffected in $E93^{Vno}$ mutants, indicating that *E93* lies downstream of the EGFR signaling pathway in bract development. We were surprised to find that E93 is expressed in a very restricted pattern in the pupal leg: expression is restricted to single cells lying just proximal to each bristle. Double labeling experiments show that these E93-expressing cells coincide with Distal-less (Dll) expressing cells, which are fated to produce bracts. I show that expression of Dll in these cells requires $E93^{+}$, and that expression of E93 in turn requires Pointed (Pnt), a transcription factor that lies at the end of the EGFR signal transduction pathway. Ectopic expression experiments show that E93 is permissive for bract formation, whereas Pnt is instructive. These findings suggest that E93 and Pnt work in concert to specify bract development, with E93 providing temporal information and Pnt providing spatial information. Still unresolved is the role

of ecdysone signaling in the bract pathway: clones expressing an RNAi construct directed against a common region of the ecdysone receptor show some loss of bracts, indicating a requirement for ecdysone signaling. However, it is not yet clear whether ecdysone is required for E93 expression in bract cells.

Rationale

The major goal of my dissertation is to determine the role of *E93* in patterning during metamorphosis. Why should *E93* be required for patterning in the pupa, but not at other times? Our hypothesis is that *E93* is a metamorphosis-specific cofactor that confers pupal specificity to the targets controlled by a number of transcription factors. Such a role would help account for numerous cases in which signaling pathways or transcription factors exert metamorphosis-specific effects (see Introduction).

To understand the role of *E93*, we focused on a single *E93*-dependent process. We decided to focus on bract formation in the leg because of its simplicity and the availability of a potential downstream target gene *Dll*. Bracts are pigmented outgrowths of unknown function that are located just proximal to each mechanosensory bristle (Fig. I-3 B arrows). They are completely absent in the $E93^{Vno}$ mutants (Fig. I-3 B'). Previous studies have shown that bristle cells, or cells associated with bristle cells, secrete the EGFR ligand Spitz, which induces a neighboring epidermal cell to become a bract through the *EGFR* signaling pathway (del Alamo et al. 2002). One potential target of EGFR signaling in these cells is the homeobox gene *Dll*, which is expressed in pre-bract cells beginning in mid pupal development, and is required for bract formation (Campbell

and Tomlinson 1998; Emmons et al. 2007). Since the *EGFR* signaling pathway activates numerous downstream targets in different contexts, we hypothesized that E93 might serve as a metamorphosis-specific cofactor that controls the specificity of EGFR signaling in the pupal leg, targeting the *Dll* gene for activation (Fig. III-1).

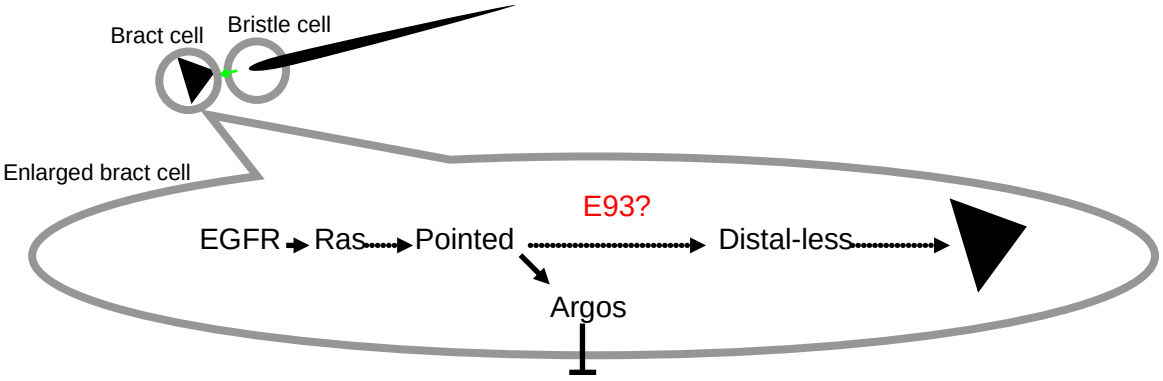


Figure III-1: Genes in the EGFR signaling pathway that may play potential roles in bract formation.

The green arrow represents the EGFR ligand sent out from the bristle cell. Dotted lines indicate that other genes are omitted from the pathway.

Methods

E93^{Vno} clones were generated by crossing *y w FLP122; FRT82B CD2 y*$^{+}$ *M(3)W Bsb / TM6B* ♀ to *FRT82B sr e*^{s} *E93*^{Vnoe47} *ca/TM6B* ♂, and then heat shocking larvae at 37˚C for 30 minutes. Adult legs were mounted in a mixture of 38% Shandon Immu-Mount, 53% filtered saturated chloral hydrate, and 10% filtered lactic acid, and placed on a slide warmer for 2 days at 45˚C. *E93*^{Vno} clones were identified by scoring the *yellow* marker in the cuticle of non-Tubby ♂ flies using a Zeiss Photomicroscope III.

For cuticular analysis, *pnt*$^{-}$ clones were generated by crossing *y w FLP122; FRT82B CD2 y*$^{+}$ *M(3)W Bsb / TM6B* ♀ to *FRT82 roe p*^{p} *cu sr pnt*$^{\Delta 88}$*/TM6B* ♂. Heat shock and preparation of legs was as described above. *pnt*$^{\Delta 88}$ is a null deletion allele of *pnt* (Scholz et al. 1993). For analysis by confocal microscopy, *pnt*$^{-}$ clones were generated by crossing *y w FLP122; FRT82 Ubi-GFP83/TM6B* ♀ with *FRT82 roe p*^{p} *cu sr pnt*$^{\Delta 88}$*/TM6B* ♂, followed by heat shocking larvae at 37°C for 30 minutes. *pnt*$^{-}$ clones were identified as GFP minus clones in non-Tubby pupae.

EcR-RNAi knock down clones were generated by crossing *y w FLP12; Act>y*$^{+}$*>Gal4 UAS-GFP* ♀ with *UAS-EcR-RNAi* ♂. *EcR-RNAi* knock down clones were identified by confocal microscopy as GFP positive clones in pupae, or as *yellow* clones in the cuticle of ♂ flies using a Zeiss Photomicroscope III.

E93 ectopic expression clones were generated in *E93*^{Vnoe47} mutant animals by crossing *y w FLP12; Act>y*$^{+}$*>Gal4 UAS-GFP; tub-Gal80*^{ts} *E93*^{Df}*/TM6B* ♀ with *UAS-E93-ORF; E93*^{Vnoe47}*/TM6B* ♂. Progeny were raised at 25°C until young larvae, and were heat shocked at 37°C for 7 min. Larvae were then moved to 19°C until the early pupal stage, when they were shifted to 30°C. Legs from non-Tubby pharate pupae were mounted as described above. *E93* ectopic expression clones were identified by scoring the *yellow* marker in the cuticle of non-Tubby ♂ flies using a Zeiss Photomicroscope III.

Antibody staining and imaging were as described in Chapter II.

Results

E93 is required in the bract cell for the bract formation

To determine whether *E93* is required in the bristle (signal sending) cell or the bract (signal receiving) cell in the bract pathway, I generated mosaics for the $E93^{Vnoe47}$ allele. In these mosaics, cells homozygous for $E93^{Vnoe47}$ are marked by yellow cuticle. The four possible genotypic combinations for bristle and bract cells are shown in Fig. III-2 A. If *E93* is required in the bristle cell, then only wild-type bristles (black bristles) can send out the right signal to the neighboring cells and induce bracts, but the bracts themselves could be either mutant (yellow) or wild type (black) (Fig. III-2 C). Conversely, if *E93* is required in bract cells, then only wild-type bracts (black bracts) will be induced, but bristles could be either mutant (yellow) or wild type (black) (Fig. III-2 D). The second result was obtained, indicating that *E93* is required in the bract cell but not the bristle cell (Fig. III-2 B).

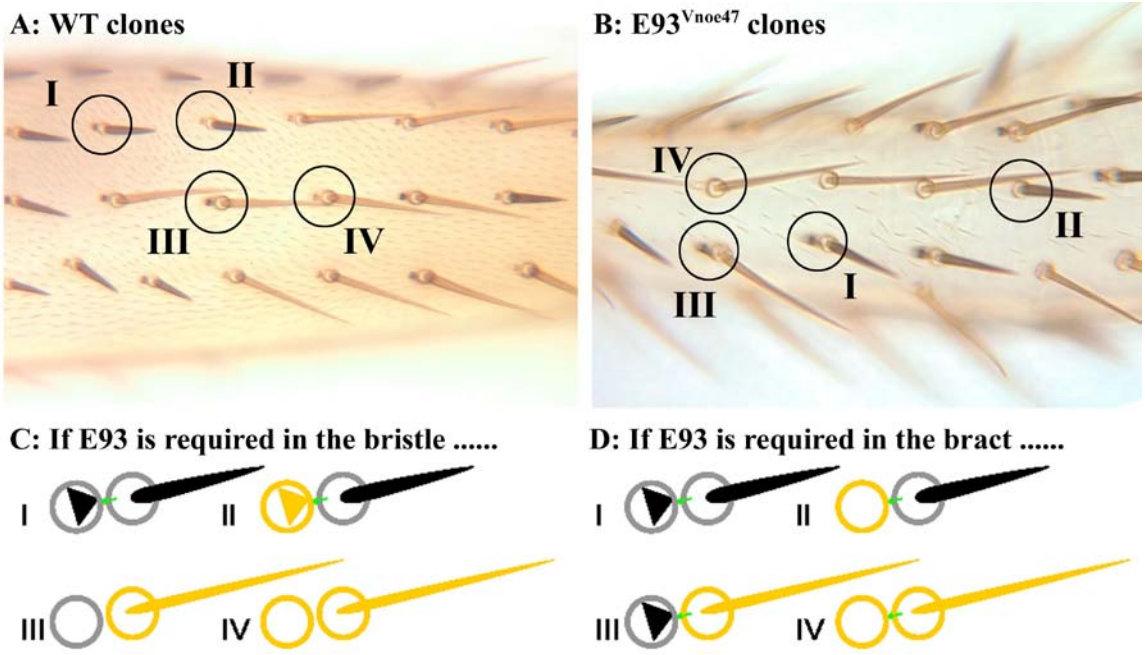


Figure III-2: Mosaic clones show E93 is required in the bract cell

A: Four possible genotype combinations of bristle and bract cells. I: black bract, black bristle. II: yellow bract, black bristle. III: black bract, yellow bristle. IV: yellow bract, yellow bristle. B: When $E93^{Vnoe47}$ clones are marked by yellow, only wild-type black bracts are seen. C: If *E93* is required in the bristle, then only black bristles will be able to induce bracts (either I or II). D: If *E93* is required in the bract, then only black bracts will be present (either I or III).

E93 is epistatic to Ras in bract formation

Since *E93* is not required in the bristle, we shifted our attention to determining where *E93* acts in the EGFR signaling pathway in bract cells. An important component of this pathway is Ras (Gilbert 2003). Previous studies have shown that ectopic expression of activated Ras between 8-12 hr AP transforms most epidermal cells of the distal leg into bracts (del Alamo et al. 2002; Held 2002). When I drove expression of $hs\text{-}Ras^{V12}$ (a constitutively active form of *Ras*) between 18-22hr AP, extra bracts were induced (Fig. III-3 A). However, when I heat shocked $hs\text{-}Ras^{V12}$ between 18-22 hr AP in an $E93^{Vno}$ mutant background, no bracts were induced (Fig. III-3 B), indicating that *E93* lies downstream of Ras in the bract pathway.

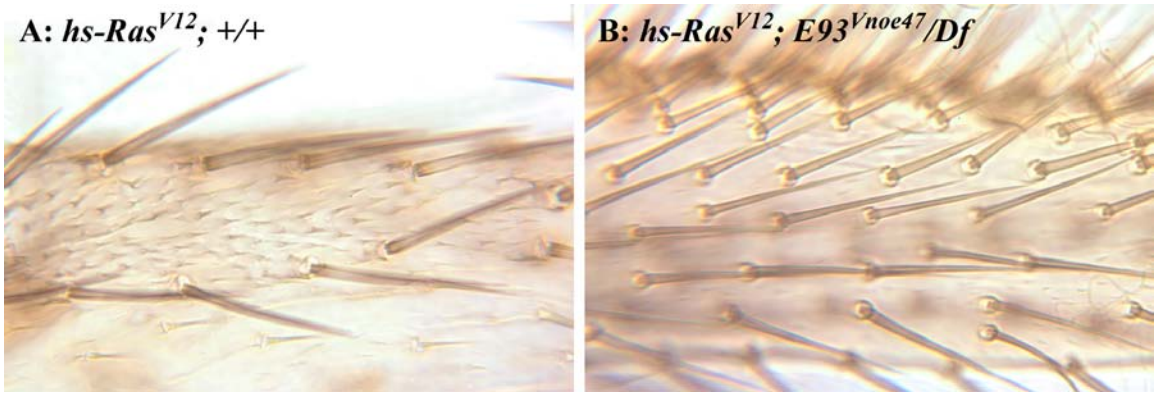


Figure III-3: Epistasis relationship of *E93* and *Ras*

A: Heat shock of *hs-Ras*^{V12} between 18-22 hr AP induces extra outgrowths of epidermal cells that look like bracts. B: Heat shock of *hs-Ras*^{V12} between 18-22 hr AP in the *E93*^{Vnoe47} mutant background does not induce bracts, indicating that *E93* is epistatic to *Ras*.

E93 functions downstream of the EGFR signal transduction pathway

A target of EGFR signaling in almost all contexts is the gene *argos*, which encodes a secreted antagonist of the Spitz EGFR ligand (Freeman et al. 1992; Schweitzer et al. 1995). To determine whether *argos* is also activated during bract induction, I examined expression of *argos-lacZ* in pupal legs. LacZ expression was found to occur immediately proximal to each bristle in the pupal leg (Fig. III-4 A). This observation suggests that the secretion of the Spitz ligand is polarized toward the proximal side of each bristle, accounting for why bracts develop only to the proximal side of bristles. I then examined *argos-lacZ* expression in the *E93*^{Vnoe47} mutant background: *lacZ* expression was unaffected relative to *E93*$^{+}$ animals (Fig. III-4 D). This finding demonstrates that the EGFR signal transduction pathway is intact in *E93*^{Vno} mutants, and suggests that *E93* acts downstream of this pathway. When I stained *argos-lacZ* pupal legs for both lacZ and

E93 expression, I found that the two co-localized to the same cells, which turn out to be pre-bract cells (Fig. III-4 C).

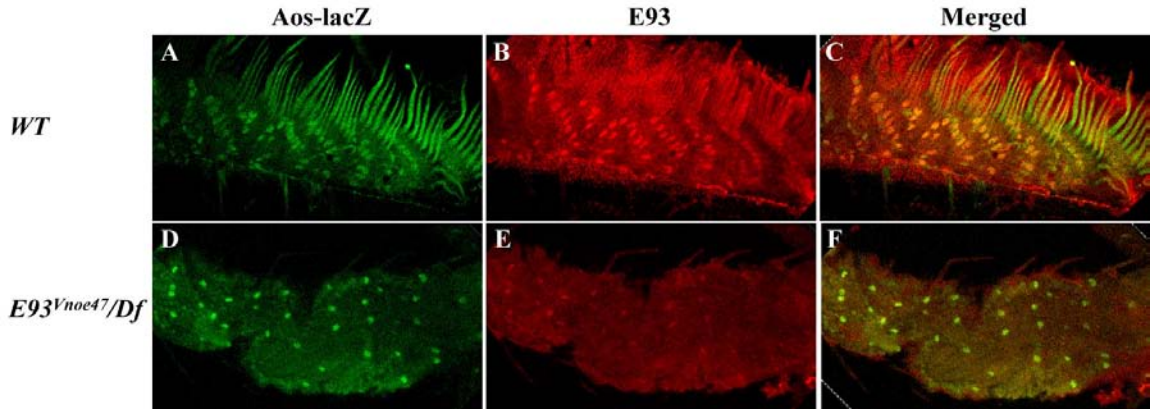


Figure III-4: Relationship of *E93* and *Arogs*

A: *argos-lacZ* expression is detected proximal to each bristle in an *argos-lacZ* $E93^{Df}$*/TM6B* pupal leg (35-45 hr AP). B: E93 is also detected proximal to each bristle. C: A merged picture shows that lacZ and E93 co-localize to the same cells. D: *argos-lacZ* expression is intact in the *E93* mutant background (*argos-lacZ* $E93^{Df}/E93^{Vnoe47}$). E: E93 expression is strongly reduced in the *E93* mutant background. F: Merged image.

E93 acts upstream of Dll in bract formation

Dll is known to be expressed in bract-associated cells in newly emerged adults, and to be required for bract formation (Gorfinkiel et al. 1997; Campbell and Tomlinson 1998). In *Dll-Gal4*; UAS-GFP newly emerged adults, GFP is expressed in bract-associated cells (Fig III-5 A). However, in the $E93^{Vnoe47}/Df$ mutant background, GFP expression is not seen in these cells (Fig III-5 B), indicating that *E93* lies upstream of *Dll* in the bract pathway. I then stained pupal legs for endogenous Dll protein, and found expression in cells located just proximal to each bristle (Figure III-5 D). Double and triple labeling

experiments demonstrated that Dll is expressed in the same cells as E93 and *argos-lacZ* Fig. III-5 D and Fig. III-6 C). When I stained for Dll expression in the $E93^{Vnoe47}$ mutant, I found that bract expression of Dll was eliminated (Fig. III-5 G and Fig. III-6 F), indicating the *E93* functions upstream of *Dll* in the bract pathway.

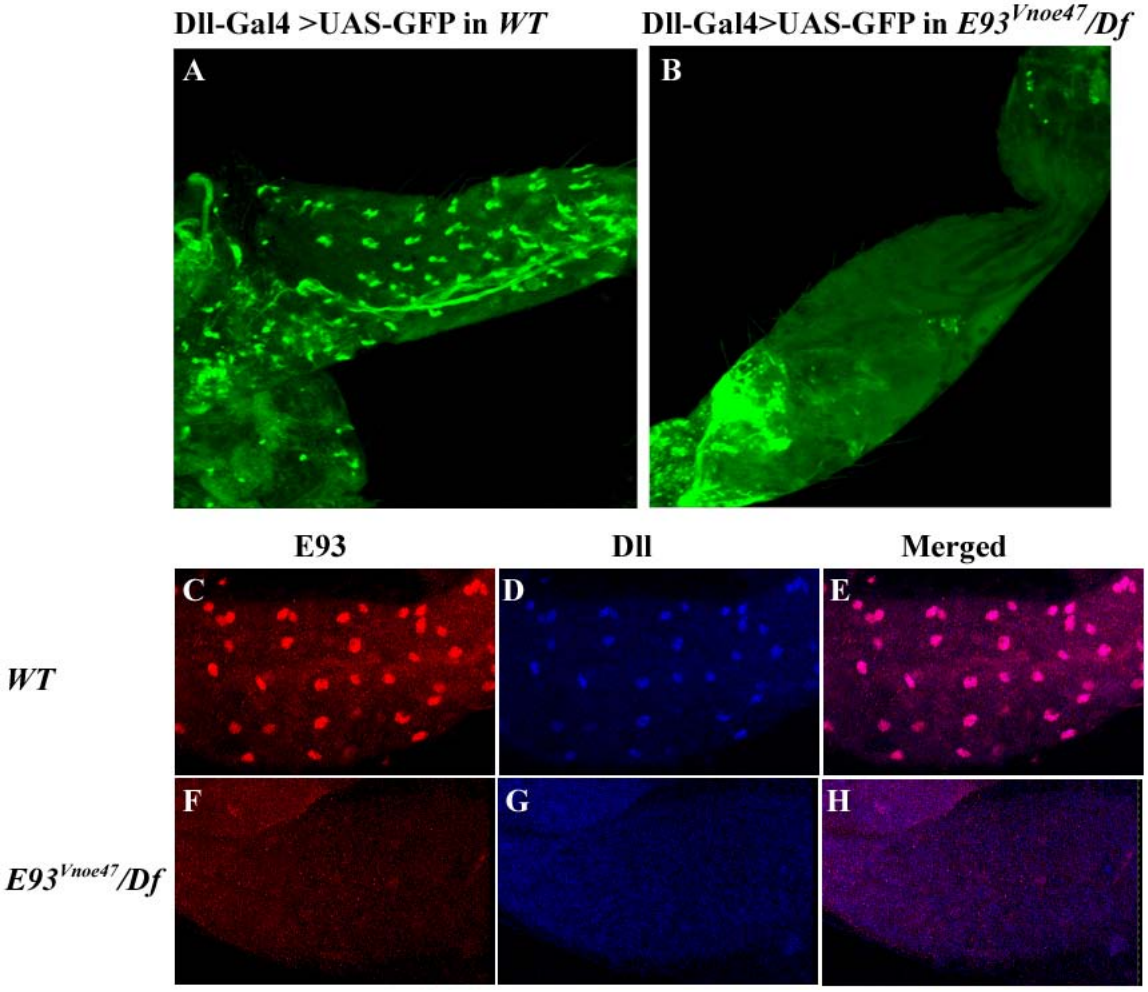


Figure III-5: Epistasis relationship of *E93* and *Dll*

A: Dll-Gal4>UAS-GFP in a wild-type pupal leg; B: Dll-Gal4>UAS-GFP in a $E93^{Vnoe47}/Df$ pupal leg; C-E: A wild-type pupal leg stained for E93 and Dll; F-H: An $E93^{Vno}$ mutant leg ($E93^{Vno}/Df$) stained for E93 and Dll; C, F: E93 expression; D, G: Dll expression; E, H: Merged images.

To confirm these experiments, I performed triple labeling with all three antibodies. In wild-type legs, all three proteins co-localize to the same cells. In the *E93*^{Vnoe47} mutant, E93 and Dll are reduced, but argos-lacZ staining is unaffected.

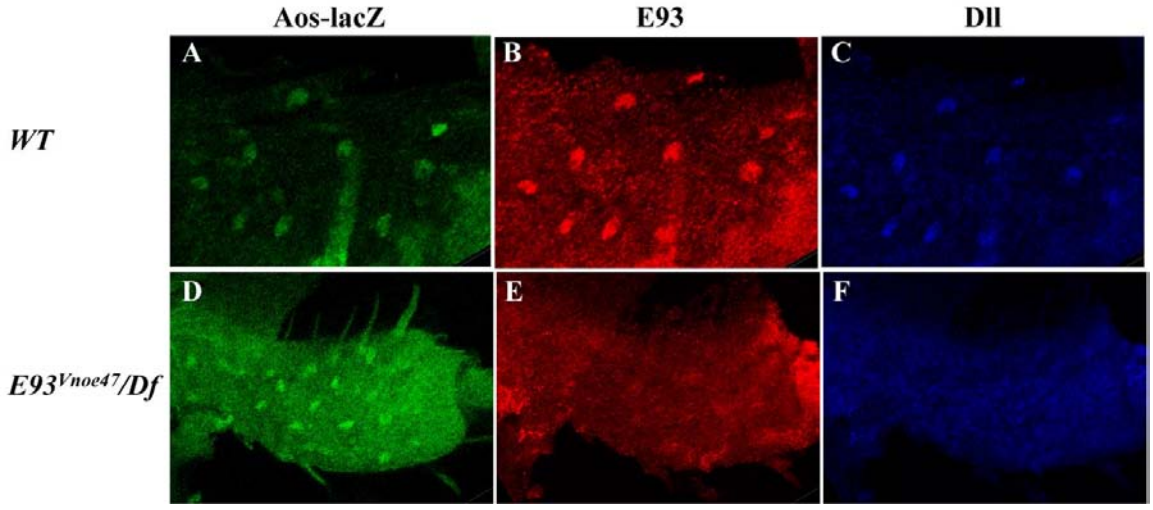


Figure III-6: Relationship of *Argos, E93,* and *Dll*

A-C: A wild-type pupal leg stained for *argos-lacZ*, E93, and Dll expression; D-F: An *E93*^{Vnoe47} mutant (*E93*^{Vnoe47}*/Df*) pupal leg stained for *argos-lacZ*, E93, and Dll.; A, D: *argos-lacZ* expression; B, E: E93 expression; C, F: Dll expression.

The temporal and spatial relationship of E93 and Pnt

In most cases, EGFR signaling is executed by the transcription factor Pointed (Pnt). To determine whether Pnt is required for bract formation, we generated *pnt*$^{-}$ clones that were marked by *yellow*. Such clones lack bracts. Although black bracts can form adjacent to yellow or black bristles, yellow bracts are never seen, indicating that *pnt*$^{+}$ is required within bract cells and not bristle cells (Fig. III-7 A). I then examined *pnt*$^{-}$ clones (marked

by loss of GFP) in pupal legs by confocal microscopy. In large *pnt*$^{-}$ clones both E93 and Dll expression are lost (Fig. III-7 D-G), indicating that both *E93* and *Dll* lie downstream of *pnt* in the bract pathway. In small *pnt*$^{-}$ clones, E93 and Dll expression are seen occasionally (Fig. III-7 H-K). Presumably this expression is due to perdurance of Pnt in these clones. Although the expression of E93 and Dll are not affected in small clones, the positional pattern of transverse rows can be altered (compare the transverse row pattern in Fig. III-7 K with Fig. III-9 I). Since E93 is required for Dll expression (see above), these results position *E93* between *Pnt* and *Dll* in the bract pathway.

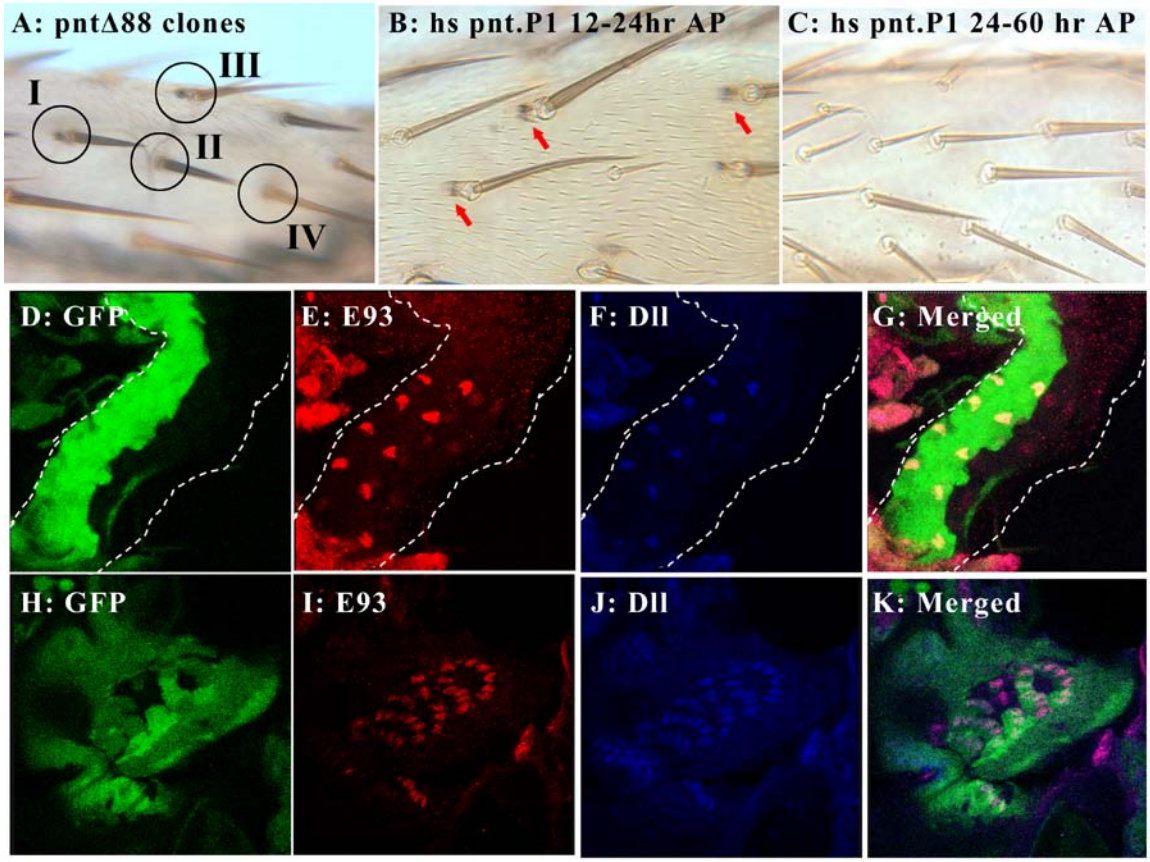


Figure III-7: *Pnt* is required for the bract formation and functions upstream of E93 and Dll

A: *pnt*$^{-}$ clones in the leg lack bracts (classes I-IV are as explained in Fig. III-2 A); B: Over expression of Pnt.P1 between 12-24 hr AP induces extra bracts (red arrows); C: Over expression of Pnt.P1 between 24-60 hr AP eliminates bracts; D, H: *pnt*$^{-}$ clones are identified by the lack of GFP; E, I: E93 expression; F, J: Dll expression; G, K: merged images. The white outlines in D, E, F and G show the twin spots: the GFP positive spot is homozygous for pnt^{+}, the GFP minus spot is homozygous for pnt^{-}.

When I over expressed UAS-pnt.P1 by heat shock, the effect on bract formation depended upon timing. Heat shocks delivered between 12-24 hr AP caused ectopic bracts to form at the base of many bristles (red arrows Fig. III-7 B). However, when heat shocks were administered at 24-60 hr AP, bract formation was suppressed (Fig. III-7 C).

To explore the relationship between Pnt and E93 further, I obtained an antiserum raised against Pnt.P1 by Dr. James Skeath. Double labeling with this antibody and anti-E93 reveals that at 30 hr AP both E93 and Pnt co-localize to bract cells (white arrows in Fig. III-8 C). However, in later pupae (35 hr AP), Pnt expression is reduced in bract cells (i.e. E93-expressing cells) but becomes elevated in two cells flanking the bract cell (white arrows in Fig. III-8 F and Fig. III-9 I). This temporal change in expression correlates with the stage specific effects of Pnt.P1 ectopic expression. In the early pupa, Pnt is expressed in the future bract cell, where it activates E93 expression. Ectopic expression

of Pnt.P1 at this time induces ectopic bract formation (Fig. III-7 B). Later, when Pnt is down regulated in bract cells, bracts are repressed by ectopic Pnt.P1, perhaps because Pnt now represses E93 (Fig. III-7 C).

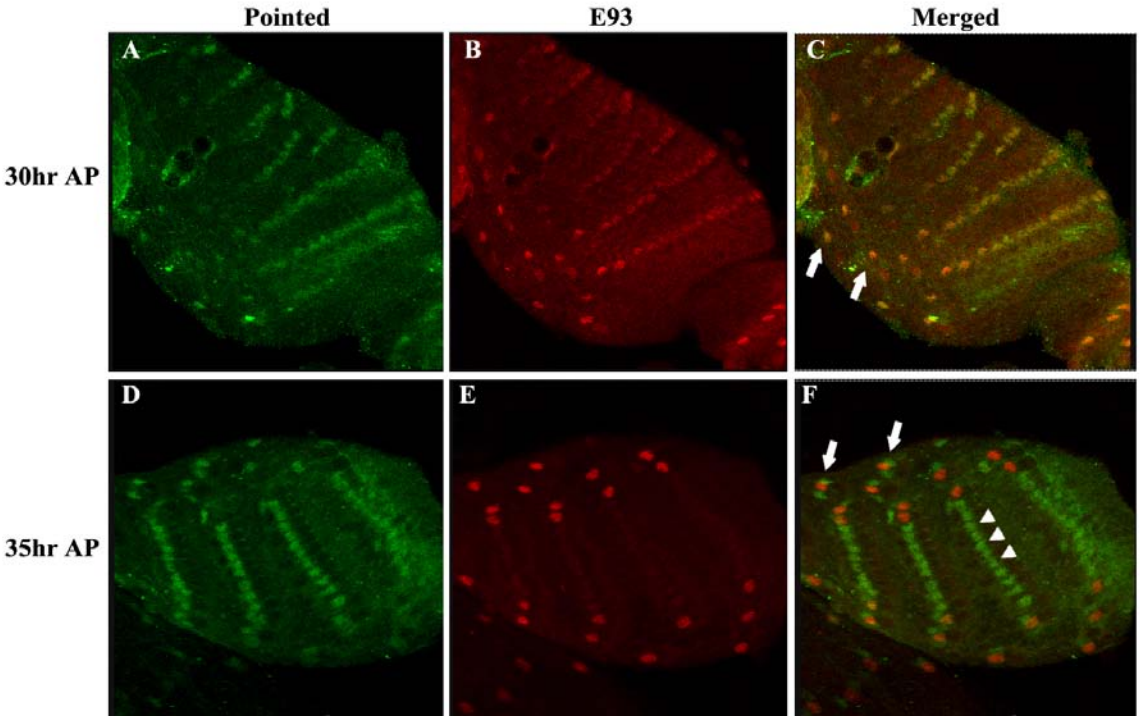


Figure III-8: The temporal specific positional relationship of Pnt and E93

A-C: The distal tibia of a wild-type first thoracic leg at 30 hr AP; D-F: The distal tibia of wild-type first thoracic leg at 35 hr AP; A, D: Pnt expression; B, E: E93 expression; C, F: merged images.

Not all bristles develop bracts in the leg. For example, the prominent transverse rows of bristles present in the distal part of the tibia of the first leg do not develop bracts, whereas transverse rows of bristles in the basitarsus do (Fig. III-9 B, Tokunaga and Stern 1965

Tokunaga 1962). By 35 hr AP, E93 expression is mostly lost from these transverse rows in the distal tibia, whereas Pnt expression is maintained (white arrow heads in Fig. III-8 F and Fig. III-9 F). In contrast, E93 expression is sustained in cells of the future transverse rows in the basitarsus, whereas Pnt expression is reduced in these cells and elevated in adjacent cells (white arrows in Fig. III-9 I). The continued expression of Pnt in cells that lose E93 expression, and the repression of Pnt in cells that maintain E93 expression, suggest that E93 or Dll may repress *pnt* at 35 hr AP.

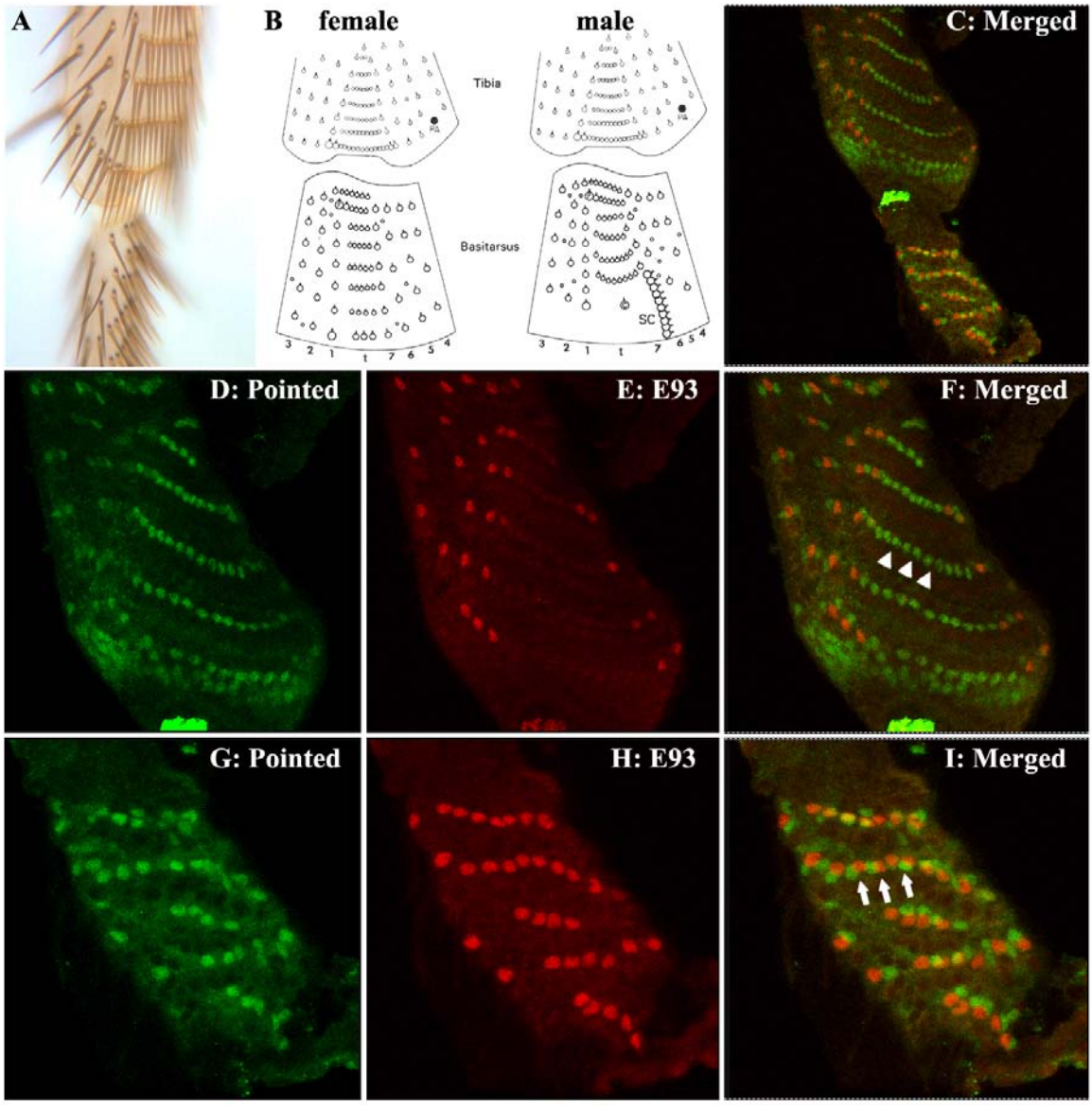


Figure III-9: The positional relationship of E93 and Pnt

A: Junction area of the tibia and basitarsus of the first leg of the fly. Transverse rows in the distal tibia have no bracts, but the transverse rows in the basitarsus do. B: Schematic summary of bristles and bracts in the distal tibia and basitarsus of the first leg (Tokunaga 1962; Tokunaga and Stern 1965). Circles represent bristles, triangles represent bracts, 1-8 represent longitudinal bristle rows, transverse rows (t), preapical bristle (PA), apical bristle (A), central bristle (C), sex comb (SC). C: A merged picture of pupal leg (34-36hr AP) with Pnt and E93 expression. D-F: The enlarged distal tibia area in C. G-I: The enlarged basitarsus area in C.

The relationship of E93 and bristle precursor cells

To determine the relationship between E93 expression and cells of the bristle lineage, I examined the *neuralized* enhancer trap A101, which is expressed in sensory organ precursor cells and their descendants (Huang et al. 1995). By 35 hr AP, sensory organ precursor cells have divided so that A101 is expressed in clusters of 3 or 4 cells (Fig. III-10 A). Double labeling reveals that E93 is expressed strongly in one of these cells, and weakly in an A101 positive cell lying just distal (cell B and cell A in Fig. III-10 N). This observation is surprising, as clonal analysis experiments show clearly that bracts are not related by lineage to bristle cells (e.g. Fig. III-2 A). Therefore, one would not expect E93 to be expressed in cells of the sensory organ precursor lineage. One possibility is that A101 expression in the strongly positive E93 cells is induced by neighboring A101 positive cells. The possibility of such induction has been raised previously (Huang et al. 1995). Pnt is expressed weakly in the E93 positive cell (cell 2 in Fig. III-10 O), but

strongly in two flanking cells (cell 4, 5 in Fig. III-10 O). In the first leg transverse row bristle rows that have no bracts, E93 expression is dramatically reduced (cell A, B in Fig. III-10 N), whereas Pnt expression remains high (cell B in Fig. III-10 O).

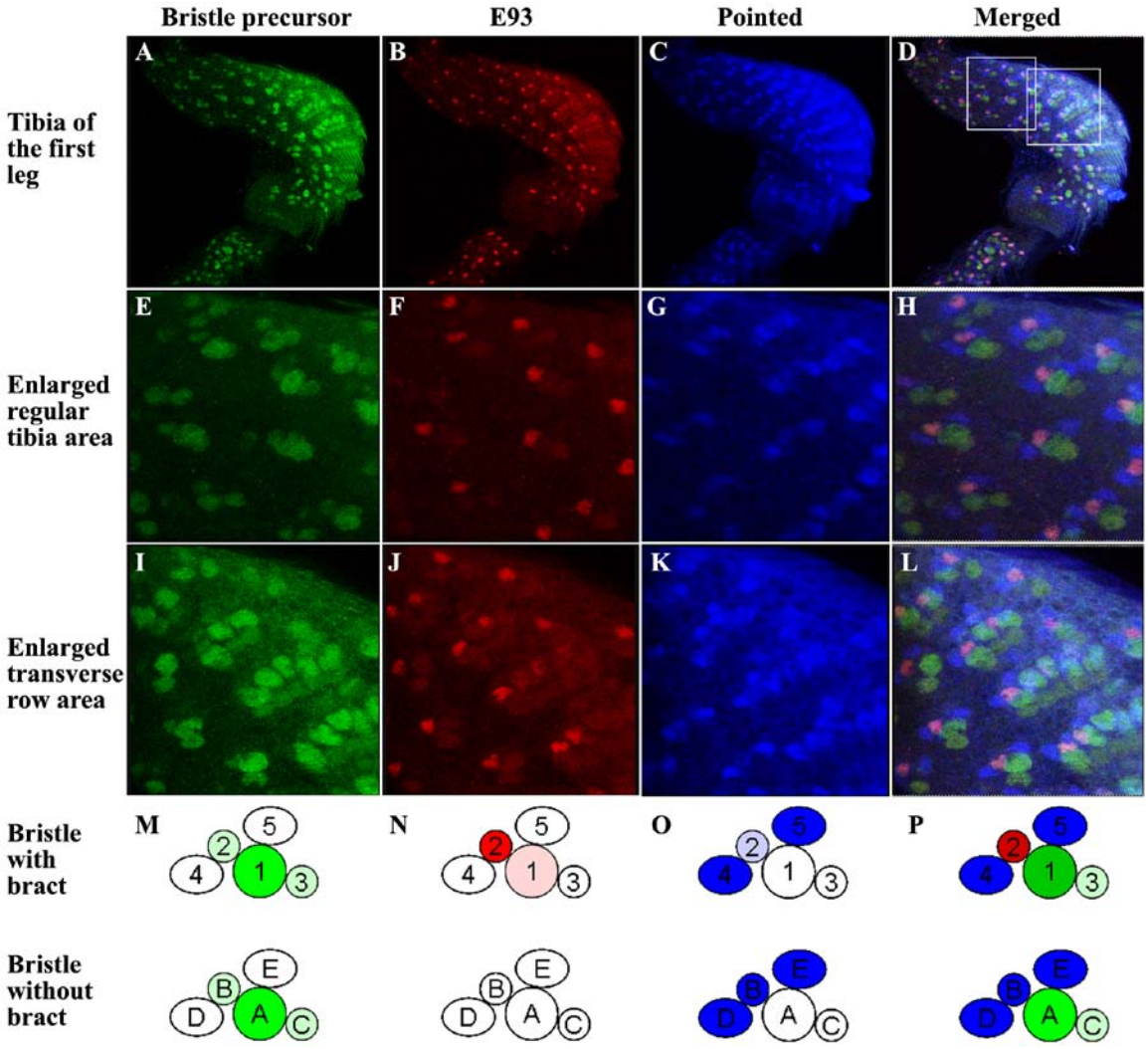


Figure III-10: The positional relationship of E93, Pnt, and A101 expression in 35hr AP legs

A-D: A wild-type leg at 35 hr AP; E-H: enlarged view of the white box on the left in D; I-L: Enlarged view of the white box on the right in D; M-P: Schematic summary of protein expression profile of bristles with or without bracts; cells 1-5 will give rise to bristles with bracts; cells A-E will give rise to bristles without bracts; A, E, I, M: A101 expression; B, F, J, N: E93 expression; C, G, K, O: Pnt expression; D, H, L, P: Merged images.

Double labeling experiments reveal that Dll has the same temporal and spatial expression pattern as E93: Dll co-localizes with E93 in the future bract cell (arrow heads in Fig. III-11 D, H), and at 35 hr AP is flanked by cells expressing Pnt (Fig. III-11 L). In addition, Dll is repressed in transverse rows that do not develop bracts (arrows in Fig. III-11 D), but expressed in transverse rows that do develop bracts (arrows in Fig. III-11 H).

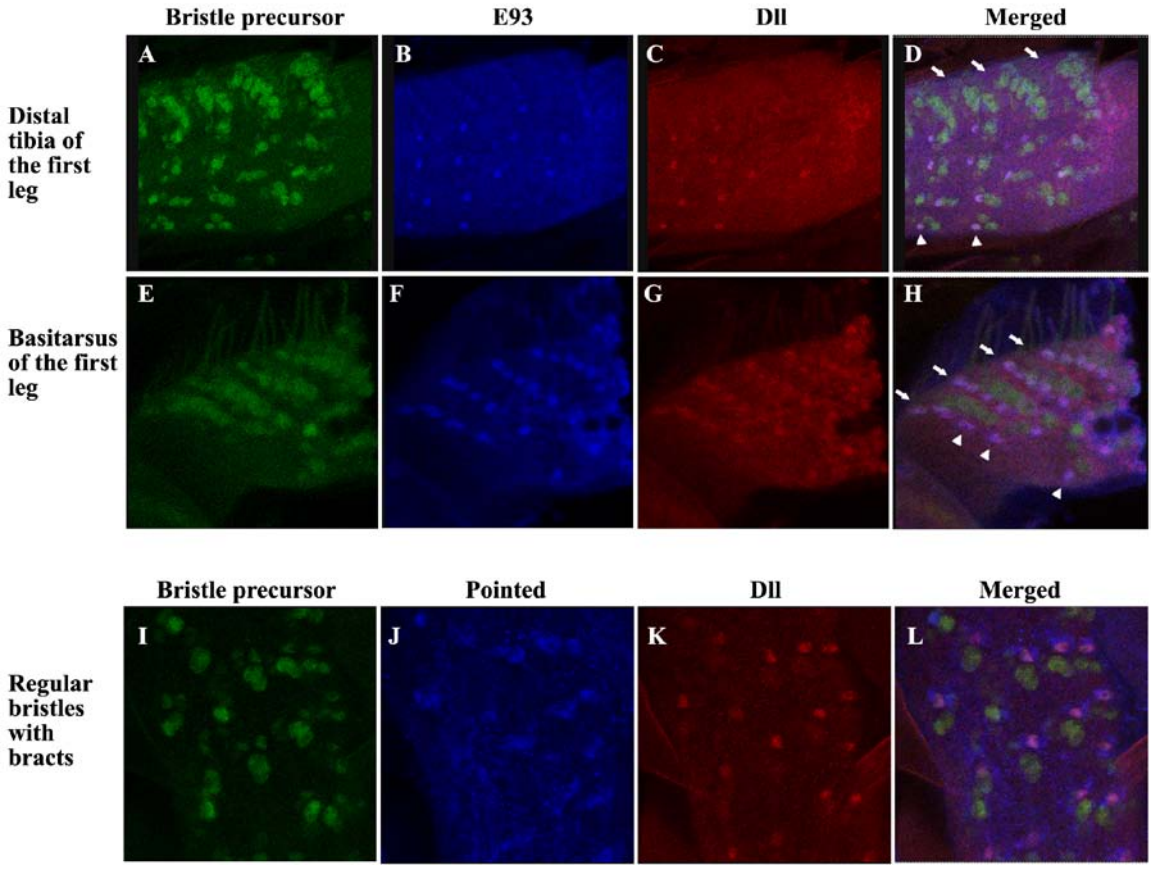


Figure III-11: The positional relationship of Pnt, E93, Dll, and A101 expression

A-L: Wild-type legs at 35hr AP; A-D: Distal tibia of the first leg; E-H: Basitarsus of the first leg; I-L: Regular bristles with bracts; A, E, I: A101 expression; B, F: E93 expression; J: Pnt expression; C, G, K: Dll expression; D, H, L: Merged images.

Ecdysone signaling is required for bract formation

The finding that *E93* is expressed specifically in bract cells and lies between *pnt* and *Dll* in the bract pathway raises the possibility that the role of *E93* in bract formation is quite distinct from its role as an ecdysone response gene. To determine whether ecdysone signaling is required for bract formation, I reduced ecdysone receptor activity by RNAi (targeting a common fragment of all EcR isoforms, Colombani et al. 2005) in mosaic

clones (in *y w FLP; Act>y*$^{+}$*>Gal4 UAS-GFP/UAS-EcR-RNAi*). Bracts are reduced in size or absent in the ecdysone receptor RNAi clones (marked by yellow), indicating that ecdysone signaling is involved in bract formation (Fig. III-12 A). To determine whether loss of ecdysone receptor activity also causes loss of E93 and Dll expression in bract cells, I examined the E93 and Dll expression in EcR knock down clones (marked by GFP positive cells). Preliminary data show that E93 and Dll are still expressed robustly in EcR knock down clones. Additional work will be required to determine whether these clones have no effect on E93 expression because EcR is not required for E93 bract expression, or because of perdurance or incomplete knock down of EcR.

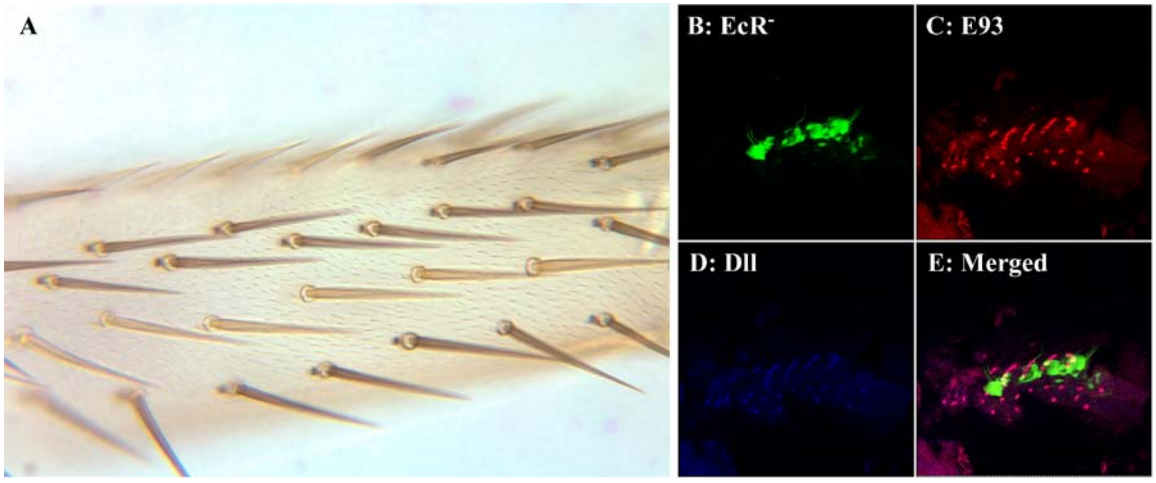


Figure III-12: Ecdysone signaling is involved in bract formation

A: Most bracts are eliminated in the ecdysone receptor knock down clones (marked by yellow); B: EcR knock down clones are marked by GFP positive cells; C: E93 expression; D: Dll expression; E: Merged images.

Is expression of E93 instructive or permissive in bract formation?

At the outset of my work on bracts, my hypothesis was that *E93* provides a temporal signal that facilitates activation of *Dll* in bract cells by other spatially restricted transcription factors (presumably Pnt). The finding that *E93* itself is restricted in expression to bract cells very much clouded the situation. Is spatially restricted expression of E93 important for bract specification? To answer this question, I generated clones that ectopically express E93 in an $E93^{Vnoe47}$ mutant background leg. The $GAL80^{ts}$ method (McGuire et al. 2004) was used to restrict ectopic expression in these clones to the pupal stage. When such clones are generated in an $E93^{Vnoe47}$ mutant background, bract development is often fully rescued proximal to bristles, but ectopic bracts are never seen (Fig. III-13). This result demonstrates that spatially restricted expression of *E93* is not required for bract formation, and that *E93* plays a permissive, not instructive, role in bract formation. Such a permissive role is consistent with our hypothesis that *E93* provides primarily temporal rather than spatial information during bract formation.

Figure III-13: E93 expression is permissive in bract formation

E93 is ectopically expressed in *E93^{Vnoe47}* mutant background by UAS-E93-ORF clones (marked by yellow in *y w FLP12; Act>y^{+}>Gal4 UAS-GFP/UAS-E93-ORF; tub-Gal80ts E93^{Df}/ E93^{Vnoe47}* animals). Bracts are formed proximal to bristles, but not elsewhere in the clone.

Discussion

My experiments establish that *E93* lies downstream of *pnt* and upstream of *Dll* in the bract induction pathway. The key lines of evidence that support these conclusions are: 1) mosaic studies demonstrate that *E93* is required within the bract cell, 2) *argos-lacZ* is activated normally in bract cells in *E93^{Vno}* mutants, indicating that *E93* acts downstream of the EGFR signal transduction pathway, 3) *pnt^{-}* clones lose bract expression of *E93*,

indicating that *E93* lies downstream of *pnt*, and 4) $E93^{Vno}$ clones lose expression of *Dll* in bract cells, indicating the *E93* lies upstream of *Dll* in the bract pathway.

Is the bract pathway a simple linear pathway in which *pnt* activates *E93*, which then activates *Dll*? The results of my E93 ectopic expression experiment would argue not. In this experiment, I demonstrated that leg clones expressing E93 in an otherwise $E93^{Vno}$ mutant background rescue bracts in their normal location proximal to bristles, but do not induce ectopic bracts. This result indicates that although *E93* expression is normally restricted to bract cells, this spatial restriction is not essential for normal bract development. That is, E93 plays a permissive, rather than instructive, role in bract specification. A very different result was obtained for Pnt: ectopic expression of Pnt at 12-24 hr AP does cause ectopic bract development, indicating that bract position is defined by spatially restricted *pnt* activity. These observations suggest that E93 and Pnt have distinct inputs to Dll activation, with Pnt providing spatial information and E93 providing temporal information. A model for the bract pathway in which Dll is activated by the combined action of E93 and Pnt is presented in Figure III-14.

The ectopic bracts produced by ectopic Pnt expression are all very near to normal bracts. Why aren't bracts produced indiscriminately by ectopic Pnt? Pnt is activated by phosphorylation as a result of the activity of the EGFR signal transduction pathway. When Pnt is expressed ectopically, presumably its activity is still restricted to the vicinity of the normal bracts because only here is it properly phosphorylated. When activity of the EGFR pathway itself is activated ectopically, bracts are in fact induced everywhere in

the leg (Fig. III-3 A, del Alamo et al. 2002; Held 2002). An unexpected feature of Pnt expression is that although Pnt is initially expressed in the bract cells, in later pupae it becomes partially repressed in these cells and activated in two flanking cells. The basis of this transition has not been determined. The loss of Pnt expression from the bract cell may result from repression by E93 or Dll. The activation of Pnt in the two flanking cells implies that these cells have received EGFR signals, but the origin of these signals is not clear. As described above, ectopic expression of Pnt in later pupae represses bract development, suggesting that Pnt may transition from an activator to a repressor of *E93* as pupal development proceeds (Fig. III-7 C).

An additional feature established in my work is that bract development is dependent upon ecdysone signaling. Although I do not know where ecdysone signaling enters into the bract pathway, it seems likely that it is required for proper *E93* expression or activity (see Fig. III-14).

A second unresolved feature of bract development is that *E93* and *Dll* are activated in cells that also express the A101 enhancer trap. This reporter labels sensory organ precursor cells and their mitotic descendants. Since bracts are clearly not related by lineage to bristles, the coexpression of A101, E93 and Dll is surprising. One possible explanation is that A101 expression in bract cells is induced by adjacent A101 positive cells. Future work will be required to resolve this issue.

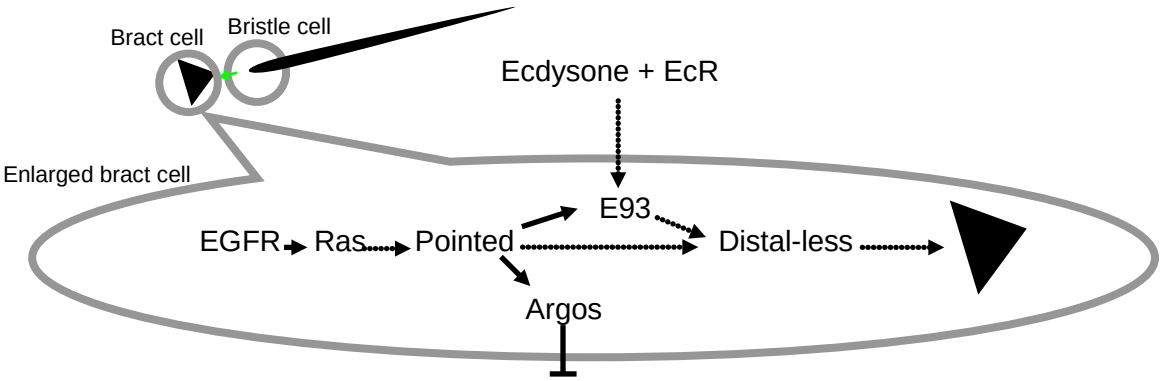


Figure III-14: A model for bract induction

In this model, bristle cell sends out EGFR ligand (green arrow) to activate the EGFR signaling pathway in an epidermal cell located at the proximal part of the bristle cell. Thus the activation of EGFR signaling provides the spatial information required for bract induction. In addition, ecdysone and EcR complex induce E93 expression during metamorphosis, which provides the temporal information required for bract induction. The inputs from both Pnt and E93 ensure the activation of Dll at the appropriate time and space. The green arrow represents the EGFR ligand sent out from the bristle cell. Dotted lines indicate that other genes are omitted from the pathway.

<u>Chapter IV</u> : Complementation of the *E93^{Vno}* alleles and *E93^{1}*, *E93^{2}*, and *E93^{3}*; are the larval cell death and imaginal patterning functions of E93 executed by different protein domains?

Abstract

As described in Chapter I, the *E93^{Vno}* alleles are defective in many imaginal patterning processes during metamorphosis, whereas the *E93^{1}*, *E93^{2}*, and *E93^{3}* alleles are defective in larval cell death during metamorphosis. These two groups of alleles fully complement one another. To understand this complementation, I first asked whether each allele type is defective in just one or the other function. Because the *E93^{1}*, *E93^{2}*, and *E93^{3}* alleles all cause death prior to imaginal disc differentiation, I assessed their effects on imaginal patterning by generating homozygous mutant clones. All of these alleles produce normal cuticular patterns in clones, indicating they all are fully functional in imaginal patterning. Eric Baehrecke's group has examined larval cell death in hemizygotes for the *E93^{Vno}* alleles: all show near normal salivary gland cell death early in metamorphosis. Why do the two groups of alleles affect specifically one function or the other? The imaginal patterning function presents few problems; *E93^{1}* is associated with a nonsense change at codon 995, and likely complements the *E93^{Vno}* alleles because the large N-terminal fragment of E93 it encodes is sufficient to execute all imaginal patterning functions. Sequence changes have not been identified for *E93^{2}* and *E93^{3}* (Lee et al. 2000), but heterozygotes for these alleles and *E93^{Vnoe47}* show almost normal E93 expression in imaginal tissues, suggesting that these alleles affect regulatory regions specific for larval cells. In contrast, the ability of the *E93^{Vno}* alleles to drive larval cell death has been

difficult to rationalize. These alleles truncate the E93 coding sequence N terminal to *E93*^{1}, and encode E93 fragments lacking all or part of the pipsq DNA-binding domain. I have considered two explanations. First, the activation of larval cell death may require only a specific domain within the E93 protein. To test this possibility, I generated UAS constructs that express fragments of the E93 (see below). Second, larval cell death may be activated by previously undescribed products of the *E93* locus. This possibility is explored in Chapter V.

Rationale

The complementation of the *E93*^{Vno} alleles and *E93*^{1}, *E93*^{2}, and *E93*^{3} has been difficult to explain. In this chapter I show that the *E93*^{1}, *E93*^{2}, and *E93*^{3} alleles affect larval cell death specifically, and are fully capable of executing the imaginal patterning functions of *E93*. Conversely, the *E93*^{Vno} alleles are defective in imaginal patterning, but are almost fully capable of executing the larval cell death functions of *E93* (Dutta and Baehrecke, personal communication). Although it is easy to rationalize why the *E93*^{1}, *E93*^{2}, and *E93*^{3} alleles are able to function in imaginal patterning, it has been difficult to understand why the *E93*^{Vno} alleles are able to function in driving larval cell death. One possibility is that only a portion of the E93 protein is required for the cell death function. In this chapter, I test this possibility by driving ectopic expression of subfragments of E93.

Methods

The generation of E93 UAS constructs

I designed six E93 UAS constructs: UAS-ORF, UAS-Clone2, UAS-*E93*^{1}Nterm, UAS-*E93*^{Vnoe47}Nterm, UAS-*E93*^{Vnoe47}Cterm, and UAS-Δpipsq (Fig. IV-1). All fragments were amplified from "LP library F211 pBluescript SK-" (the Clone2 cDNA I obtained from cDNA screening - see Chapter V). Primers were designed so that a consensus translation initiation sequence in Drosophila (CAAC) was present at the beginning of each ORF. Each E93 fragment was subcloned into PCR8/GW/TOPO entry clone by PCR8/GW/TOPO TA cloning kit (Invitrogen, Cat # k2500-20), and then recombined into pTW destination vector for injection (primers used to generate each fragment and sequence information for each fragment is included in the Appendix). The UAS-LP0 construct was constructed by Dianne Duncan using the pUAST vector. The UAS-E93(Baehrecke) construct was made by Eric Baehrecke, and was cloned into the pUAST vector (Lee et al. 2000).

UAS-E93(Baehrecke): *E93* full length ORF from Dr. Eric Baehrecke. Although my sequencing shows that the normal initiating ATG is absent from this construct, antibody staining (see below) indicates that this construct is translated, presumably using an alternate ATG.

UAS-ORF: *E93* full length ORF

UAS-Clone2: *E93* full length ORF with leading sequence of clone2

UAS-LP0: *LP0* full length ORF with leading sequence of LP0

UAS-E93^{1}Nterm: *E93* ORF truncated at the position of the *E93*^{1} nonsense mutation (codon 995)

UAS-*E93*^{Vnoe47}Nterm: *E93* ORF truncated at the position of *E93*^{Vnoe47} nonsense mutation (codon 360)

UAS-*E93*^{Vnoe47}Cterm: *E93* ORF starting from the first in frame ATG after the *E93*^{Vnoe47} mutation (codon 371)

UAS-Δpipsq: *E93* ORF with a deletion of codons 750-898. This deletion removes the pipsq DNA-binding domain (codons 761-814) and surrounding sequences. The deletion is bigger than the conserved pipsq domain because of my miscalculation

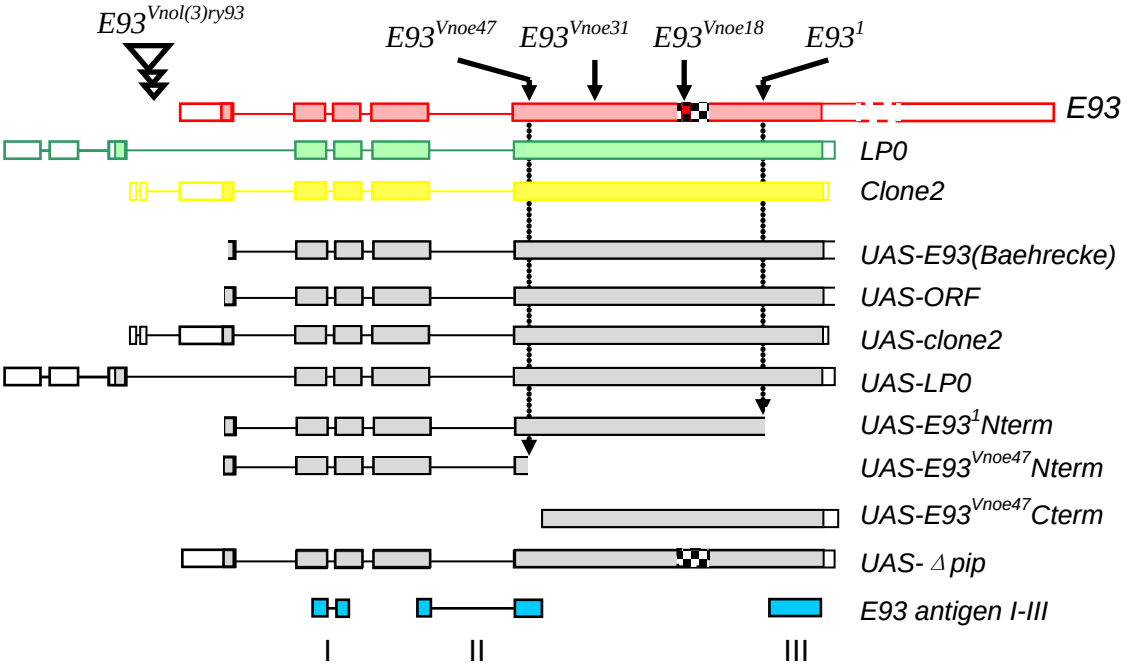


Figure IV-1: Molecular map of *E93* transcripts, mutants, *UAS-E93* constructs and E93 antigen

The boxes represent exons, and shaded areas represent the predicted coding regions. The transcript inferred by Baehrecke is in red, the *LP0* transcript is in green, and the *Clone2* transcript is in yellow. Black arrows indicate the stop codon positions of the *E93*^{Vnoe47}, *E93*^{Vnoe31}, *E93*^{Vnoe18}, and *E93*^{1} mutations, respectively. The black triangles are insertions

of two Doc elements and a P-element in the *E93*$^{Vnol(3)ry93}$ allele. The E93 UAS constructs are indicated in gray. Coding regions used for antibody production are in blue.

Stock construction:

I built two groups of stocks. In one group, I included a Gal4 driver (*dpp-Gal4*, *fkh-Gal4*, or *act>y+>Gal4*), a temperature-sensitive Gal4 repressor (*tub-Gal80ts)*, a clone marker (*UAS-GFP*), and *E93*^{Df}. In the other group, I included one of the UAS-E93 constructs together with *E93*^{vnoe47} *or E93*^{1}.

Gal4 driver stocks	
fkh-Gal4	w; tub-Gal80^{ts} UAS-GFP; fkh-Gal4 E93^{Df} /TM6B
dpp-Gal4	w; tub-Gal80^{ts} UAS-GFP; dpp-Gal4 E93^{Df}/TM6B
act>y+>Gal4	yw FLP; act>y+>Gal4 UAS-GFP; tub-Gal80ts E93^{Df}/TM6B
E93 UAS construct stocks	
UAS-E93 (Baehrecke) UAS-E93-ORF (line A1) UAS-E93-clone2 (line B2) UAS-E93-LP0 (line I2e) UAS-E93^{1}Nterm (line D1)	w; UAS-E93-X; E93^{e47}/TM6B
UAS-E93^{Vnoe47}Nterm (line H1) UAS-E93^{Vnoe47}Cterm (line A2) UAS-E93-Δpip (line A3)	w; UAS-E93-X; E93^{1}/TM6B

Other stocks and methods:

Clones homozygous for *E93*^{1}, *E93*^{2}, or *E93*^{3} were generated by crossing *y w FLP122; FRT82B CD2 y*$^{+}$ *M(3)W Bsb / TM6B* ♀ with *FRT82B cu E93*$^{1/2/3}$ *ca / TM6B* ♂, and then heat shocking progeny larvae at 37℃ for 30min. Homozygous E93 -mutant clones were identified in non-Tubby progeny and were recognized by their long bristles in both ♀ and ♂, or by their long bristles and yellow cuticle in ♂ flies.

Acridine Orange Staining: imaginal discs are stained in 1.6 uM freshly prepared Acridine Orange solution for 5 min, rinsed with Ringer's solution, and mount in Ringer's solution for immediate examination.

Results:

E93^{Vno} *alleles and E93*$^{1/2/3}$ *alleles are normal in the function that is defective in the other complementation group*

As described above, the *E93*^{Vno} mutants are defective in numerous imaginal patterning processes that take place during metamorphosis. Since homozygotes for *E93*^{1}, *E93*^{2}, or *E93*^{3} die at the early pupal stage, I assessed the ability of these mutants to execute imaginal patterning by generating homozygous mutant clones. All three alleles show normal patterning in such clones: no patterning defects are found in the leg, the eye, the antenna, the arista or the abdominal tergites (Fig. IV-2 B-E), all of which show abnormal phenotypes in the *E93*^{Vno} mutants. These findings demonstrate that *E93*^{1}, *E93*^{2}, and *E93*^{3} are not defective in the imaginal patterning functions of *E93*.

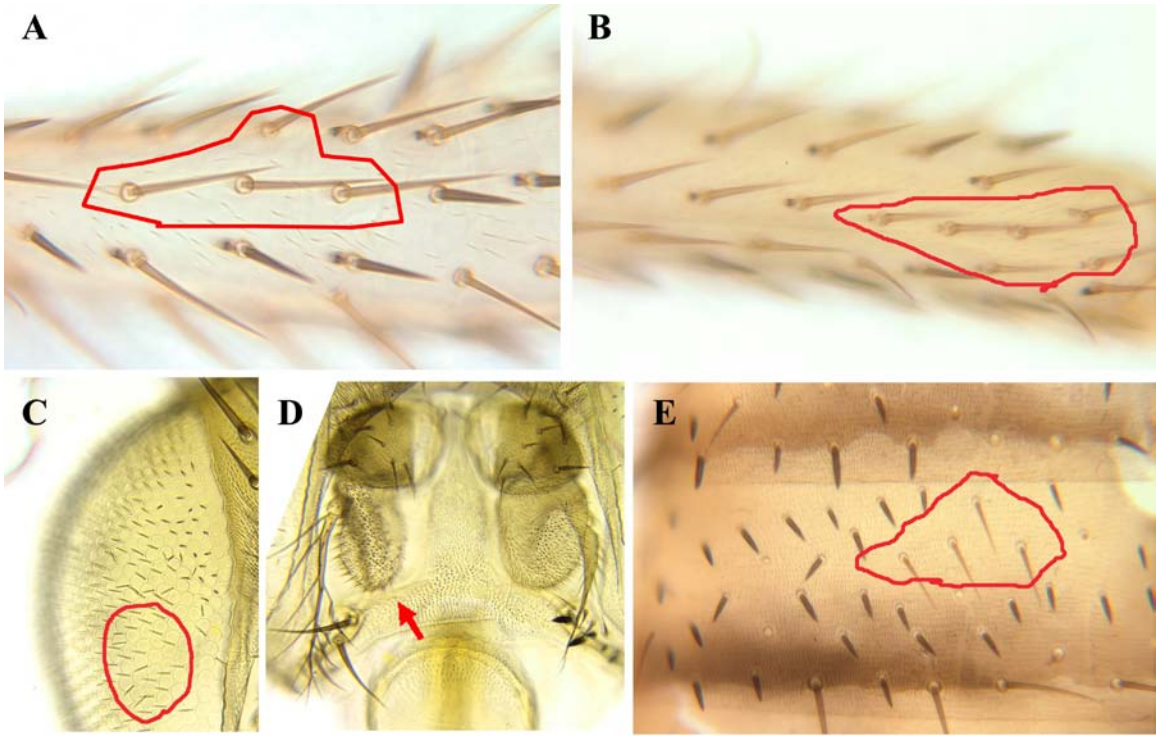


Figure IV-2: *E93*$^{1/2/3}$ alleles are normal in imaginal patterning function

A: An *E93*^{Vnoe47} mutant clone; this is a positive control to show that there are no bracts in *E93*^{Vnoe47} mutant clones; B-E: *E93*^{1} mutant clones in the leg, eye, antenna, and abdomen; Clone boundaries in A, B, C, and E are indicated in red. The red arrow in D indicates an *E93*^{1} antenna; the other antenna is heterozygous for the markers *M(3)w* and *Bsb*, which cause a reduction in bristle size and in the aristal branches. *E93*^{2} and *E93*^{3} clones behave identically.

Baehrecke's group has shown that the *E93*^{1}, *E93*^{2}, and *E93*^{3} mutants are all defective in the death of larval cells in the midgut and salivary gland during metamorphosis (Lee et al. 2000). In wild-type pupae, salivary glands are completely degraded by 24hr AP, whereas in hemizygotes for *E93*^{1}, *E93*^{2}, or *E93*^{3}, salivary glands remain intact at this time (Table IV-1, personal communication from Baehrecke's lab). For her thesis work, Sudeshna

Dutta (a graduate student in Eric Baehrecke's lab) examined salivary gland death in selected $E93^{Vno}$ genotypes. Her results are reproduced in Table IV-1. Hemizygotes for the $E93^{Vnoe31}$ and $E93^{Vnoe18}$ alleles show mildly impaired salivary gland death, with 90 - 100% of 24 hr AP pupae showing absence or fragmentation of salivary glands. Curiously, the three heterozygous combinations of the $E93^{Vno}$ alleles and the one homozygote examined ($E93^{Vnoe31}/E93^{Vnoe31}$) show a more severe phenotype, with no pupae showing complete loss of salivary glands at 24 hr AP, 29%-66% showing salivary gland fragmentation, and 34 - 71% showing intact salivary glands. The weak phenotypes seen in hemizygotes indicate that the $E93^{Vno}$ alleles are only weakly impaired in the cell death function, but the stronger phenotypes shown by the homozygous or heteroallelic genotypes suggest that the $E93^{Vno}$ alleles encode proteins that actively interfere with the cell death function.

Table IV-1: The percent of 24 hr AP pupae with the indicated salivary gland phenotypes

(n> 20 pupae/genotype) (personal communication from Sudeshna Dutta and Dr. Eric Baehrecke)

Genotype	**Intact/condensed salivary glands**	**Fragmented salivary glands**	**No glands**
$E93^{Vnoe18}/+$	0	20	80
$E93^{1}/Df$	100	0	0
$E93^{Vnoe18}/Df$	10	40	50

$E93^{Vnoe31}/Df$	0	35	65
$E93^{1}/E93^{Vnoe18}$	13	20	67
$E93^{Vnoe18}/E93^{Vnoe31}$	71	29	0
$E93^{Vnoe31}/E93^{Vnoe47}$	54	46	0
$E93^{Vnoe47}/E93^{Vnoe18}$	34	66	0
$E93^{Vnoe31}/E93^{Vnoe31}$	36	64	0

E93 expression in complementing heterozygotes:

The larval midgut

$E93^{Vno}/Df$ animals ($E93^{Vnoe18}/Df$, $E93^{Vnoe31}/Df$, and $E93^{Vnoe47}/Df$) show a strong reduction in E93 staining in the midgut around 1-5hr AP(Fig. IV-3 B, C, D), despite showing near-normal salivary gland apoptosis (personal communication from Baehrecke's lab). $E93^{1}/Df$, $E93^{2}/Df$, and $E93^{3}/Df$ animals also show a strong reduction in E93 staining in the 1-5 hr AP midgut (Fig. IV-3 E, F, G). I also examined E93 staining in heterozygotes for the $E93^{Vnoe47}$ allele and $E93^{1}$, $E93^{2}$, and $E93^{3}$. $E93^{Vnoe47}/E93^{1}$ heterozygotes show very little E93 staining in the 1-5 hr AP midgut. In contrast, almost normal E93 staining is seen in the 1-5 hr AP midgut in $E93^{Vnoe47}/E93^{2}$ and in $E93^{Vnoe47}/E93^{3}$ heterozygotes (Fig. IV-3 I, J). The latter results are striking because neither $E93^{Vnoe47}$ nor the $E93^{2}$ or $E93^{3}$ mutants show much staining in midgut cells on their own when hemizygous. One possibility is that $E93^{2}$ and $E93^{3}$ are mutations in larval-cell specific enhancer(s), and that the normal larval enhancers carried by the $E93^{Vnoe47}$ allele can act in trans to activate expression of the intact E93 coding region from the $E93^{2}$ and $E93^{3}$ alleles. Such trans

action of enhancers (transvection) has been described for several other genes in Drosophila. Although transvection may help account for the ability of the $E93^{Vno}$ alleles to complement $E93^{2}$ and $E93^{3}$, transvection cannot explain the complementation of these alleles with $E93^{1}$, nor can it explain why the $E93^{Vno}$ alleles are able to execute near-normal larval cell death on their own.

Imaginal patterning

The ability of the $E93^{2}$ and $E93^{3}$ alleles to execute the imaginal patterning functions of *E93* is consistent with the expression of E93 protein in heterozygotes of these alleles and $E93^{Vno}$ alleles. $E93^{Vno}/Df$ animals ($E93^{Vnoe18}/Df$, $E93^{Vnoe31}/Df$, and $E93^{Vnoe47}/Df$) show a strong reduction in E93 staining in bract cells in pupal legs (Fig. IV-3 B', C', D'). However, $E93^{2}/E93^{Vnoe47}$ and $E93^{3}/E93^{Vnoe47}$ heterozygotes show almost normal levels of E93 expression in bract cells (Fig. IV-3 I', J'). This result suggests that the $E93^{2}$ and $E93^{3}$ mutations may be defective specifically in larval cell expression. The $E93^{1}$ allele (a nonsense change at codon 995) behaves differently: $E93^{1}/E93^{Vnoe47}$ heterozygotes show some staining of E93 in pupal legs, but it is not as strong as in $E93^{2}/E93^{Vnoe47}$ or $E93^{3}/E93^{Vnoe47}$ heterozygotes (Fig. IV-3 H'). One possibility is that the large N-terminal fragment encoded by $E93^{1}$ is competent to execute the imaginal patterning functions of E93, even when present at low levels.

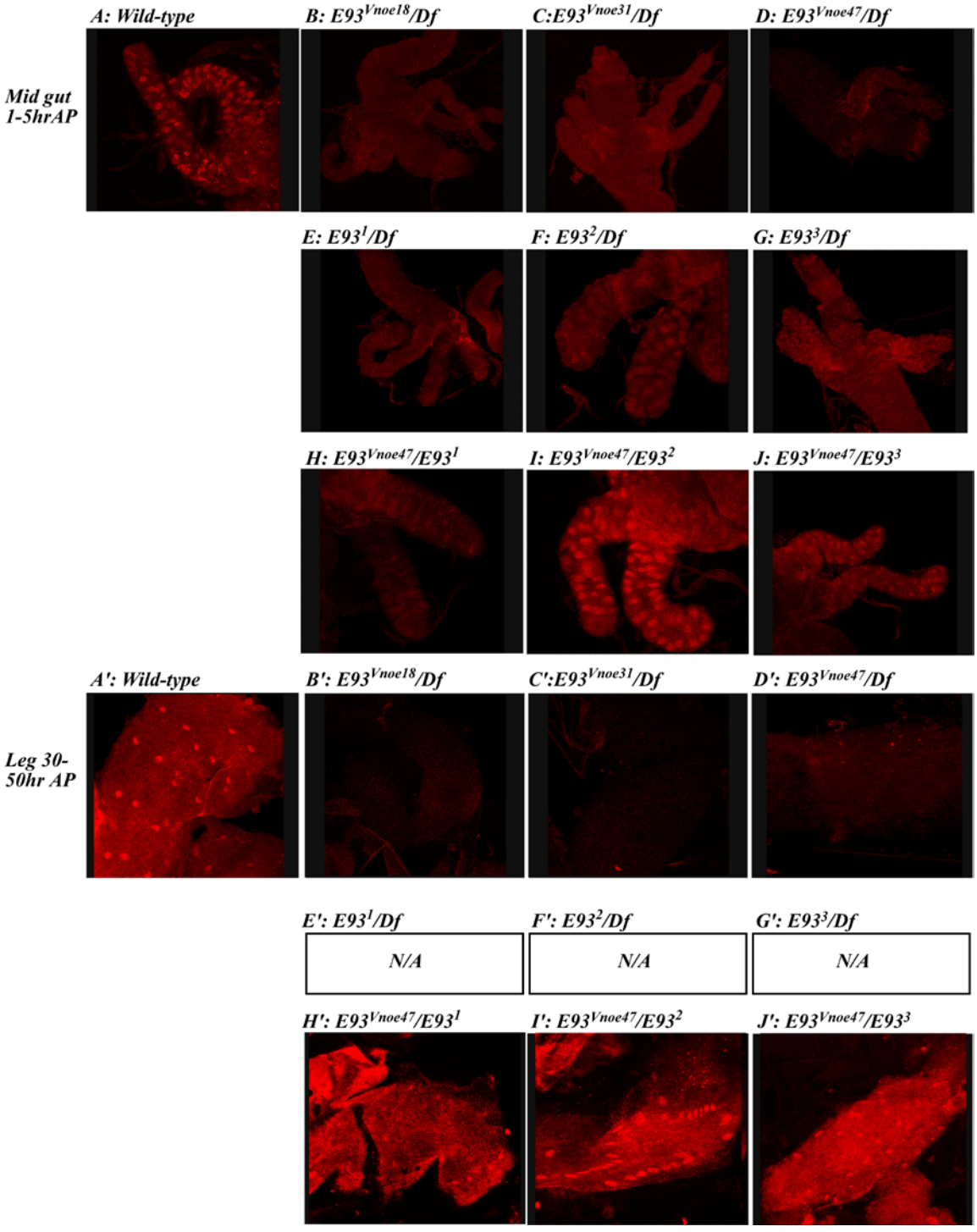

A: Wild-type
B: E93Vnoe18/Df
C:E93Vnoe31/Df
D: E93Vnoe47/Df
Mid gut 1-5hrAP
E: E93¹/Df
F: E93²/Df
G: E93³/Df
H: E93Vnoe47/E93¹
I: E93Vnoe47/E93²
J: E93Vnoe47/E93³
A': Wild-type
B': E93Vnoe18/Df
C':E93Vnoe31/Df
D': E93Vnoe47/Df
Leg 30-50hr AP
E': E93¹/Df
F': E93²/Df
G': E93³/Df
N/A
N/A
N/A
H': E93Vnoe47/E93¹
I': E93Vnoe47/E93²
J': E93Vnoe47/E93³

Figure IV-3: Expression of E93 in different allelic combinations during metamorphosis

A-J: Midgut at 1-5hr AP; A'-J': Pupal leg at 30-50hr AP; A & A': Wild-type; B & B': *E93^{Vnoe18}/Df;* C & C': *E93^{Vnoe31}/Df;* D & D': *E93^{Vnoe47}/Df*; E: *E93^{1}/Df;* F: *E93^{2}/Df (coming soon);* G: *E93^{3}/Df;* E, F and G genotypes were not survive to late pupal stage, so E', F' and G' are Not Available (N/A); H &H': *E93^{Vnoe47}/E93^{1}*; I & I': *E93^{Vnoe47}/E93^{2}*; J & J': *E93^{Vnoe47}/E93^{3}*.

The execution of imaginal patterning function and larval cell death function

Generation of UAS constructs

To test whether specific subdomains of E93 are responsible for the imaginal patterning and cell death functions of the locus, I generated UAS constructs that express fragments of the E93 coding sequence (see Methods). I also generated constructs expressing alternate transcripts from the locus. The molecular maps of the UAS constructs I examined are summarized in Fig. IV-1. To make sure that the constructs I produced are capable of being expressed, I tested each transformant line by crossing it to en-Gal4 and staining the progeny embryos for E93 expression. All eight constructs analyzed produce striped embryos, indicating that the constructs are correct and have been engineered into transformants successfully (Fig. IV-4).

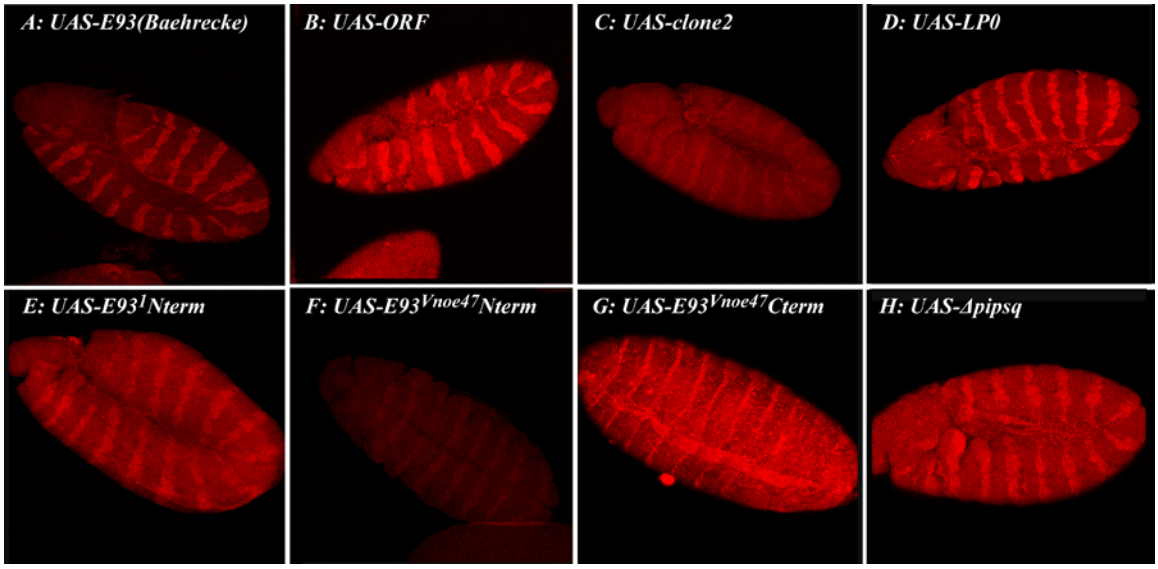


Figure IV-4: Expression of E93 UAS constructs in embryos when driven by en-Gal4

A: UAS-E93(Baehrecke); B: UAS-ORF A1 line: C: UAS-clone2 B2 line; D: UAS-LP0 I2e line; E: UAS-E93^{1}Nterm D1 line; F: UAS-E93^{Vnoe47}Nterm H1 line; G: UAS-E93^{Vnoe47}Cterm A2 line; H: UAS-Δpipsq A3 line; A-H are all stained by E93 antibody from rabbit B, except F, which is stained by E93 antibody from mouse 5.

Imaginal patterning function

My work to test the ability of the UAS constructs to rescue the imaginal patterning functions of *E93* has only just begun. The one result I have is described in Chapter III: in this experiment I showed that clones expressing the UAS-ORF construct during pupal development in *E93^{Vnoe47}* mutants are able to rescue bract formation proximal to bristles in the legs, but do not cause additional cells in the leg to become bracts (Fig. III-13). This observation demonstrates that *E93* plays a permissive rather than instructive role in bract formation. Tests of the rescue ability of the other UAS constructs are in progress.

Larval cell death function

It has been difficult to understand why the *E93^{Vno}* mutants are almost fully competent to drive larval cell death. All three of these mutations are nonsense alleles that truncate the coding sequence N-terminal to the *E93^{1}* allele, which is defective in larval cell death. They also lie N-terminal to the pipsq DNA binding domain. One possibility is that the portion of the protein N-terminal to the 5'-most of the *E93^{Vno}* truncations (*E93^{Vnoe47}*) is capable of executing the cell death function. Another possibility is that translation reinitiation occurs subsequent to termination at the *E93^{Vno}* nonsense changes, so as to produce C-terminal fragments of E93 capable of executing the cell death function.

As an initial test of these possibilities, I drove expression of each UAS construct using fkh-Gal4, which is expressed in salivary glands in the embryo and larva (Henderson and Andrew 2000; Berry and Baehrecke 2007). Although there was some variation among different lines tested for each construct, in general, each construct had a consistent effect. The results are summarized in Table IV-2. Salivary glands were absent in late larvae when fkh-Gal4 was combined with the full length variants UAS-ORF, UAS-Clone2, and UAS-LP0. Expression of the full-length construct UAS-E93 (Baehrecke) did not cause ablation of salivary glands, but I have shown that this construct has an introduced mutation in its initiating ATG. Among the partial E93 constructs tested, UAS-E93^{1}Nterm and UAS-E93^{Vnoe47}Cterm caused deletion of salivary glands, but UAS-E93^{Vnoe47}Nterm and UAS-E93-Δpipsq did not. If we assume that larval salivary gland ablation is a good proxy for the larval cell death function of E93, then the finding that UAS-E93^{Vnoe47}Nterm does not affect salivary glands argues that the normal larval cell

death seen in *E93*^{Vnoe47} mutants is not due to activity of the N-terminal nonsense fragment encoded by this mutation. The finding that UAS-E93^{Vnoe47}Cterm does cause deletion of salivary glands suggests that larval cell death in *E93*^{Vnoe47} mutants may be executed by a C-terminal fragment of E93 produced by translation reinitiation. The finding that UAS-E93^{1}Nterm causes salivary gland ablation was surprising because salivary gland death is defective in E93^{1} mutants, which presumably produce this fragment endogenously. A likely explanation is that this fragment is produced at higher levels in fkh-Gal4/UAS-E93^{1}Nterm heterozygotes than in *E93*^{1} mutants. The complete lack of effect of UAS-E93-*Δ*pipsq suggests that the DNA-binding pipsq domain is essential for the cell death functions of E93.

Table IV-2: The effects of *UAS-E93* constructs when expression driven by fkh-Gal4

	Observed salivary gland
UAS-E93 (Baehrecke)	present
UAS-ORF	deleted
UAS-E93-Clone2	deleted
UAS-E93-LP0	deleted
UAS-E93^{1}*Nterm*	deleted
UAS-E93^{Vnoe47}*Nterm*	present
UAS-E93^{Vnoe47}*Cterm*	deleted

UAS-E93-Δpipsq	present

However it is not clear that the loss of larval salivary glands is a valid assay for the larval cell death function of E93. To determine whether E93 can cause death of imaginal cells, I drove UAS-LP0 by dpp-Gal4 in larvae and used tub-Gal80ts to control the timing of expression. Acridine orange staining after 22 hr of expression revealed cell death induction along the compartment boundaries of the imaginal discs, whereas the wild-type control group had much less cell death in discs (Fig. IV-5). Thus, it is possible that the deletion of salivary glands in fkh-Gal4>UAS-E93 constructs is caused by cell death as well.

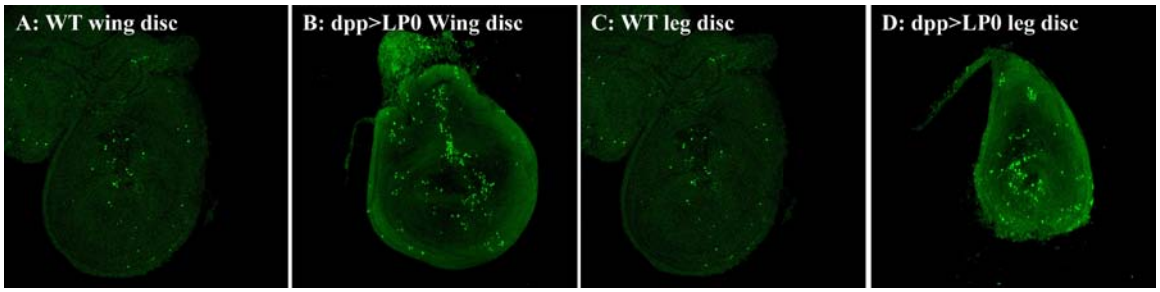


Figure IV-5: UAS construct causes cell death in imaginal discs

A: Wild-type wing disc; B: dpp-Gal4>UAS-LP0 expressed in a wing disc for 22hrs; C: Wild-type leg disc; D: dpp-Gal4>UAS-LP0 expressed in a leg disc for 22hrs; A-D: dpp-Gal4 is repressed by tub-Gal80ts during embryonic and early larval stages, and activated in 3rd instar larvae. All discs stained with acridine orange.

To look directly for a C-terminal fragment encoded by the *E93*^{Vnoe47} mutant, I took advantage of the antigen specificity of the E93 antibodies produced by Mouse 5 and

Mouse 6. The Mouse 5 antibody was raised against antigen II, which is in the N-terminal half of the coding sequence, whereas the Mouse 6 antibody was raised against antigen III, which is in the C-terminal half (Fig. II-1 I, L and Fig. IV-1). If the larval cell death function in the *E93*^{Vnoe47} mutant is executed by a C-terminal fragment of E93 produced by translation reinitiation, then this protein should be detected by the antibody from Mouse 6, but not from Mouse 5. The result was that neither antibody detected any signal in the 2hr AP midgut or 13hr AP salivary gland (Fig. IV-6 C, D, G, H) of *E93*^{Vnoe47} mutants. Even the rabbit antibody raised against all three antigens failed to detect any signal (Fig. IV-6 B, F). If a C-terminal fragment is produced in the *E93*^{Vnoe47} mutant, it is in amounts below detection by our antibodies.

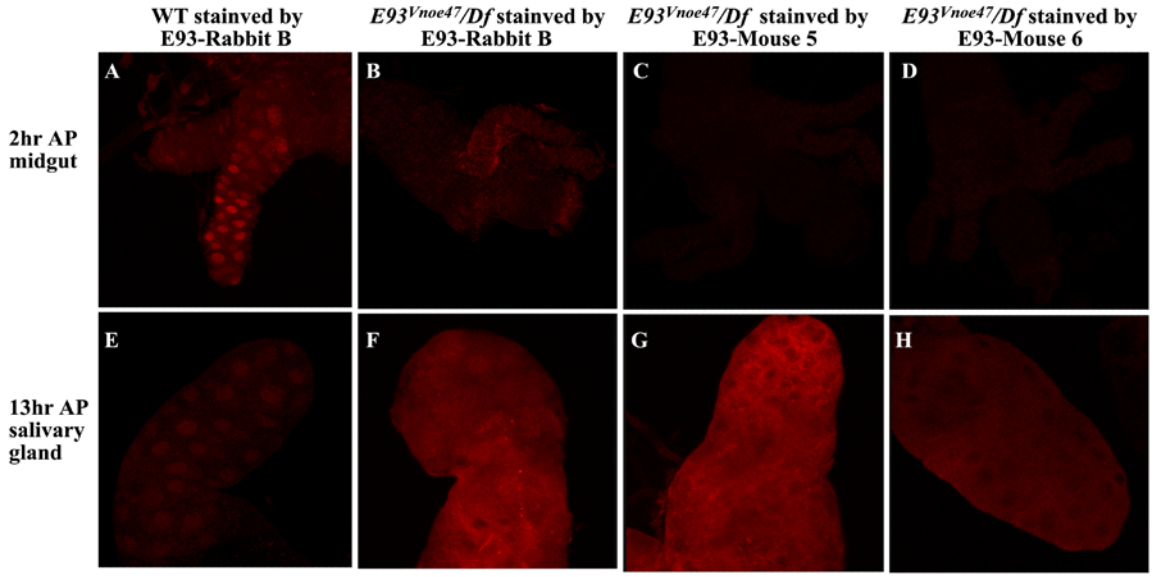


Figure IV-6: The relationship of E93 functional domain and the execution of larval cell death

A-D: 2hr AP midgut; E-H: 13hr AP salivary gland; A, E: Wild-type animal; B-D, F-H: $E93^{Vnoe47}/Df$ mutant; A, B, E, F: stained using antibody from Rabbit B; C, G: stained using antibody from Mouse 5; D, H: stained using antibody from Mouse 6.

Discussion

In this chapter I attempt to understand the complementation of the $E93^{1}$, $E93^{2}$, and $E93^{3}$ alleles, which are defective in larval cell death, and the $E93^{Vno}$ alleles, which are defective in many imaginal patterning processes during metamorphosis. In my work, I showed that clones of cells homozygous for $E93^{1}$, $E93^{2}$, or $E93^{3}$ show completely normal imaginal patterning, demonstrating that these alleles are defective only in driving larval cell death. Conversely, Baehrecke's group has shown that the $E93^{Vno}$ mutants show near normal larval cell death, indicating that these alleles more or less specifically affect the imaginal patterning function of *E93*. Given that these two groups of alleles affect different functions of *E93*, their ability to complement clearly does not require a special interaction between alleles such as transvection or trans-splicing. Rather, the problem lies in understanding why the two groups of alleles specifically affect primarily one or other function.

The requirements for the imaginal patterning function of *E93* seem fairly straightforward: The $E93^{Vno}$ alleles are nonsense changes N-terminal to or within the pipsq DNA-binding domain, suggesting that imaginal patterning is executed by the E93 protein first defined

by Baehrecke's group. Antibody stainings suggest that the $E93^{2}$ and $E93^{3}$ alleles express this protein properly in imaginal tissues, accounting for the ability of these alleles to complement the $E93^{Vno}$ alleles. The $E93^{1}$ allele likely complements the $E93^{Vno}$ alleles because the large N-terminal fragment it encodes (which contains amino acids 1 - 994 and includes the pipsq domain) is sufficient to execute the imaginal patterning functions of *E93*.

The requirements for the larval cell death function of *E93* are much less clear. A major impediment has been that sequence changes have not been identified for the $E93^{2}$ and $E93^{3}$ alleles; neither affects the known coding sequences of *E93.* Possibly, these alleles affect regulatory sequences required for expression in larval cells. The $E93^{1}$ allele does affect the coding sequence (it is a nonsense change at codon 995). This allele causes dramatically reduced expression of E93 in imaginal and larval cells, likely because it causes nonsense-mediated decay (reviewed in Wen and Brogna 2008) of *E93* transcripts. This reduced expression is likely responsible at least in part for the loss of larval cell death in this mutant, since expression of the $E93^{1}$ N-terminal fragment driven by the UAS system causes the ablation of larval salivary glands.

The ability of the $E93^{Vno}$ mutants to execute the larval cell death function is particularly difficult to explain. My finding that expression of the N-terminal fragment encoded by the $E93^{Vnoe47}$ allele does not ablate larval salivary glands suggests that this fragment cannot drive cell death. My finding that the coding sequence C-terminal to the $E93^{Vnoe47}$ truncation does cause larval salivary gland ablation suggests that translation reinitiation

in this mutant might produce an E93 C-terminal fragment capable of driving larval cell death. However, such a reinitiation product was not identified by antibody staining. Perhaps the level of protein expression is too low to be detected by our staining, or translation reinitiation is in a different reading frame, and therefore not detectable by our antibodies. Taken together, these observations raise the possibility that the larval cell death function of *E93* may be executed by a novel product or products from the locus. In Chapter V, I describe my search for such products.

Chapter V : Search for alternate products from *E93*

Abstract

Northern blotting, RTPCR, cDNA screening, and Ribonuclease Protection Assays (RPA) were used to search for alternate transcripts of *E93* that might function specifically in its larval cell death or imaginal patterning functions. Northern blotting confirmed the presence of the 9.5kb *E93* transcript described by Baehrecke and Thummel (1995). The *LP0* transcript was also confirmed, but this RNA and the *E93* RNA are expressed in both larval and imaginal cells, indicating neither is specific for imaginal patterning or larval cell death. I identified a novel *E93* transcript (called *clone2*) by cDNA screening, but it contains the same coding region as *E93*. Using probes from the 3' portion of the *E93* coding region, I also detected three small polyadenylated transcripts on Northern blots. These RNAs appear just prior to salivary gland cell death, suggesting they may be involved in executing larval cell death. However, RPA experiments failed to detect novel splice sites for these RNAs, suggesting they may be degradation products of the larger *E93* RNAs.

Rationale

In Chapter IV, I showed that the $E93^{1}$, $E93^{2}$, and $E93^{3}$ alleles are able to execute the imaginal patterning functions of *E93* normally, but are defective in larval cell death. Conversely, the $E93^{Vno}$ alleles are defective in imaginal patterning during metamorphosis, but can execute the larval cell death function almost normally. One explanation for the specificity of these two allele types is that they affect alternate products of the locus that

execute the two functions. In this Chapter, I describe my efforts to identify alternate products from *E93*.

Methods

Northern Blots

Pupae were staged by collection of white prepupae. Total RNA was extracted from larvae or pupae using the TRI Reagent (Sigma Cat# T9424). PolyA mRNA was purified using the PolyATract mRNA Isolation System IV (Promega Cat# Z5310). All samples were normalized by their O.D. value (measured using a NanoDrop ND-1000 Spectrophotometer), and stored at -70˚C before use.

RNA samples were separated on 1.5% Agarose (ISCBioExpres Cat# E-3120-500) gels in 2.2 M formaldehyde and 1X MOPS buffer (20mM MOPS pH 7.0, 2mM NaOAc, 1mM EDTA pH 8.0), transferred to a Nitrocellulose Transfer Membrane (Micron Separations INC. Cat# E04HY00010), fixed on the membrane by 90 seconds of U.V. irradiation, incubated with radioactive probes, and exposed to X-ray film to detect signals. For reuse with other probes, membranes were stripped in 0.1% SDS or Strip-EZ™ RNA (Ambion, Cat# 1360-1368).

Probe sequences were amplified by PCR, purified by MinElute Gel Extraction Kit (QIAGEN Cat#28604), ligated with the pSTBlue-1 AccepTor™ Vector (Novagen Cat# 70595-4DFRZ), and transformed into NovaBlue Singles™ competent Cells (Novagen Cat# 70181-4PKG) or Electrocompetent Cells (Lucigen Corporation, Cat# 60052-4). The

molecular map of each probe is shown in Fig. V-1. The primers used to generate each probe are included in the Appendix. For rp49, DNA probes were labeled with ^{32}P by Oligolabeling using random primers (Pharmacia Cat# 27-9250-01). DNA probes were also used for the 1st half of pipsq (P1) and the 2nd half of pipsq (P2); these were labeled with ^{32}P by PCR (Fig. V-9). All other probes were RNA, and were labeled with ^{32}P by in vitro transcription from the SP6/T7 promoter (MAXIscript SP6/T7 kit Ambion Cat# 1320). Probes were purified on Sephadex G-50 columns pre-equilibrated with STE (0.1M NaCl, 10M Tris-Cl, 1mM EDTA pH8.0), and denatured before use.

Densitometry was measured by BIO-RAD Fluor-S MultiImager and normalized by the background and rp49 loading control in the same lane with equal area. For example, the normalized-$Band^{density}$ = ($Band^{density}$ - $Background^{density}$) / ($rp49^{density}$ - $Background^{density}$)

Reverse Transcriptase PCR (RTPCR)

2ug total RNA was used for reverse transcription, which was conducted at 42°C or 1 hour, followed by 10 min 92°C heat inactivation of the reverse transcriptase (RETROscriptTM Kit, Ambion Cat#1710). Reaction without reverse transcriptase was used as negative control to ensure no contamination from genomic DNA.

5ul of reverse transcription products were used for the next PCR reaction. Different primers were tested to avoid unwanted side products. The final primers used in RTPCR are shown in Fig. V-4, sequences are included in Appendix. Different cycle times (20-30 cycles) were tested to ensure that the amount of product was below the saturation. The

optimized thermocycle profile was: 95°C 4min one cycle; 94°C 0.5min, 61°C 25sec, 72°C 1.5min (1min per kb of target) 30 cycles; 72°C 5min one cycle, 4°C forever.

cDNA screening

The cDNA LP library were constructed from pupal stage RNA using the Uni-ZAP XR Vector Kit and Uni-ZAP XR Gigapack Cloning Kits. The E93 C-terminal fragment (5exoIII) was labeled by Oligolabeling (Pharmacia Cat# 27-9250-01) and used to screen the LP library. Positive clones were purified, and the pBluescript phagemid excised using the ExAssist Interference-Resistant Helper Phage into the SOLR bacteria strain (Stratagene Cat#200253). The pBluescript phagemid clones were stored in glycerol stock at -80˚C. The molecular map of identified cDNA clones are shown in Fig. V-9. Their sequences are included in the Appendix.

Ribonuclease Protection Assay (RPA)

RNA probes were labeled by in vitro transcription (MAXIscript SP6/T7 kit, Ambion Cat# 1320). RNA samples were hybridized with RNA probes and digested by RNase A/RNase T1 Mix (RPA III^{TM} kit, Ambion Cat# 1415). Samples were separated in 6% acrylamide. Signals were detected by X-ray film.

Results

Northern blotting

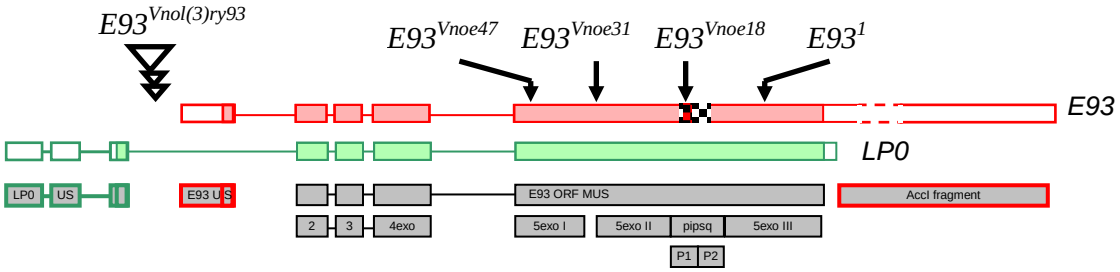


Figure V-1: The molecular map of probes used in Northern blots

The green and red boxes represent *LP0* and *E93* transcripts respectively. The shaded areas represent the predicted ORFs. The dashed lines in *E93* represent omitted sequences. The relative positions of $E93^{Vnoe47}$, $E93^{Vnoe31}$, $E93^{Vnoe18}$ *and* $E93^{1}$ are indicated above the transcripts. The $E93^{Vnol(3)ry93}$ is inserted before the 1st exon of *E93*, and between the 3rd and 4th exon of *LP0*. The grey boxes are the probes used in Northern blots. *LP0 US* (LP0 unique start) is specific to *LP0* (grey box with green outline). *E93 US* and *AccI* fragment are specific to *E93* (grey boxes with red outline). The other probes come from shared common exons of *LP0* and *E93*. The P1 (the 1st half of pipsq) and P2 (the 2nd half of pipsq) probes are DNA probes, all the other probes are RNA probes (rp49 DNA probe is not included in this picture, because it is from a different gene).

I first examined the expression profile of *E93* by Northern blotting. Baehrecke and Thummel (1995) detected a 9.5 kb transcript using an *AccI* fragment from the *E93* 3' UTR as probe. I confirmed this result and found a similar expression profile: the 9.5 kb transcript is expressed at 12hr AP and disappears by 48hr AP in wild-type total RNA probed by AccI RNA probe (Fig. V-2). The same result was obtained when the RNA probe from the 5' unique start region of the *LP0* transcript *(LP0 US)* was used (Fig. V-2). The lower band visible in the *LP0 US* blot is probably ribosomal RNA (Fig. V-2). When

the RNA probes from the 5' start region unique to *E93 (E93 US)* or the *E93 ORF Minus the Unique Start (E93 ORF MUS)* were used, a heavy smear is detected, probably due to the presence of repetitive regions in these probes (Fig. V-2).

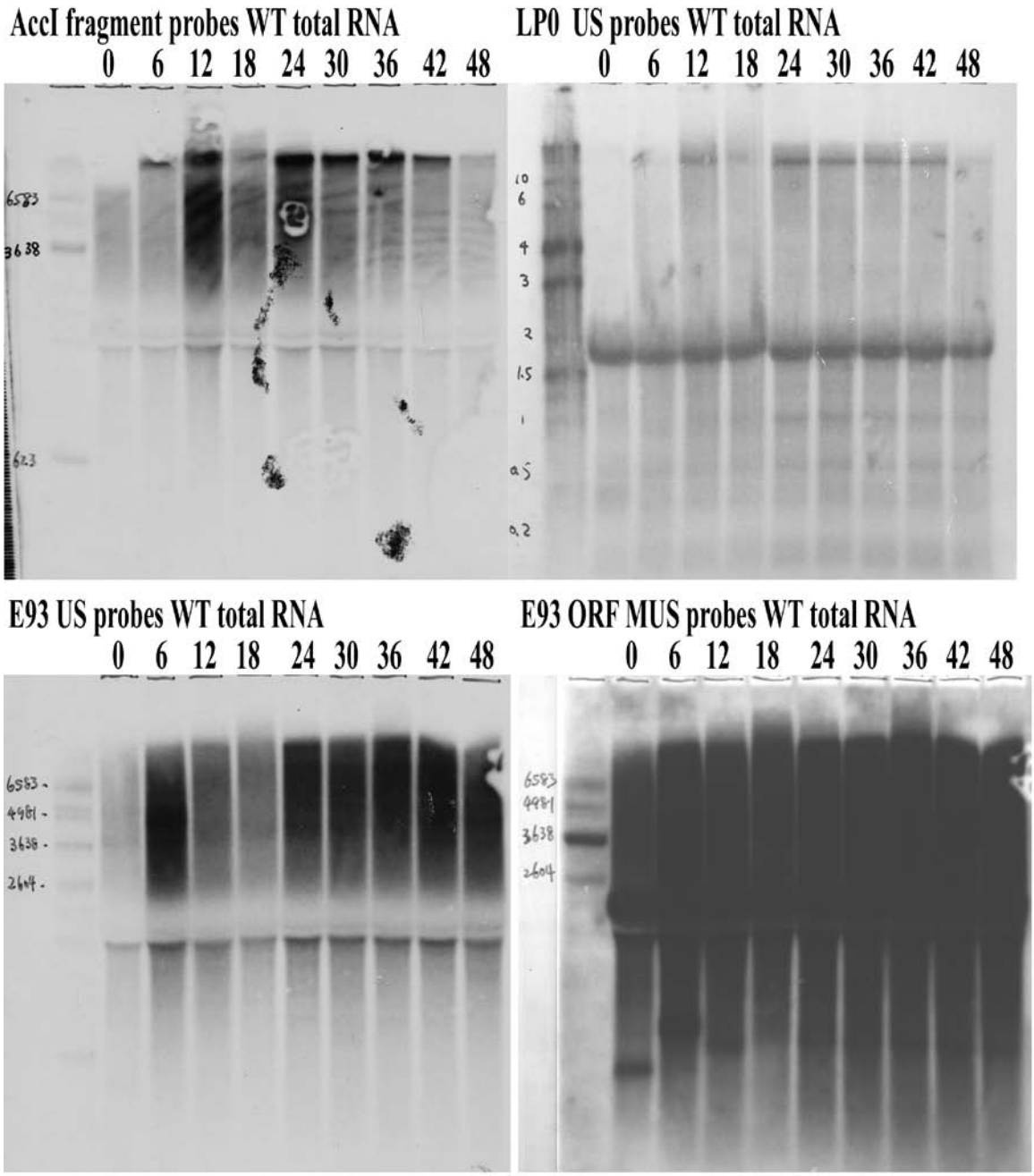


Figure V-2: The expression profile of wild type Total RNA probed by *AccI, LP0 US, E93 US,* and *E93 ORF MUS*

Total RNA of wild-type staged pupae (0-48hr AP) was extracted and 40ug total RNA was loaded per lane. RNA probes of *AccI, LP0 US, E93 US,* and *E93 ORF MUS* fragments were used to probe the blots.

The LP0 and E93 transcripts show no tissue specific distribution

The P-element insertion in the allele $E93^{Vnol(3)ry93}$ lies 5' to the *E93* transcript, and within an intron of the *LP0* transcript (see Fig. V-1, Fig. IV-1). This allele is defective in the imaginal patterning function of *E93* but does not appear to affect the cell death function. We wondered whether this allele might specifically affect expression of the LP0 transcript. The P-element in the $E93^{Vnol(3)ry93}$ insertion carries $rosy^{+}$ (ry^{+}) as a marker. The ry^{+} fragment in this element includes a portion of an adjacent gene, *l(3)S12*. When inserted within an intron, this fragment of *l(3)S12* can act as a splicing acceptor and transcriptional terminator (Horowitz and Berg 1995). Thus, one might expect the *l(3)S12* insertion to cause truncation of the *LP0* transcript, but to have no effect on the *E93* RNA. Such a finding would support the model that the *LP0* transcript is responsible for the imaginal patterning functions of *E93*, while the *E93* transcript is responsible for its cell death functions. Surprisingly, when *LP0 US* was used as an RNA probe, $E93^{Vnol(3)ry93}$ homozygotes showed a 9.5kb band, as in wild type, indicating that termination does not always occur within the insertion. Bands of 5kb and >9.5 kb are also present (Fig. V-3). To determine whether any of these bands contained *l(3)S12* sequences, the blots were reprobed with *l(3)S12* RNA probe. This probe detects the 9.5 kb and >9.5 kb bands plus

a new band at ~6kb, but did not detect the 5kb band, whose origin remains unexplained (Fig. V-3). None of these bands are endogenous *l(3)S12* transcripts, as these are only 0.7kb in size.

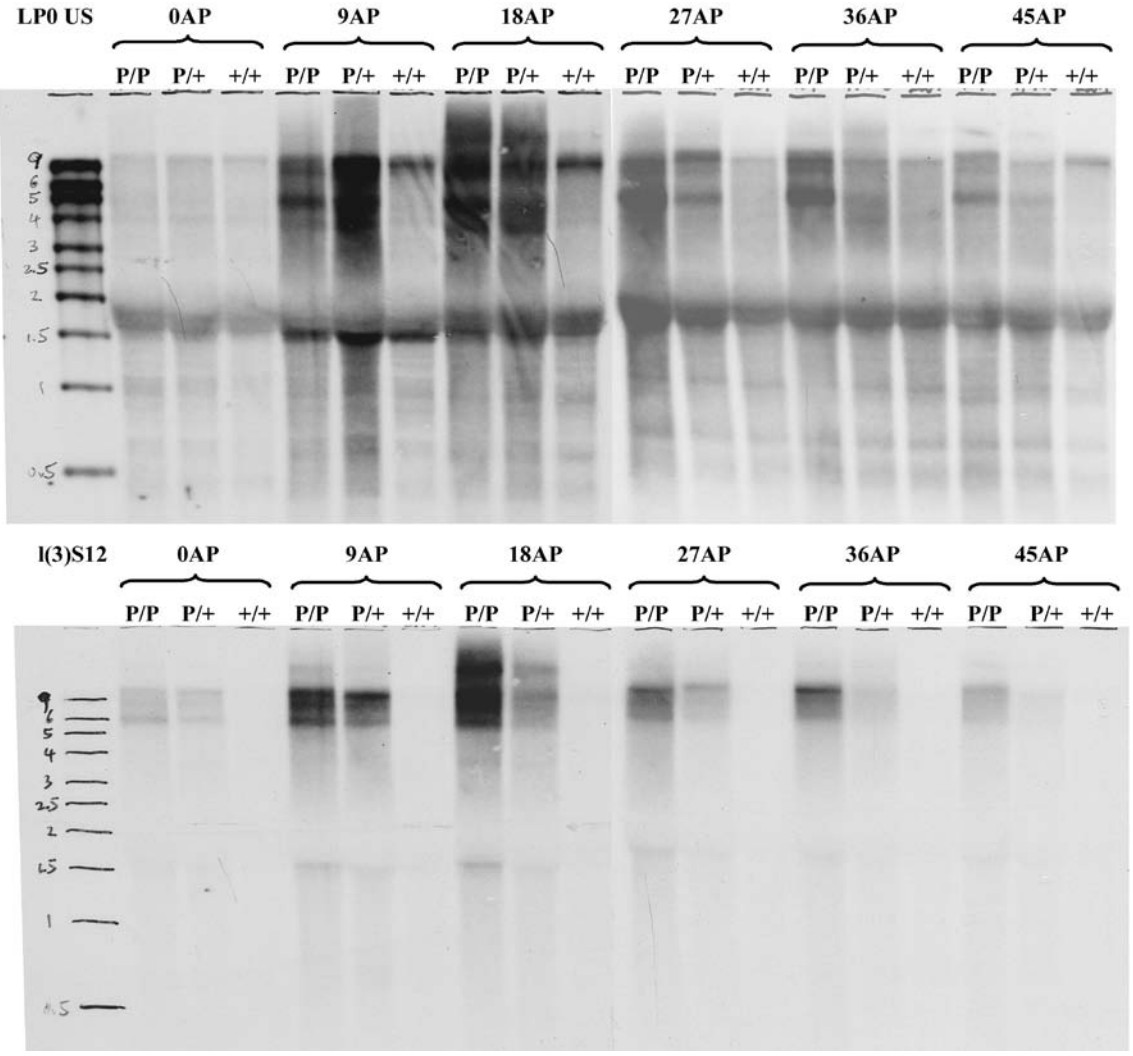


Figure V-3: The expression profile of wild type and *E93*$^{Vnol(3)ry93}$ mutant

Total RNA of *E93*$^{Vnol(3)ry93}$ and wild-type staged pupae (0-45hr AP) was probed by *LP0 US* and *l(3)S12* RNA probes. P/P represents *E93*$^{Vnol(3)ry93}$ homozygotes; P/+ represents *E93*$^{Vnol(3)ry93}$ heterozygotes; +/+ represents wild-type. 40ug total RNA per lane.

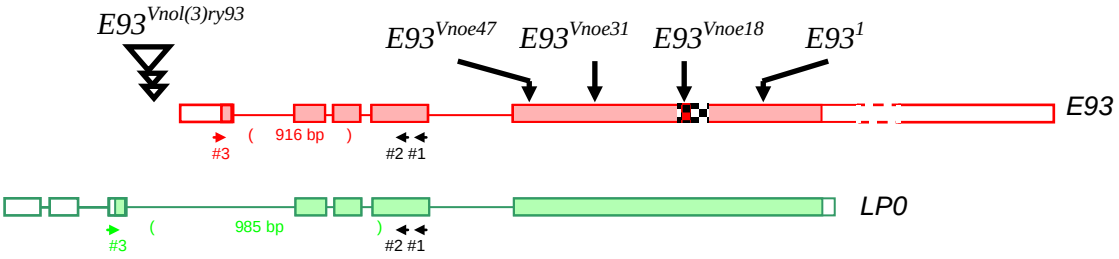


Figure V-4: The molecular map of primers used in RT PCR

The *LP0* and *E93* transcripts are as described in Fig. V-1. The arrows below the *LP0* and *E93* cDNA are the primers used in RTPCR. The sizes of expected RTPCR products are indicated in the parentheses.

To determine whether the $E93^{Vnol(3)ry93}$ P-element sequences are ever correctly spliced out of the mature *LP0* transcript, we performed RTPCR to amplify transcript sequences spanning the insertion site. As indicated in Fig. V-4, a primer complementary to the end of the third common exon of *E93* and *LP0* was used to prime reverse transcription (the black arrow labelled #1 in Fig. V-4; the sequences of this and all other primers are included in the Appendix). PCR was then carried out using a 5' primer specific for the LP0 transcript (the green arrow #3) and an internal backward primer (the black #2 arrow). PCR was also carried out using a 5' primer specific for the *E93* transcript (the red arrow #3) and the internal backward primer (black arrow #2). The PCR products for *LP0* and *E93* are distinguishable by size (985bp for *LP0*, 916bp for *E93*). We were surprised to find that $E93^{Vnol(3)ry93}$ mutant homozygotes produce normal sized RTPCR products for *LP0* (Fig. V-5). These products were sequenced and no differences were found relative to the same PCR products from wild type. This observation indicates that the P and Doc elements do not cause strong transcriptional termination and are correctly excised from

the LP0 transcript in the *E93*$^{Vnol(3)ry93}$ mutant. I also used RTPCR to examine the expression profiles of *LP0* and *E93*. These experiments show that in wild type *LP0* is weakly expressed at 0hr AP, increasing gradually from 9-45hr AP, whereas *E93* is expressed robustly all the way through the period 0-45hr AP (Fig. V-5, whole animal +/+). In *E93*$^{Vnol(3)ry93}$ homozygotes, *LP0* expression is weak in 0-18hr AP, and increases from 27-45hr AP; *E93* is present in all stages (Fig. V-5, whole animal P/P). Compared to wild type, *E93*$^{Vnol(3)ry93}$ homozygotes show a delay in expression of *LP0* and may show an overall reduction in expression of *LP0* and *E93*. I also used RTPCR to examine the tissue specificity of *LP0* and *E93* expression (Fig. V-5). The results indicate that both *LP0* and *E93* are expressed in both imaginal discs and salivary glands. Taken together, my results argue against the hypothesis that the *LP0* transcript is responsible for the imaginal patterning function of *E93* or that the *E93* transcript functions specifically to drive larval cell death.

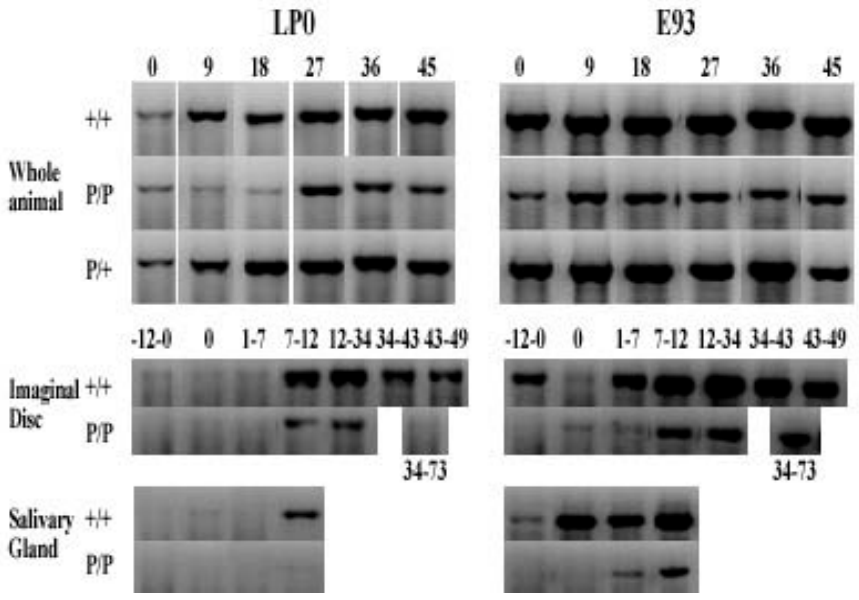


Figure V-5: RTPCR of wild type and *E93*$^{Vnol(3)ry93}$ mutant.

P/P represents *E93*$^{Vnol(3)ry93}$ homozygotes; P/+ represents *E93*$^{Vnol(3)ry93}$ heterozygotes; +/+ represents wild type.

Three small transcripts detected by Northern blotting

To look for additional alternate transcripts, I returned to Northern blotting. Instead of using the entire coding region as probe, which produces a heavy smear, we dissected the coding region into smaller fragments and used these as probes on blots of total and polyA+ RNA from wild type. Two RNA probes from the C-terminal region of the coding sequence (the *Pipsq* probe and *5exoIII* probe see Fig. V-1) provided striking results. These probes detected three strong bands of 1.7kb, 1.4kb and 0.6kb in RNA from pupae at 9hr AP (Fig. V-6). These bands are detectable in total RNA, but are strongly enriched in polyA+ RNA (Fig. V-6). The *5exoI* and *LP0 US* probes appear to detect very weakly these same bands. Other fragments from the ORF detected smeary patterns, with bands of 2.5kb and 4kb visible in polyA+ RNA blots (Fig. V-6).

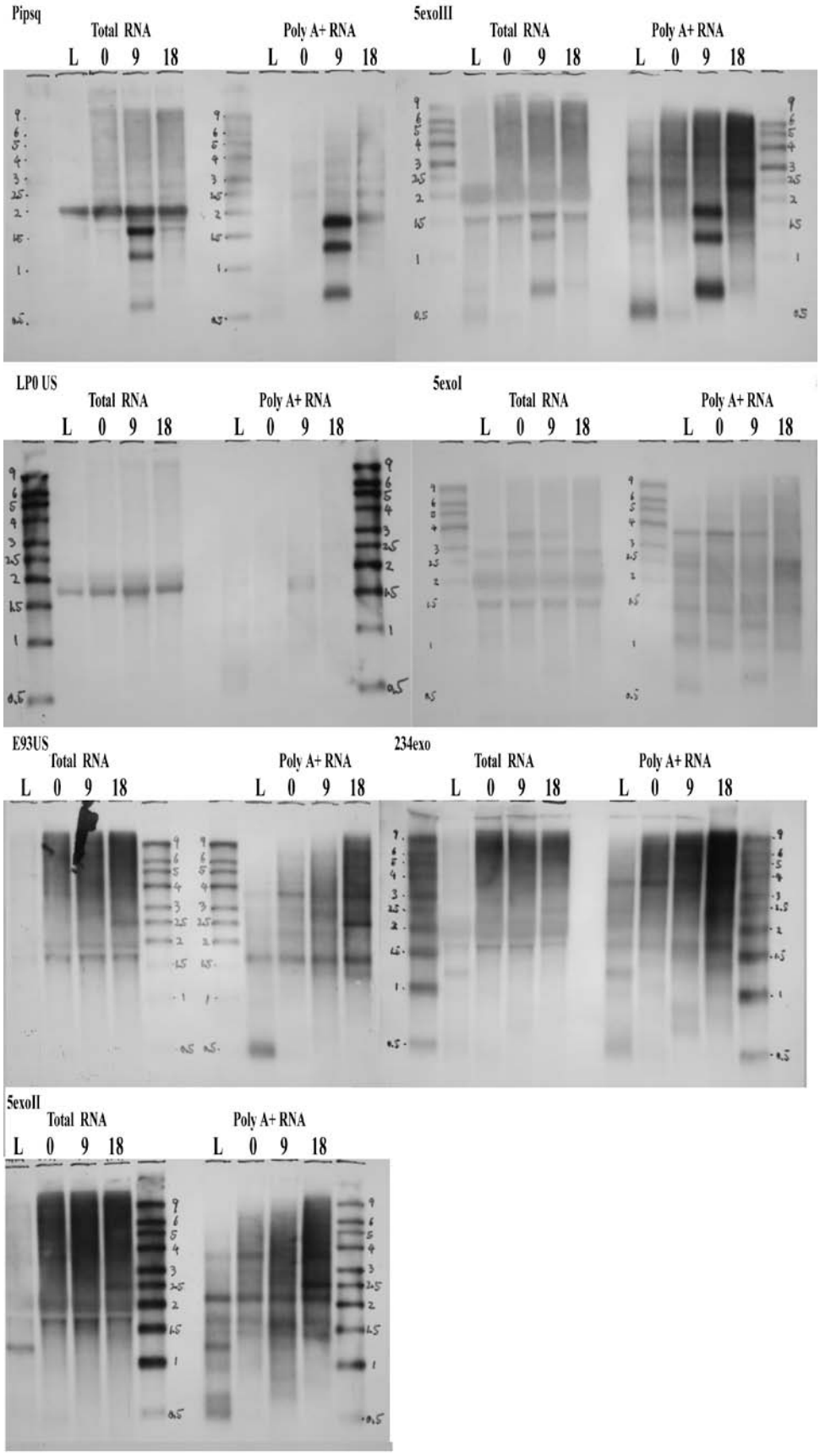

Pipsq
Total RNA
Poly A+ RNA
L 0 9 18
5exoIII
LP0 US
5exoI
E93US
234exo
5exoII

Figure V-6: The expression profile of wild type Total RNA and PolyA+ RNA probed by *Pipsq, 5exoIII, LP0 US, E93 5exo I, E93 US, E93 234exo,* and *E93 5exoII*.

L represents 3rd instar larvae; 0, 9, 18 represent 0-18hr AP. 5ug Total RNA or 0.5ug PolyA+ RNA per lane.

I then used the pipsq probe to check the expression of these small transcripts throughout the period from late third instar larva to 48hr AP in 3 hr intervals. In this period, these RNAs appear only around 9 hr AP (Fig. V-7). Northern blotting of samples taken every half hour from 6.5 hr to 11 hr AP shows that the small transcripts are induced at 7AP and disappear by 11AP (Fig. V-7).

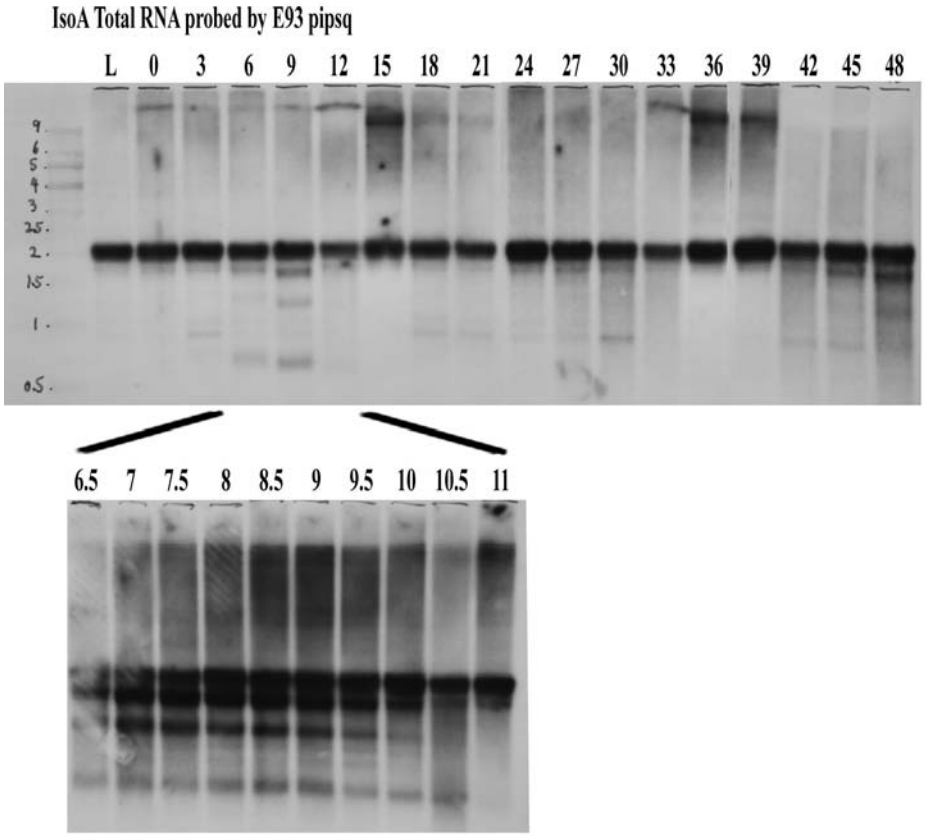


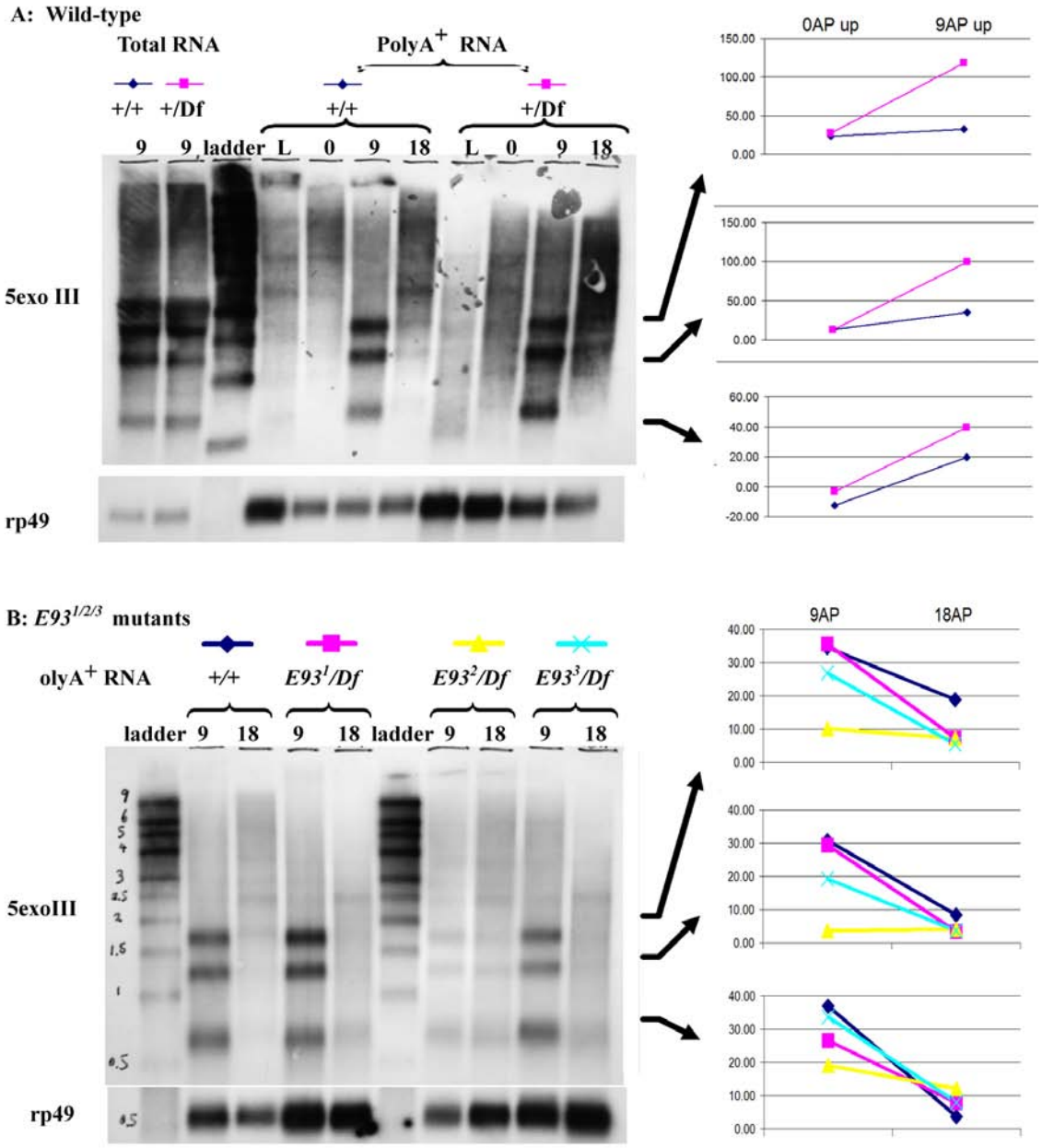
Figure V-7: The expression profile of three small transcripts in WT animal

L represents larvae, numbers above the lanes represent 0-48hr AP, 5ug Total RNA per lane

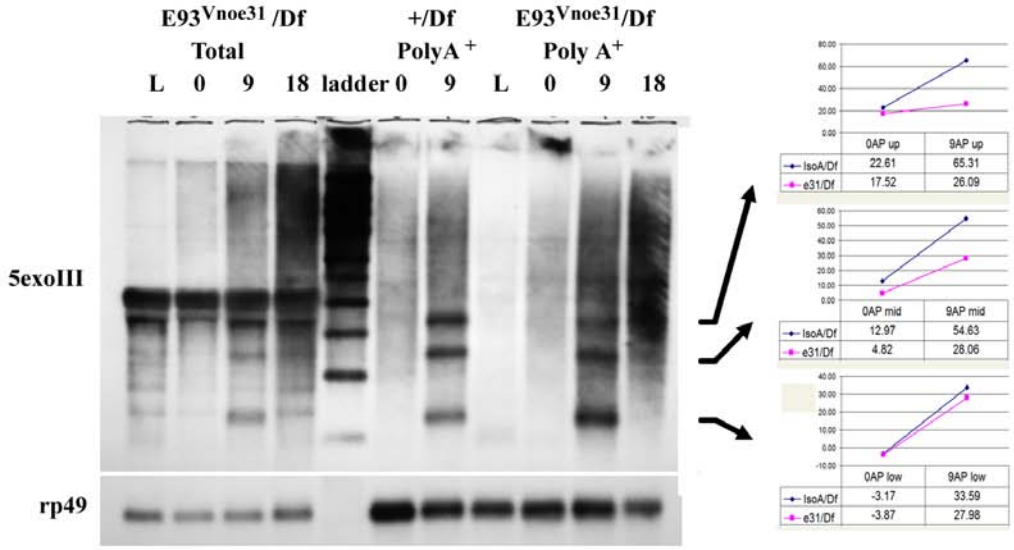
One or more of the 1.7kb, 1.4kb and 0.6kb transcripts might be responsible for the cell death functions of *E93*. There are four reasons to suspect such a role: first, these RNAs include the C-terminal region of *E93* where the $E93^{1}$ allele is located. Second, these RNAs do not appear to contain most sequences from the N-terminal portion of *E93*, where the $E93^{Vno}$ alleles are located. Third, these RNAs are produced just prior to salivary gland cell death. Fourth, the transcripts detected by the C-terminal probes are below the size cutoff of most cDNA libraries, perhaps accounting for why they have not been detected previously.

If the small RNAs are responsible for the cell death function of *E93*, one might expect their levels or size to be affected by alleles that are defective in this function. As a control, I first used 5exoIII to probe Northern blots of RNA from wild-type hemizygotes and wild-type homozygotes. All three bands showed a reduction in intensity by about half in hemizygotes (Fig. V-8 A), suggesting that the small RNAs are encoded by the *E93* locus and are not being detected by cross hybridization. I then examined $E93^{1}$, $E93^{2}$, and $E93^{3}$ mutant hemizygotes. The three small RNAs are reduced in these three genotypes relative to wild type, but no more than expected from the hemizygosity of the locus (Fig. V-8 B). The $E93^{Vnoe31}$ and $E93^{Vnoe47}$mutants also showed small reduction in upper and middle bands (Fig. V-8 C, D), but $E\ 93^{Vnoe18}$ and $E93^{VnoPry93}$ showed similar intensity

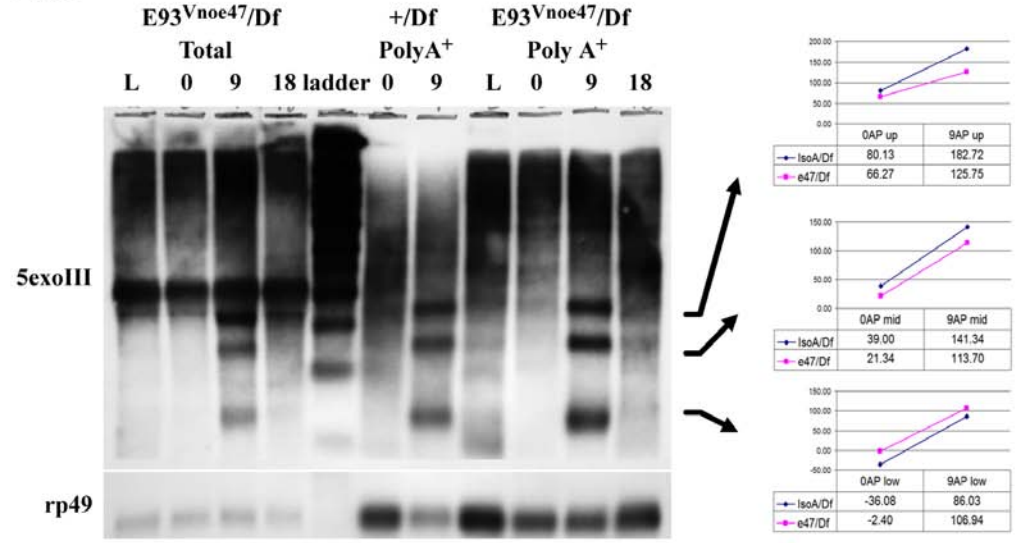
compared with wild type (Fig. V-8 E, F). The *Tp(3;3)Vno* mutants showed a smeary pattern (Fig. V-8 G).



C: E93^{Vnoe31}



D: E93^{Vnoe47}



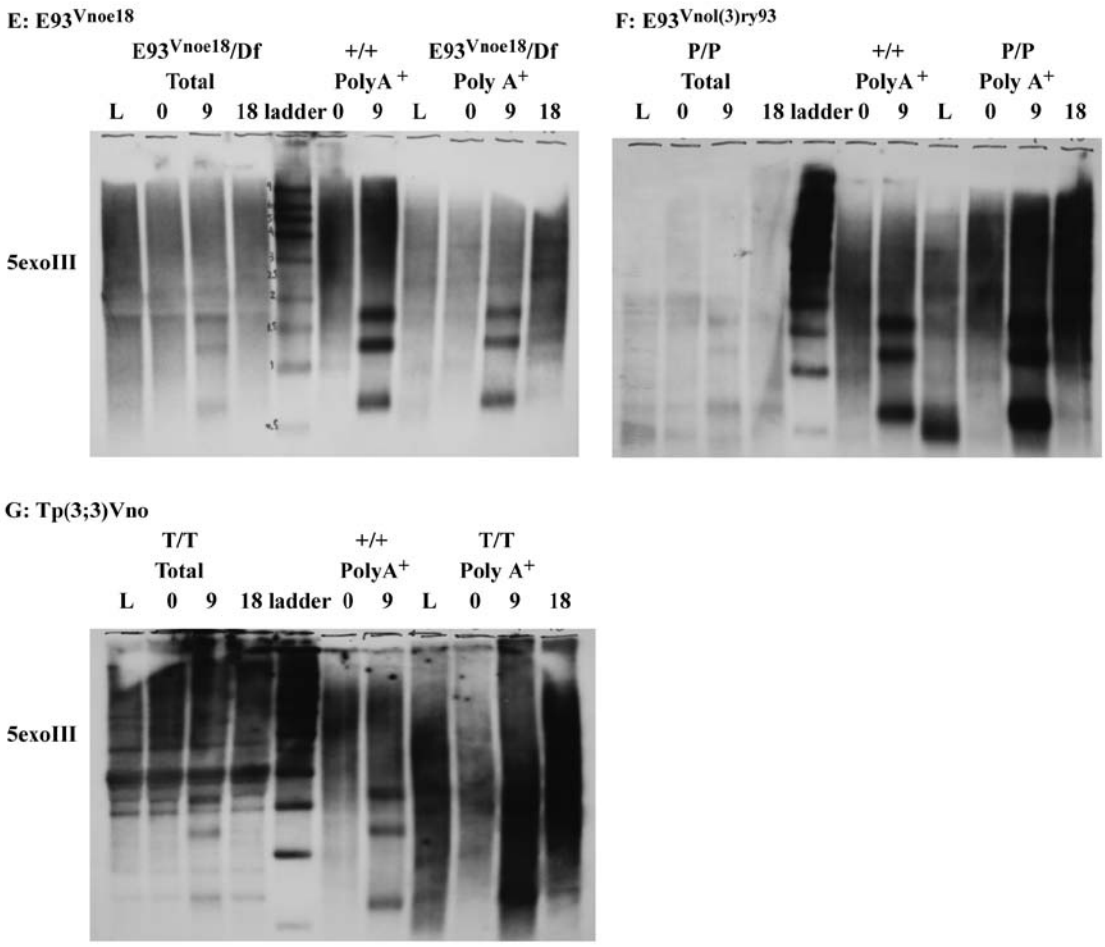


Figure V-8: The reduction of small RNA transcripts in *E93*$^{1/2/3}$ mutants, *E93*^{Vno} mutants, and wild-type hemizygotes

rp49, a ribosomal protein gene, was used as loading control (prepared as DNA probe by Oligolabeling using random primers). The right panel shows the densitometry of the three small bands after normalization. Wild type and the mutants are color coded as indicated above the lanes. 5ug Total RNA or 0.5ug PolyA+ RNA per lane.

I also used sub-fragments of the E93 pipsq probe to see whether these bands are specific to a smaller region. The 1st half of pipsq DNA probe (P1 in Fig. V-1) did not detect any

signal after 98hr exposure (Fig. V-9 A). The 2nd half of pipsq DNA probe (P2 in Fig. V-1) detected the two smaller bands (Fig. V-9 B).

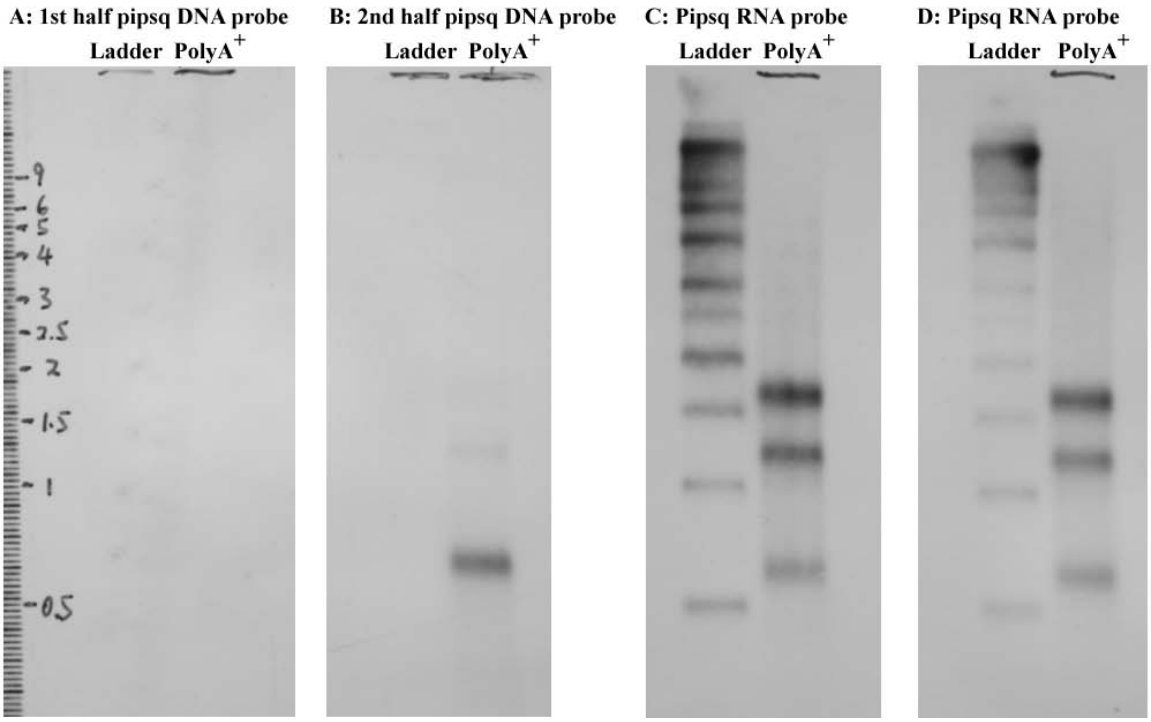


Figure V-9: Authenticity test of the three small transcripts by subfragments of pipsq probe

A: Wild-type 9hr AP PolyA+ RNA (0.5ug) probed by the 1st half of pipsq DNA probe; B: Wild-type 9hr AP PolyA+ RNA (0.5ug) probed by the 2nd half of pipsq DNA probe; C & D: The blots used in A & B were stripped and reprobed by pipsq RNA probe to confirm the RNA sample is still intact.

To determine whether RNAs were transcribed from the sense strand of pipsq and 5exoIII, I used sense strand RNA probes from these regions to probe Northern blots. The sense strand of 5exoIII did detect a small RNA after 4hr exposure (Fig. V-10 B). However, the

sense strand of pipsq did not detect any obvious band, even after 20hr exposure (Fig. V-10 C).

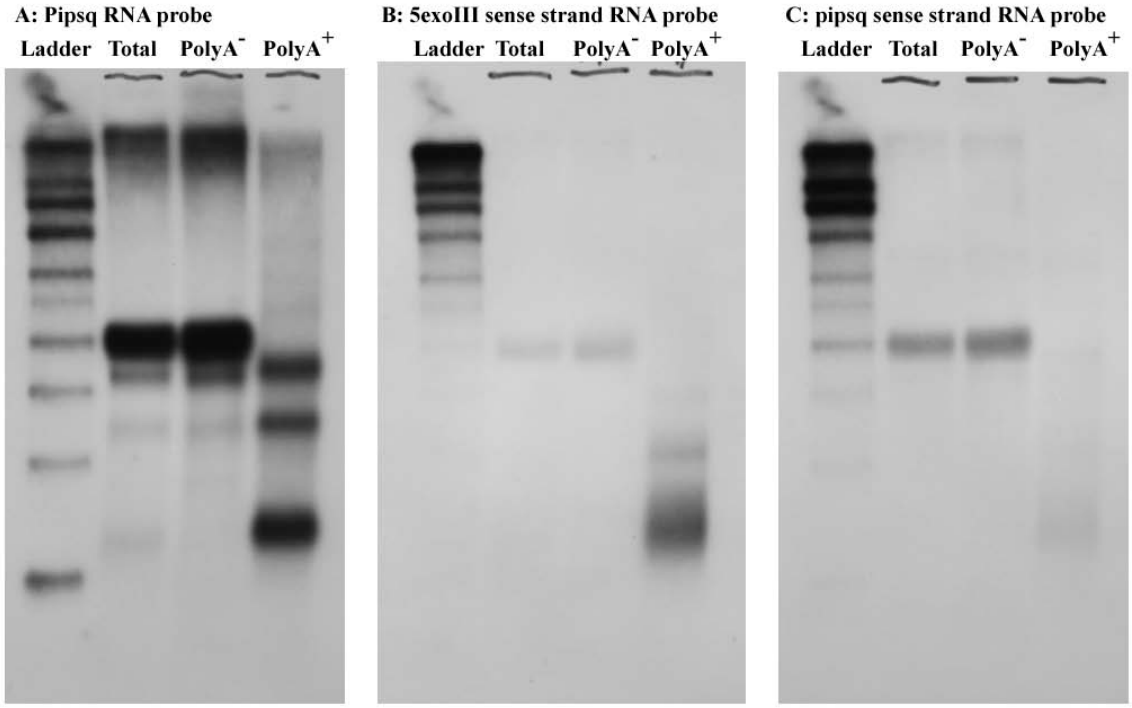


Figure V-10: Authenticity tests of the three small transcripts by sense strand RNA probes

A: The wild-type 9hr AP Total RNA (5ug), PolyA- RNA (5ug), and PolyA+ RNA (0.5ug) probed by pipsq RNA probe; B: The blot used in A was stripped and reprobed by 5exoIII sense strand RNA probe; C: the blot used in A and B was stripped and reprobed by pipsq sense strand RNA probe.

Attempt to identify the three small transcripts by RPA

The three small RNAs that appear at 9 hr AP suggest that novel splice sites may be present in the 3' portion of the *E93* coding sequence. To search for such sites, I used the *Pipsq* and *5exoIII* probes in Ribonuclease Protection Assays (RPA). When I used the

pipsq probe, I saw two small protection bands in Total and PolyA- RNA lanes, but not in PolyA+ RNA (Fig. V-11 lane 3-5). Since Northern blotting indicates that the three small transcripts are enriched in PolyA+ RNA (see above), it seems unlikely that the protected bands represent these transcripts. No specific protection was detected for the 5exoIII probe (Fig. V-11 lane C-E). A probe covering the boundary of E93 5exon (which protects the first 240bp of E93 5exon) was used as a positive control to ensure the RPA assay was working properly (data not shown).

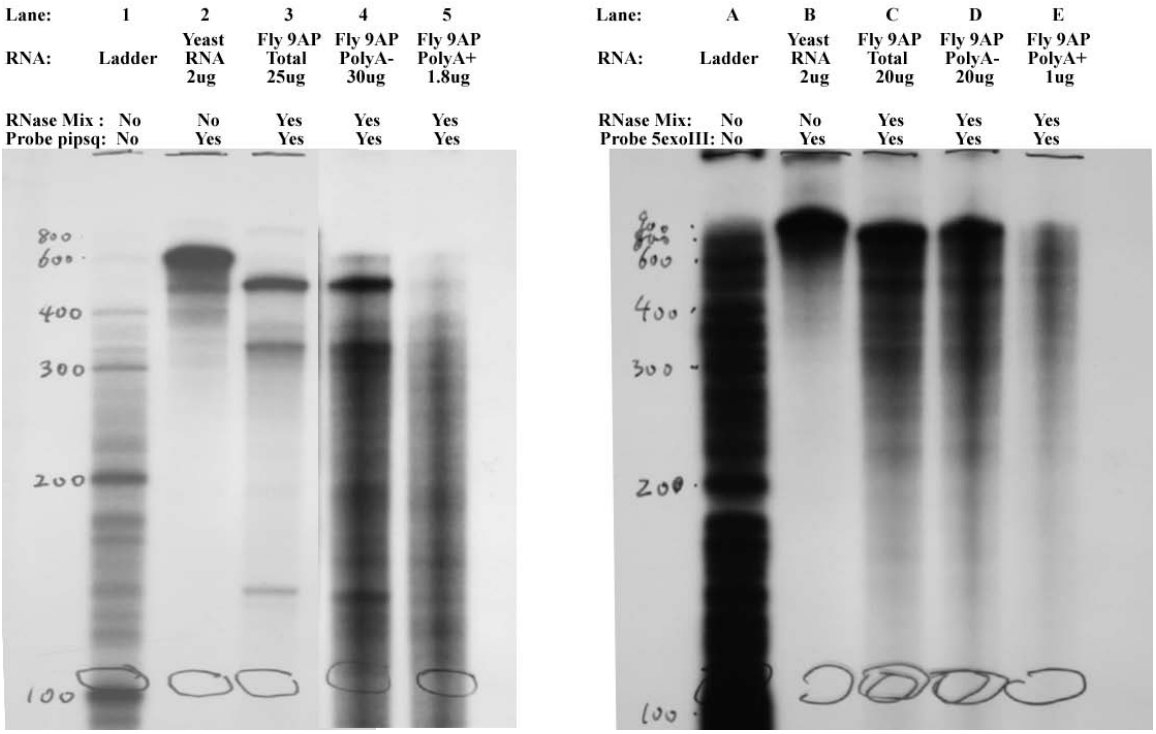


Figure V-11: RPA assay

The blot on the left was probed by Pipsq, the blot on the right by 5exoIII; Lane 1 and A are RNA ladders; Lane 2 is Yeast RNA (2ug) plus Pipsq probe (560bp expected); Lane 3 is Pipsq protected by Fly 9 hr AP Total RNA (25ug) (445bp expected); Lane 4 is Pipsq protected by Fly 9 hr AP PolyA- RNA (30ug); Lane 5 is Pipsq protected by Fly 9 hr AP

PolyA+ RNA (1.8ug); Lane B is the 5exoIII probe plus Yeast RNA (2ug) (904bp expected); Lane C is 5exoIII protected by Fly 9 hr AP Total RNA (20ug) (802bp expected); Lane D is 5exoIII protected by Fly 9 hr AP PolyA- RNA (20ug); Lane E is 5exoIII protected by Fly 9 hr AP PolyA+ RNA (1ug). The origin of the two small bands (~150 bp and ~350 bp) in lanes 3&4 is not known, but is unlikely to be due to the small RNAs detected by Northern blotting.

cDNA screening for additional alternate transcripts

As a second approach to identifying alternate transcripts, I screened a fly cDNA library constructed from pupal stage RNA (the LP library) using a C-terminal fragment (*5exoIII*) as probe. I recovered 6 clones, but none are alternatively spliced and all are at least 2kb long (Fig. V-12, white boxes, detailed sequence information is available in Appendix). However, I did make two new discoveries. First, I found two new 5' exons in *clone 2*. In genomic DNA, these exons extend 3.2kb upstream of the *E93* transcript and 1.7kb upstream of the $E93^{Vnol(3)ry93}$ insertion. Second, all six clones end at the same place, 300bp downstream of the ORF, suggesting that most *E93* transcripts do not contain the large 3' UTR present in the 9.5kb *E93* transcripts.

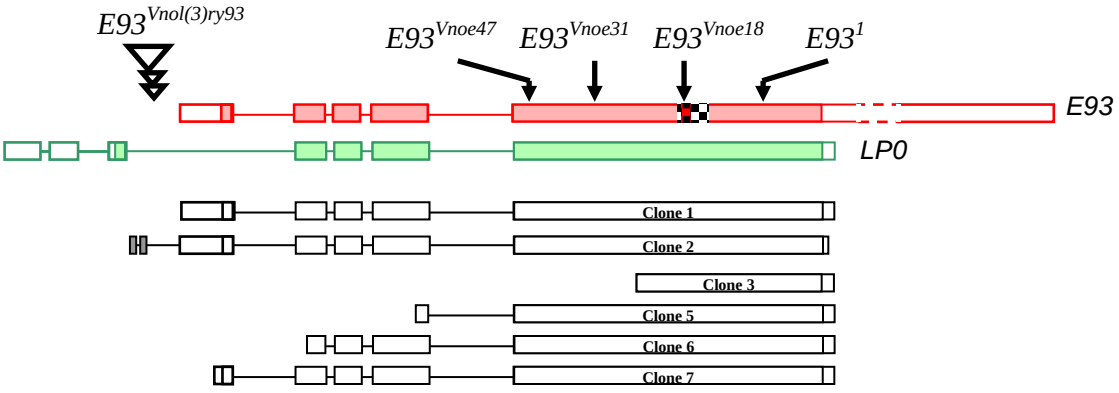


Figure V-12: The molecular map 6 clones discovered from cDNA screening.

The *LP0* and *E93* transcripts are as described in Fig. V-1. The white boxes are the 6 clones identified from cDNA screening. *Clone 2* has two novel exons presented by grey boxes.

Discussion

In this chapter, I search for alternate products of E93 that might be responsible for its larval cell death and imaginal patterning functions. Northern blotting confirmed the 9.5kb *E93* transcript described by Baehrecke and Thummel (1995) and provided a profile for its expression. Probes specific for the *LP0* transcript also detect RNAs of 9.5 kb. The *LP0* transcript appeared initially to be a good candidate for executing the imaginal patterning functions of *E93*. $E93^{Vnol(3)ry93}$ is a *Vno*-type allele associated with the insertion of a P element and two Doc elements within the third intron of the *LP0* transcript, just upstream of the E93 transcript. The P-element in the $E93^{Vnol(3)ry93}$ mutant carries a fragment of *l(3)S12* that in other contexts causes termination of transcription (Horowitz and Berg 1995). Therefore, an attractive model was that premature termination of the *LP0* transcript in this allele was responsible for its effects on imaginal patterning. However, to my surprise, I found that the *LP0* transcript is not eliminated in the $E93^{Vnol(3)ry93}$ mutant, indicating that transcriptional termination by the *l(3)S12* sequence does not occur in this context, or occurs with low efficiency. In addition, RTPCR experiments demonstrated that the P and Doc sequences are correctly excised from the *E93* primary transcript. RTPCR experiments also demonstrated that the *LP0* and *E93* transcripts are expressed in

both larval and imaginal tissues, arguing against the possibility that either specifically executes the larval cell death or imaginal patterning functions of *E93*.

Northern blots also demonstrated the existence of three small transcripts in PolyA+ RNA (Fig. V-6, Fig. V-7). These are good candidates for the larval cell death function of *E93*, as they appear just prior to salivary gland death and are detected by the 3' portion of the coding sequence, near where the $E93^{1}$ allele is located. Attempts to identify these RNAs by RPA were inconclusive; no novel splice sites were detected using the pipsq and 5exoIII regions of the *E93* coding sequence as probes (Fig. V-10). The small RNAs may be degradation products of *E93* produced during apoptosis and their functions, if any, may be unrelated to cell death.

Besides the three small RNAs, transcripts of 2.5kb and 4kb were detected by many probes (E93US, 234exo, 5exoI, 5exoII, and 5exoIII, Fig. V-6). Although these bands are variable in intensity, they are very robust compared with the original 9kb band detected by the 3' AccI probe, suggesting that some E93 transcripts may not contain the long 3' UTR region. In an attempt to identify these potential alternate transcripts, I screened for additional cDNAs using 3' coding sequences as probe. Of six cDNAs identified, one (*clone2*) has a novel 5' exon, but it contains the same coding region as *E93*.

Chapter VI : Future directions

The study of the role of *E93* in imaginal patterning during metamorphosis promises to provide important insights into how stage-specificity is achieved during development. Here are some questions I think future work should be directed toward answering:

A. How does *E93* regulate *Dll* expression? I have shown that *Dll* lies downstream of *E93* in the bract pathway, but we do not know whether E93 regulates *Dll* through direct binding or through other genes and/or cofactors. Identification of the *Dll* bract enhancer and study of its interaction with E93 and Pnt is a high priority, and could serve as a model for understanding how *E93* provides temporal specificity during metamorphosis.

B. Where in the bract pathway does ecdysone signaling act? So far, clones that are knocked down for the ecdysone receptor have failed to show any effect on *E93* expression in bract cells. Clones having a more severe knockdown of receptor activity, either *usp* mutant clones or EcR RNAi clones, need to be generated to test whether ecdysone signaling is required for *E93* expression in bract cells and other imaginal tissues. If ecdysone does not control imaginal expression of *E93*, then it will become a key to determine whether EcR interacts directly with the *Dll* bract enhancer and/or with Pnt.

C. Are distinct domains of E93 involved in executing the imaginal patterning and larval cell death functions of *E93*? I have generated stocks which should allow testing the abilities of portions of E93 to rescue the cell death and imaginal patterning functions of

E93. These experiments could provide an explanation for the different phenotypes of the *Vno* and cell-death alleles of *E93*.

D. What is the relationship of *E93* and *pnt* during bract formation? My data suggest that *pnt* activates *E93* expression early in metamorphosis, but that these genes engage in mutual repression at later stages. The changing roles of these genes should be explored further by generating ectopic expression and loss of function clones of *E93* and *pnt*.

E. What is the nature of $E93^{2}$ and $E93^{3}$ alleles? My results suggest that $E93^{2}$ and $E93^{3}$ may be mutations in larval-cell specific regulatory sequences of *E93*. To test this idea, the changes present in the $E93^{2/3}$ alleles should be determined by a combination of genetic mapping and DNA sequence analysis.

F. Are alternate *E93* transcripts responsible for the larval cell death and imaginal patterning functions? My work did not resolve the role of the small *E93* RNAs detected by 3' probes. Further work should be conducted to determine their structure and expression patterns.

G. What is the role of *E93* in patterning other tissues? Much work remains to determine the role of *E93* in patterning tissues other than bracts. Because so much is known about eye development, the role of *E93* in patterning the 2^{nd} and 3^{rd} pigment cells is likely tractable; a first approach here would be to use existing *lacZ* markers for specific cell types to determine which cells in the eye express *E93*. The role of *E93* in wing vein

formation is likely also tractable, as the wing vein enhancer of *dpp* has been defined (Sotillos and de Celis 2006). *E93* is known to be expressed in the heart during remodeling (Zeitouni et al. 2007), but its role here is unknown. As a first approach, heart remodeling should be examined in the $E93^{Vno}$ mutants to determine if abnormalities are present. The different cases of neuronal remodeling should also be examined in $E93^{Vno}$ mutants.

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Appendix

Stocks used in the experiments

hs-rasV12/CyO perimon
w^{118}; Df(3R) Exel 6188 / TM6B (E93 Df stock)
Tp(3;3)Vno / TM6B
w/+; +; $E93^{Vnol(3)ry93}$ / TM6B BL# 10616
UAS-pnt.P1: w[1118]; P{w[+mC] pnt[P1.UAS]=UAS-pnt.P1}3 (BL# 869)
Aos-lacZ: P{ry[+t7.2]=PZ}argos[05845] CG33158[05845] ry[506]/TM3, ry[RK] Sb[1] Ser[1] (BL# 11674)

y w FLP122; FRT82B CD2 y^{+} M(3)W Bsb / TM6B
y w FLP122; FRT82 Ubi-GFP83/TM6B
FRT82B sr e^{s} $E93^{Vnoe47}$ ca/TM6B
FRT82 roe p^{p} cu sr $pnt^{\Delta 88}$/TM6B
FRT82B cu $E93^{1/2/3}$ ca / TM6B ♂

y w FLP12; Act>y^{+}>Gal4 UAS-GFP
y w FLP12; Act>y^{+}>Gal4 UAS-GFP; tub-Gal80^{ts} E93^{Df}/TM6B
UAS-EcR-RNAi: w[1118]; P{w[+mC]=UAS-EcR-RNAi}97 on 3rd (BL#9326)
UAS-E93-ORF; E93^{Vnoe47}/TM6B

E93 antigen I-III sequence and primers used for amplification

E93 antigen-I
(543..782 Gly47-Ala126)
E93 antigen-I (F)543-558: CCGGAATTCggttccgaggacgagc
E93 antigen-I (B)782-768: CGCAAGCTTGggccgacggtctcca

GGTTCCGAGGACGAGCCATCGCAATACAACCACAGCAGCAAGGAGATCAGC
CAGAGCAATCCCAACCACTGTAAGACAGAGAACCACCGTCTGGAGCAGCAA
CACAACGGCAGCCAGCTATTGGAAGAAGAAGATTCTGAGAACAACCAAACA
TCACACGATTCATCACGTACACCAACACCGGGAGCCACCAGTACACCATCAC
CACCGCCAGAACCCATCGATTGGAGACCGTCGGCC

E93 antigen-II
(1257..1604 Ala285-Ala400)
E93 antigen-II (F)1257-1272: CCGGAATTCgcactcaaagatacac
E93 antigen-II (B)1604-1590: CGCAAGCTTGcgcattgctgtccac

GCACTCAAAGATACACCCAGTCCCAGTGTGGATGCACCGCTCGATCTTAGCA
GCAAACCATCGCCGAACTCATCGATTAGCGGCGATGTGAAGTCCGTCAGAGC
CTGTGCCACGCCCACGCCGTCGGGAAGAAGGGCGTACAGTGAAGAGGATCT
GAGCCGGGCCCTACAGGATGTGGTGGCCAACAAGCTAGATGCCCGGAAATC
GGCTAGCCAGCACCATGAGCAGCGCTCCATTCTGGACAACCGGCTGTTCAAG
ATGAAACACCATGACCAGGAGCAGGATCATGATGGCGACGAGCTCGAGGAC
TCCAACGATGATGCTGAGGCGGAAGTGGACAGCAATGCG

E93 Antigen-III
(3399..3779 Asn999-His1125)
E93 antigen-III (F)3399-3414: CCGGAATTCaaccatcggaacaatg
E93 antigen-III (B)3779-3765: CGCAAGCTTGgtgactgctgctgct

AACCATCGGAACAATGACTACGCCGCCACCTGTTCGAGTGCCAGCGGGGAGA
GCGTAAAGCGGTCGGGCAGTCCCATGGGCAACTATGCAGACATCAAGCGGG
AGCGCCTGAGCGCCGACAGCGGCGGCAGCAGCGATGAGGAGCACTCGGCCA
GCCACATCAACAACAACAACAGCGATTTGGCGCACAACAAGAACAAGAGCG
GCGGCGGCGGCGGCGGCGGCGGCAATGGCCAGACCAATGGGAACGGCAGGA
GCAGCCGGATGACGTCGCGGGATGATTCCGAAACGGATGCCAGCAGCTTGAA
GAGCGGCGAAAGTGGCGGCCAGCAAAACCACAAAATGATGGATCTCAATGG
CGGCAGCAGCAGCAGCAGTCAC

Sequence of E93 UAS constructs

Note: Boxed capital letters are predicated ORF of each construct. Small letters are flanking sequences that inserted into the vector with the predicated ORF.

UAS-ORF

Primers:
E93 CAAC-ORF start primer (E93_CAAC-ORF(f) (CAAC-405-424)):
caacatgcacatcagcagctatga
#13 E93-clone2-end (4007-4026)(4599-4618, clone2 numbering):tttgtatggtactatcaaaa

Sequence:

caac

ATGCACATCAGCAGCTATGAAATATCCCTAGAACGCGTTGCTGAAGAATGTA

TGGGTCGCAGGCAATGGAAACATTATCAAGACAAACTGACGTGCAGCCACTT

GAATATCGAGGAGCAACAGCCCATAGCAATAGCCGGTTCCGAGGACGAGCC

ATCGCAATACAACCACAGCAGCAAGGAGATCAGCCAGAGCAATCCCAACCA

CTGTAAGACAGAGAACCACCGTCTGGAGCAGCAACACAACGGCAGCCAGCT

ATTGGAAGAAGAAGATTCTGAGAACAACCAAACATCACACGATTCATCACGT

ACACCAACACCGGGAGCCACCAGTACACCATCACCACCGCCAGAACCCATCG

ATTGGAGACCGTCGGCCAAGTGCAACTTCTGTGTTAACGGTCGCCTGCTAAC

GGTTAACGCCCAGGGCAAGTTGGTGGCCGAGTCAGCAGCAACTGCCACTAGT

AGTAGCACTAGTAATAGTCACATTCATCAGCACGACAGTGACAGCAACTCGA

GTGCATCACTGCCCCACCACATCAGCAGCAGCAGCAGCAGCAACAACAATA

GCAGTGGCAACAGGGCACGCCACATTGCTGCTGCAAGTGCAAGAGCAACAC

CAGCAGCGGCCACACCCGCCAACTCCCTTGAACTCTACAAGCTGCTGACCCA

GCGGGCAGCCAAAATGACATCGATGGACTCGATGGCCGCCCAGCTGGCGCA

ATTCTCACTGCTGGCCGACTTCAATCTGATCAACTCGCTGGCCAGCCAACAGC

AGCAGCAGCAGCAGCAACAGATCGCTAGTGCGGTAACGCCAACTACCTCAG

AAGTATCTGCAGCCGCAATCAGTCCCGCACTCAAAGATACACCCAGTCCCAG

TGTGGATGCACCGCTCGATCTTAGCAGCAAACCATCGCCGAACTCATCGATT

AGCGGCGATGTGAAGTCCGTCAGAGCCTGTGCCACGCCCACGCCGTCGGGAA

GAAGGGCGTACAGTGAAGAGGATCTGAGCCGGGCCCTACAGGATGTGGTGG

CCAACAAGCTAGATGCCCGGAAATCGGCTAGCCAGCACCATGAGCAGCGCTC

CATTCTGGACAACCGGCTGTTCAAGATGAAACACCATGACCAGGAGCAGGAT

CATGATGGCGACGAGCTCGAGGACTCCAACGATGATGCTGAGGCGGAAGTG

GACAGCAATGCGTCGACACCGGTGTATCCGGCAGAGTTTGCAAGGGCACAAC

TGCGCAAACTGAGCCACCTGTCCGAGCACAATGGCAGCGATCTGGGCGAGG

ATGTGGATCGTGGATCGCCGAAAATGGGGCGACATCCGGCCTGTGGCAATGC

CAGTGCCAATCAGGGCGCACCGCCATCCATTCCGCTGGATGCCAATGTCCTG

CTGCACACTCTGATGCTGGCTGCTGGGATTGGTGCAATGCCGAAGCTGGATG

AAACGCAAACGGTGGGCGACTTTATCAAGGGTCTGCTGGTGGCCAACAGTGG

TGGCATAATGAACGAGGGACTGCTAAATCTGCTGTCCGCCAGTCAGGAGAAC

AGCAATGGCAATGCCTCGCTGCTGCTGCAACAGCAACAGCATCAGCAACACC

ATCAGCAACACCATCAGCAGCAGCAGCAGCAGCAACATGTCGCCGCCTACCG

GCATCGCCTGCCCAAGTCGGAGACTCCGGAAACGAACTCCTCGTTGGATCCG

AACGATGCCAGCGAGGATCCCATACTGAAGATTCCGTCCTTCAAGGTCAGCG

GTCCGGCCAGCAGCAGCAGCCTGTCGCCGGGCGGACTGGTTGGTGGTCACCA

CCATCCGCTGAACAACAACAACAGCCTCAGCATCAGCAACAACAGCAACCA

CAGCAGCAACAGCCATCGGAACGGCAGCAATCGCAGCCCGCATTCCGCATCG

CCCATGCTGGCCGCGGCCGTGGCCCAAGGTGGCTACTCCGCCGGCAACAGTT

TGCTGACCTCATCCTCGTCTAGCATACAGAAGATGATGGCCAGCAATATCCA

GCGCCAGATCAACGAACAGAGTGGCCAGGAGAGTCTCAGGAACGGAAATGT

TAGCGATTGCAGCAGCAACAATGGCGGCTCCTCCTCGCTGGGATACAAGAAG

CCGAGCATTTCGGTGGCCAAGATCATTGGCGGAACGGACACCTCACGGTTCG

GAGCCTCGCCCAATCTGCTGTCCCAACAGCACCATTCGGCTCACCACCTGAC

CCACCAGCAACAGCAGCAACAGCTGAGCGCCCAGGAGGCATTGGGCAAGGG

AACGCGACCAAAGAGGGGCAAGTATCGCAACTATGACCGCGACAGTTTGGT

GGAGGCGGTCAAGGCGGTGCAGAGAGGTGAAATGTCGGTTCATCGAGCGGG

TAGCTACTACGGCGTACCGCATTCCACGCTGGAGTACAAGGTCAAGGAACGT

CACCTGATGCGACCGCGCAAGCGAGAGCCCAAGCCGCAGCCCGATCTCGTCG

GCCTGACCGGACCAGCCAACAAGCTGCAGCTGGACAAACTGAAGGCGGGAC

CACATGGTGGCTCCAAGCTGAGCAATGCCCTCAAGAACCAAAACAATCAGGC

GGCTGCGGCGGCGGCGGCGGCAGCAGCAGCAGCGGCCGCTGCCACGCCCAA

CGGCCTGAAACTGCCCCTTTTCGAGGCGGGTCCACAGGCGTTATCCTTTCAGC

CGAACATGTTCTGGCCCCAGACGAACGCCACGAATGCCTACGGCCTGGACTT

CAATCGCATCACGGAGGCGATGCGGAATCCCCAGGCCTCCAATCACCACGGC

CTGATGAAGAGTGCCCAGGACATGGTGGAGAACGTGTACGATGGCATCATCA

GGAAGACGCTGCAGGCGAGCGAGGGCAATGGCAGTGCGGCGGGTAATGGCA

GCAACGGTAGCAATGGCAACGGGCATGGGCACGGGCATGGCCATGGACACG

CCCTGCTCGATCAGCTGCTGGTGAAGAAGACCCCCTTGCCGTTCACCAACCA

TCGGAACAATGACTACGCCGCCACCTGTTCGAGTGCCAGCGGGGAGAGCGTA

AAGCGGTCGGGCAGTCCCATGGGCAACTATGCAGACATCAAGCGGGAGCGC

CTGAGCGCCGACAGCGGCGGCAGCAGCGATGAGGAGCACTCGGCCAGCCAC

ATCAACAACAACAACAGCGATTTGGCGCACAACAAGAACAAGAGCGGCGGC

GGCGGCGGCGGCGGCGGCAATGGCCAGACCAATGGGAACGGCAGGAGCAGC

CGGATGACGTCGCGGGATGATTCCGAAACGGATGCCAGCAGCTTGAAGAGC

GGCGAAAGTGGCGGCCAGCAAAACCACAAAATGATGGATCTCAATGGCGGC

AGCAGCAGCAGCAGTCACATCAAGTGCGAATCGGAGGCGGCCACCGGACAT

CACAGTCCTGGACACCACACCACGTCCATACTGCACGAGAAGCTGGCCCAGA

TCAAGGCCGAGCAAGTGGACCAGGCGGATCAGTTATAG

gagcagccgatggccgcgaatccagcgttcgcctggccaccgctggccgcccactactacagcttctaggcggagggaggg

ggaacaccaaattaagccacgtttttgatagtaccatacaaa

UAS-clone2

Primers:
#12 Clone_2(f) (1-20): gca gaa ata caa cgc atc cg
#11 E93 ORF downstream (3988-3969) (4580-4561, clone2 numbering):
gttccccctccctccgccta

Sequence:

gcagaaatacaacgcatccgatatgcaacggaacgcattttcggacgcggcccgagtcgttccgaggtgtagcgtccgattccg
attcagattcagattccgacgatcgcgtatatcaatcatattaaacaaaaacaacaactgcaacaacaacggctacacttttgttagt
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ggaac

<u>UAS-E93[1]Nterm</u>

Primers:

E93 CAAC-ORF start primer (E93_CAAC-ORF(f) (CAAC-405-424)):
caacatgcacatcagcagctatga
#3.3 stop-CD1 (f): catcggctgctcctaggggtcttcttcac
E93 ORF triple stops (3979-3998)(4571-4591, clone2 numbering):ttaatttggtgttccccctc
#2.3 CD1-stop(b): gtgaagaagacccctaggagcagccgatg

Sequence:

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gagcagccgatggccgcgaatccagcgttcgcctggccaccgctggccgcccactactacagcttctaggcggagggaggg

ggaacaccaaattaa

UAS-E93^{Vnoe47}Nterm

Primers:
E93 CAAC-ORF start primer (E93_CAAC-ORF(f) (CAAC-405-424)):
caacatgcacatcagcagctatga
#6.2 stop-e47(f): gctgctcctactcatggtgctggctagccg
E93 ORF triple stops (3979-3998)(4571-4591, clone2 numbering):ttaatttggtgttccccctc
#5.3 e47-stop(b): gcaccatgagtaggagcagccgatggccgc

Sequence:

caac

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ggaacaccaaattaa

UAS-E93^{Vnoe47}Cterm

Primers:
caac-ATG(after e47 at 1515): CAACatgaaacaccatgacc
E93 ORF triple stops (3979-3998)(4571-4591, clone2 numbering): ttaatttggtgttccccctc

Sequence:

caac

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ATCCGGCAGAGTTTGCAAGGGCACAACTGCGCAAACTGAGCCACCTGTCCGA

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ggaacaccaaattaa

UAS-Δpipsq

Primers:

E93 CAAC-ORF start primer (E93_CAAC-ORF(f) (CAAC-405-424)):
caacatgcacatcagcagctatga
#8.5 flank-pipsq(f): ctggggccagaacatctgttgctgctgttg
E93 ORF triple stops (3979-3998)(4571-4591, clone2 numbering):ttaatttggtgttccccctc
#7.5 flank-pipsq(b): caacagcagcaacagatgttctggccccag

Sequence:

caac

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UAS-LP0 in pUAST (made by Dianne Duncan)

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ACGCCAACTACCTCAGAAGTATCTGCAGCCGCAATCAGTCCCGCACTCAAAG

ATACACCCAGTCCCAGTGTGGATGCACCGCTCGATCTTAGCAGCAAACCATC

GCCGAACTCATCGATTAGCGGCGATGTGAAGTCCGTCAGAGCCTGTGCCACG

CCCACGCCGTCGGGAAGAAGGGCGTACAGTGAAGAGGATCTGAGCCGGGCC

CTACAGGATGTGGTGGCCAACAAGCTAGATGCCCGGAAATCGGCTAGCCAGC

ACCATGAGCAGCGCTCCATTCTGGACAACCGGCTGTTCAAGATGAAACACCA

TGACCAGGAGCAGGATCATGATGGCGACGAGCTCGAGGACTCCAACGATGA

TGCTGAGGCGGAAGTGGACAGCAATGCGTCGACACCGGTGTATCCGGCAGA

GTTTGCAAGGGCACAACTGCGCAAACTGAGCCACCTGTCCGAGCACAATGGC

AGCGATCTGGGCGAGGATGTGGATCGTGGATCGCCGAAAATGGGGCGACAT

CCGGCCTGTGGCAATGCCAGTGCCAATCAGGGCGCACCGCCATCCATTCCGC

TGGATGCCAATGTCCTGCTGCACACTCTGATGCTGGCTGCTGGGATTGGTGCA

ATGCCGAAGCTGGATGAAACGCAAACGGTGGGCGACTTTATCAAGGGTCTGC

TGGTGGCCAACAGTGGTGGCATAATGAACGAGGGACTGCTAAATCTGCTGTC

CGCCAGTCAGGAGAACAGCAATGGCAATGCCTCGCTGCTGCTGCAACAGCAA

CAGCATCAGCAACACCATCAGCAACACCATCAGCAGCAGCAGCAGCAGCAA

CATGTCGCCGCCTACCGGCATCGCCTGCCCAAGTCGGAGACTCCGGAAACGA

ACTCCTCGTTGGATCCGAACGATGCCAGCGAGGATCCCATACTGAAGATTCC

GTCCTTCAAGGTCAGCGGTCCGGCCAGCAGCAGCAGCCTGTCGCCGGGCGGA

CTGGTTGGTGGTCACCACCATCCGCTGAACAACAACAACAGCCTCAGCATCA

GCAACAACAGCAACCACAGCAGCAACAGCCATCGGAACGGCAGCAATCGCA

GCCCGCATTCCGCATCGCCCATGCTGGCCGCGGCCGTGGCCCAAGGTGGCTA

CTCCGCCGGCAACAGTTTGCTGACCTCATCCTCGTCTAGCATACAGAAGATG

ATGGCCAGCAATATCCAGCGCCAGATCAACGAACAGAGTGGCCAGGAGAGT

CTCAGGAACGGAAATGTTAGCGATTGCAGCAGCAACAATGGCGGCTCCTCCT

CGCTGGGATACAAGAAGCCGAGCATTTCGGTGGCCAAGATCATTGGCGGAAC

GGACACCTCACGGTTCGGAGCCTCGCCCAATCTGCTGTCCCAACAGCACCAT

TCGGCTCACCACCTGACCCACCAGCAACAGCAGCAACAGCTGAGCGCCCAGG

AGGCATTGGGCAAGGGAACGCGACCAAAGAGGGGCAAGTATCGCAACTATG

ACCGCGACAGTTTGGTGGAGGCGGTCAAGGCGGTGCAGAGAGGTGAAATGT

CGGTTCATCGAGCGGGTAGCTACTACGGCGTACCGCATTCCACGCTGGAGTA

CAAGGTCAAGGAACGTCACCTGATGCGACCGCGCAAGCGAGAGCCCAAGCC

GCAGCCCGATCTCGTCGGCCTGACCGGACCAGCCAACAAGCTGCAGCTGGAC

AAACTGAAGGCGGGACCACATGGTGGCTCCAAGCTGAGCAATGCCCTCAAG

AACCAAAACAATCAGGCGGCTGCGGCGGCGGCGGCGGCAGCAGCAGCAGCG

GCCGCTGCCACGCCCAACGGCCTGAAACTGCCCCTTTTCGAGGCGGGTCCAC

AGGCGTTATCCTTTCAGCCGAACATGTTCTGGCCCCAGACGAACGCCACGAA

TGCCTACGGCCTGGACTTCAATCGCATCACGGAGGCGATGCGGAATCCCCAG

GCCTCCAATCACCACGGCCTGATGAAGAGTGCCCAGGACATGGTGGAGAAC

GTGTACGATGGCATCATCAGGAAGACGCTGCAGGCGAGCGAGGGCAATGGC

AGTGCGGCGGGTAATGGCAGCAACGGTAGCAATGGCAACGGGCATGGGCAC

GGGCATGGCCATGGACACGCCCTGCTCGATCAGCTGCTGGTGAAGAAGACCC

CCTTGCCGTTCACCAACCATCGGAACAATGACTACGCCGCCACCTGTTCGAG

TGCCAGCGGGGAGAGCGTAAAGCGGTCGGGCAGTCCCATGGGCAACTATGC

AGACATCAAGCGGGAGCGCCTGAGCGCCGACAGCGGCGGCAGCAGCGATGA

GGAGCACTCGGCCAGCCACATCAACAACAACAACAGCGATTTGGCGCACAA
CAAGAACAAGAGCGGCGGCGGCGGCGGCGGCGGCGGCAATGGCCAGACCAA
TGGGAACGGCAGGAGCAGCCGGATGACGTCGCGGGATGATTCCGAAACGGA
TGCCAGCAGCTTGAAGAGCGGCGAAAGTGGCGGCCAGCAAAACCACAAAAT
GATGGATCTCAATGGCGGCAGCAGCAGCAGCAGTCACATCAAGTGCGAATC
GGAGGCGGCCACCGGACATCACAGTCCTGGACACCACACCACGTCCATACTG
CACGAGAAGCTGGCCCAGATCAAGGCCGAGCAAGTGGACCAGGCGGATCAG
TTA

taggagcagccgatggccgcgaatccagcgttcgcctggccaccgctggccgcccactactacagcttctaggcggagggag
ggggaacaccaaattaagccacgttttttgatagtaccatacaaatcactaaatagaattatatatatatatatatatatatatacata
tataataatttatgccagccagctgaccgatgtgcgtggtaaatgtgcgctagctcttagttaaatgtgtaatcaactgcatagggga
aaaacaaaaccacaggaaatcataaacaacaacaaacaaacaaaaaaaaaaaaaaaaa

UAS-E93 in pUAST (made by Baehrecke)

The pUAST sequence preceding the ORF should be “agatctt” followed by the first “ATG”. The predicated ORF should start from the first ATG in grey shading, like this:

agatcttATGCACATCAGCAGCTATGAAATATCCCTAGAACGCGTTGCTGAAGAA
TGTATG……

However, the red “tAT” mutated into “G” at the beginning of ORF, so the predicated ORF may start from the next in frame ATG in grey shading, like this:

agatctGGCACATCAGCAGCTATGAAATATCCCTAGAACGCGTTGCTGAAGAAT
GTATGGGTCGCAGGCAATGGAAACATTATCAAGACAAACTGACGTGCAGCC
ACTTGAATATCGAGGAGCAACAGCCCATAGCAATAGCCGGTTCCGAGGACG
AGCCATCGCAATACAACCACAGCAGCAAGGAGATCAGCCAGAGCAATCCCA
ACCACTGTAAGACAGAGAACCACCGTCTGGAGCAGCAACACAACGGCAGCC
AGCTATTGGAAGAAGAAGATTCTGAGAACAACCAAACATCACACGATTCATC
ACGTACACCAACACCGGGAGCCACCAGTACACCATCACCACCGCCAGAACCC
ATCGATTGGAGACCGTCGGCCAAGTGCAACTTCTGTGTTAACGGTCGCCTGC
TAACGGTTAACGCCCAGGGCAAGTTGGTGGCCGAGTCAGCAGCAACTGCCAC

TAGTAGTAGCACTAGTAATAGTCACATTCATCAGCACGACAGTGACAGCAAC

TCGAGTGCATCACTGCCCCACCACATCAGCAGCAGCAGCAGCAGCAACAACA

ATAGCAGTGGCAACAGGGCACGCCACATTGCTGCTGCAAGTGCAAGAGCAA

CACCAGCAGCGGCCACACCCGCCAACTCCCTTGAACTCTACAAGCTGCTGAC

CCAGCGGGCAGCCAAAATGACATCGATGGACTCGATGGCCGCCCAGCTGGC

GCAATTCTCACTGCTGGCCGACTTCAATCTGATCAACTCGCTGGCCAGCCAAC

AGCAGCAGCAGCAGCAGCAACAGATCGCTAGTGCGGTAACGCCAACTACCT

CAGAAGTATCTGCAGCCGCAATCAGTCCCGCACTCAAAGATACACCCAGTCC

CAGTGTGGATGCACCGCTCGATCTTAGCAGCAAACCATCGCCGAACTCATCG

ATTAGCGGCGATGTGAAGTCCGTCAGAGCCTGTGCCACGCCCACGCCGTCGG

GAAGAAGGGCGTACAGTGAAGAGGATCTGAGCCGGGCCCTACAGGATGTGG

TGGCCAACAAGCTAGATGCCCGGAAATCGGCTAGCCAGCACCATGAGCAGC

GCTCCATTCTGGACAACCGGCTGTTCAAGATGAAACACCATGACCAGGAGCA

GGATCATGATGGCGACGAGCTCGAGGACTCCAACGATGATGCTGAGGCGGA

AGTGGACAGCAATGCGTCGACACCGGTGTATCCGGCAGAGTTTGCAAGGGCA

CAACTGCGCAAACTGAGCCACCTGTCCGAGCACAATGGCAGCGATCTGGGCG

AGGATGTGGATCGTGGATCGCCGAAAATGGGGCGACATCCGGCCTGTGGCA

ATGCCAGTGCCAATCAGGGCGCACCGCCATCCATTCCGCTGGATGCCAATGT

CCTGCTGCACACTCTGATGCTGGCTGCTGGGATTGGTGCAATGCCGAAGCTG

GATGAAACGCAAACGGTGGGCGACTTTATCAAGGGTCTGCTGGTGGCCAACA

GTGGTGGCATAATGAACGAGGGACTGCTAAATCTGCTGTCCGCCAGTCAGGA

GAACAGCAATGGCAATGCCTCGCTGCTGCTGCAACAGCAACAGCATCAGCAA

CACCATCAGCAACACCATCAGCAGCAGCAGCAGCAGCAACATGTCGCCGCCT

ACCGGCATCGCCTGCCCAAGTCGGAGACTCCGGAAACGAACTCCTCGTTGGA

TCCGAACGATGCCAGCGAGGATCCCATACTGAAGATTCCGTCCTTCAAGGTC

AGCGGTCCGGCCAGCAGCAGCAGCCTGTCGCCGGGCGGACTGGTTGGTGGTC

ACCACCATCCGCTGAACAACAACAACAGCCTCAGCATCAGCAACAACAGCA

ACCACAGCAGCAACAGCCATCGGAACGGCAGCAATCGCAGCCCGCATTCCG

CATCGCCCATGCTGGCCGCGGCCGTGGCCCAAGGTGGCTACTCCGCCGGCAA

CAGTTTGCTGACCTCATCCTCGTCTAGCATACAGAAGATGATGGCCAGCAAT

ATCCAGCGCCAGATCAACGAACAGAGTGGCCAGGAGAGTCTCAGGAACGGA

AATGTTAGCGATTGCAGCAGCAACAATGGCGGCTCCTCCTCGCTGGGATACA

AGAAGCCGAGCATTTCGGTGGCCAAGATCATTGGCGGAACGGACACCTCACG

GTTCGGAGCCTCGCCCAATCTGCTGTCCCAACAGCACCATTCGGCTCACCACC

TGACCCACCAGCAACAGCAGCAACAGCTGAGCGCCCAGGAGGCATTGGGCA

AGGGAACGCGACCAAAGAGGGGCAAGTATCGCAACTATGACCGCGACAGTT

TGGTGGAGGCGGTCAAGGCGGTGCAGAGAGGTGAAATGTCGGTTCATCGAG

CGGGTAGCTACTACGGCGTACCGCATTCCACGCTGGAGTACAAGGTCAAGGA

ACGTCACCTGATGCGACCGCGCAAGCGAGAGCCCAAGCCGCAGCCCGATCTC

GTCGGCCTGACCGGACCAGCCAACAAGCTGCAGCTGGACAAACTGAAGGCG

GGACCACATGGTGGCTCCAAGCTGAGCAATGCCCTCAAGAACCAAAACAATC

AGGCGGCTGCGGCGGCGGCGGCGGCAGCAGCAGCAGCGGCCGCTGCCACGC

CCAACGGCCTGAAACTGCCCCTTTTCGAGGCGGGTCCACAGGCGTTATCCTTT

CAGCCGAACATGTTCTGGCCCCAGACGAACGCCACGAATGCCTACGGCCTGG

ACTTCAATCGCATCACGGAGGCGATGCGGAATCCCCAGGCCTCCAATCACCA

CGGCCTGATGAAGAGTGCCCAGGACATGGTGGAGAACGTGTACGATGGCAT

CATCAGGAAGACGCTGCAGGCGAGCGAGGGCAATGGCAGTGCGGCGGGTAA

TGGCAGCAACGGTAGCAATGGCAACGGGCATGGGCACGGGCATGGCCATGG

ACACGCCCTGCTCGATCAGCTGCTGGTGAAGAAGACCCCCTTGCCGTTCACC

AACCATCGGAACAATGACTACGCCGCCACCTGTTCGAGTGCCAGCGGGGAGA

GCGTAAAGCGGTCGGGCAGTCCCATGGGCAACTATGCAGACATCAAGCGGG

AGCGCCTGAGCGCCGACAGCGGCGGCAGCAGCGATGAGGAGCACTCGGCCA

GCCACATCAACAACAACAACAGCGATTTGGCGCACAACAAGAACAAGAGCG

GCGGCGGCGGCGGCGGCGGCGGCAATGGCCAGACCAATGGGAACGGCAGGA

GCAGCCGGATGACGTCGCGGGATGATTCCGAAACGGATGCCAGCAGCTTGA

AGAGCGGCGAAAGTGGCGGCCAGCAAAACCACAAAATGATGGATCTCAATG

GCGGCAGCAGCAGCAGCAGTCACATCAAGTGCGAATCGGAGGCGGCCACCG

GACATCACAGTCCTGGACACCACACCACGTCCATACTGCACGAGAAGCTGGC

CCAGATCAAGGCCGAGCAAGTGGACCAGGCGGATCAGTTATAG

gagcagccgatggccgcgaatccagcgttcgcctggccaccgctggccgcccactactacagcttctaggcggagggaggg

ggaacaccaaattaagccacgttttttgatagtaccatacaaatcactaaatagaattatatatatatatatatatatatacatatat

aataatttatgccagccagctgaccgatgtgcgtggtaaatgtgcgctagctcttagttaaatgtgtaatcaactgcataggggaaa

aacaaaaccacaggaaatcataaacaacaacaaacaaacaaaaaaaacaaaaataacaagaaccgcaagcaaagaaacata

catttgtgccccggagtgtacgatgtatattttgtttcgttttgacaatcgacaaataggcattctcttgtacaaactttcttaaaagcta

acaacaaaacaaatctaaaaccttaagaccaaaaaaaacaaaaaatgaaaaaaaa

Probes used in Northern and RPA

E93 unique start (E18set3 clone1.4 pSTBlue1)
Forward primer (17792211-31) ACAAAATAAG TGCAGCAGTA C
Backward primer (17792840-20) CTCGTTAATT ATTTATGACT C

Flanking intron (101)
ACAAAATAA GTGCAGCAGT ACAACAACAA AAAGCATTAC ACAATTGAAC
AAACAACATACAAAAAAAAA ATATTGCCAC CAAATAAAAA AAAACCCGAA
AG

E93 exone aaaattcgcg……tatgaaatat(1-429)
AAAATTCGCGCTTCATTTTCACGCGCTGCATATAATTTACTCTGTGCTCCACA
TATATATATATATATATATATATAATAAATAAAAACCAACCAATAGCCCAAAAA
AAAAGAAATGAAAAGTCCGCATCAACAATAAAAATCTGCCTGCATTTTTGCC
TTTTGTGTGAGCTGCCCACCAGAACGAGAGAAGCACTTTTATTGTATATAAAA
ATTATATACATCGCCGGGAGGAGCTGCAGCAACCCACTCCAAGCCAGGGTTG
CCACGTCCTGAGCTGCTGTAAGCTCTCCGCAGCAGCTGCAGCAGCATCAGCA
TCGCAGCAGCATCAGCAGCAGCGCAGCAACCGCCGCAGCATCAATTTGGCTT
TTGGGCAGAGATAATTTAAGACAAATATATGTGATGCTATGCACATCAGCAG
CTATGAAATAT

Flanking intron (99)
GTACGTATGCCAAATTGCTTTTTTTTTCAAATAATCGCAAATAAACTAAAAAT
ATATTTAATGATTAATTGTAATTAAT GAGTCATAAATAATTAACGA

LP0 US (LP08695 unique F made by Dianne)
CGGTTCGCTTCGGTCCCGGCCCCGCTGATTTGGATTTGGATTCGGATTCT
CGGTTTCTCGGTTTCTCGGCTTCACGCTTCCTCGGATTCTCGGTTTCTCG
GATACTTGGCTACTCCGATAGTGGGATATTCTCGCCAGACTCGGCTTCTC
TGCGAGATTCTCCATTCTCCTCACTGCCCAGACGGGCGATTCTTGTGCCC

GACCGATTTGGGTTCGGATCGAAGGCGGCGTTCGCTGCTGCAGCGTTTGC
CGGCGTCGCTGCCTGCGCTCTTGCGTATTAGTAACGTCGTTGACATGCCA
GGCCACTAAAAACGAAAAAAAAAAATAAAAAAGTAAAAAGAGCTGAAAAT
GCAAAAGTGCTGTGACAACCACAAATAAATGATAGAAGGAAAAAAGTTTA
ATACATTAAAAACAAATACCACAAACAAAGTCAACGTTTTCATCGTACTG
CAAAAAAACCACAAATTTTTTACATATTTACATTAATGAGCTTGTAAATA
TTGGGACGCATTGCCACGAAAAGTACAGTTGCGATCTAACAATATCGCCA
ATATATCCTCAAAAAGAAAGCTATGGCCGATTGTTCATATGTGAGATGTC
AGCAAGAGCGTCGGCTCATCAAAAAGAAGCTATTGAAATGGTCCACAGAT
ATGCTGCATATTGTG

E93 ORF MUS (E93 ORF minus unique start)
CCCTAGAACG……GGCGGATCAG (430-3896=>3467)
CCCTAGAACGCGTTGCTGAA E93NUSI(F)
AAGTGGACCAGGCGGATCAG >> CTGATCCGCCTGGTCCACTT E93NUSII(B)
CCCTAGAACGCGTTGCTGAAGAATGTATGGGTCGCAGGCAATGGAAACATTA
TCAAGACAAACTGACGTGCAGCCACTTGAATATCGAGGAGCAACAGCCCATA
GCAATAGCCGGTTCCGAGGACGAGCCATCGCAATACAACCACAGCAGCAAG
GAGATCAGCCAGAGCAATCCCAACCACTGTAAGACAGAGAACCACCGTCTG
GAGCAGCAACACAACGGCAGCCAGCTATTGGAAGAAGAAGATTCTGAGAAC
AACCAAACATCACACGATTCATCACGTACACCAACACCGGGAGCCACCAGTA
CACCATCACCACCGCCAGAACCCATCGATTGGAGACCGTCGGCCAAGTGCAA
CTTCTGTGTTAACGGTCGCCTGCTAACGGTTAACGCCCAGGGCAAGTTGGTGG
CCGAGTCAGCAGCAACTGCCACTAGTAGTAGCACTAGTAATAGTCACATTCA
TCAGCACGACAGTGACAGCAACTCGAGTGCATCACTGCCCCACCACATCAGC
AGCAGCAGCAGCAGCAACAACAATAGCAGTGGCAACAGGGCACGCCACATT
GCTGCTGCAAGTGCAAGAGCAACACCAGCAGCGGCCACACCCGCCAACTCCC
TTGAACTCTACAAGCTGCTGACCCAGCGGGCAGCCAAAATGACATCGATGGA
CTCGATGGCCGCCCAGCTGGCGCAATTCTCACTGCTGGCCGACTTCAATCTGA
TCAACTCGCTGGCCAGCCAACAGCAGCAGCAGCAGCAGCAACAGATCGCTA
GTGCGGTAACGCCAACTACCTCAGAAGTATCTGCAGCCGCAATCAGTCCCGC
ACTCAAAGATACACCCAGTCCCAGTGTGGATGCACCGCTCGATCTTAGCAGC
AAACCATCGCCGAACTCATCGATTAGCGGCGATGTGAAGTCCGTCAGAGCCT
GTGCCACGCCCACGCCGTCGGGAAGAAGGGCGTACAGTGAAGAGGATCTGA
GCCGGGCCCTACAGGATGTGGTGGCCAACAAGCTAGATGCCCGGAAATCGGC
TAGCCAGCACCATGAGCAGCGCTCCATTCTGGACAACCGGCTGTTCAAGATG
AAACACCATGACCAGGAGCAGGATCATGATGGCGACGAGCTCGAGGACTCC
AACGATGATGCTGAGGCGGAAGTGGACAGCAATGCGTCGACACCGGTGTATC
CGGCAGAGTTTGCAAGGGCACAACTGCGCAAACTGAGCCACCTGTCCGAGCA
CAATGGCAGCGATCTGGGCGAGGATGTGGATCGTGGATCGCCGAAAATGGG
GCGACATCCGGCCTGTGGCAATGCCAGTGCCAATCAGGGCGCACCGCCATCC
ATTCCGCTGGATGCCAATGTCCTGCTGCACACTCTGATGCTGGCTGCTGGGAT
TGGTGCAATGCCGAAGCTGGATGAAACGCAAACGGTGGGCGACTTTATCAAG
GGTCTGCTGGTGGCCAACAGTGGTGGCATAATGAACGAGGGACTGCTAAATC
TGCTGTCCGCCAGTCAGGAGAACAGCAATGGCAATGCCTCGCTGCTGCTGCA

ACAGCAACAGCATCAGCAACACCATCAGCAACACCATCAGCAGCAGCAGCA
GCAGCAACATGTCGCCGCCTACCGGCATCGCCTGCCCAAGTCGGAGACTCCG
GAAACGAACTCCTCGTTGGATCCGAACGATGCCAGCGAGGATCCCATACTGA
AGATTCCGTCCTTCAAGGTCAGCGGTCCGGCCAGCAGCAGCAGCCTGTCGCC
GGGCGGACTGGTTGGTGGTCACCACCATCCGCTGAACAACAACAACAGCCTC
AGCATCAGCAACAACAGCAACCACAGCAGCAACAGCCATCGGAACGGCAGC
AATCGCAGCCCGCATTCCGCATCGCCCATGCTGGCCGCGGCCGTGGCCCAAG
GTGGCTACTCCGCCGGCAACAGTTTGCTGACCTCATCCTCGTCTAGCATACAG
AAGATGATGGCCAGCAATATCCAGCGCCAGATCAACGAACAGAGTGGCCAG
GAGAGTCTCAGGAACGGAAATGTTAGCGATTGCAGCAGCAACAATGGCGGCT
CCTCCTCGCTGGGATACAAGAAGCCGAGCATTTCGGTGGCCAAGATCATTGG
CGGAACGGACACCTCACGGTTCGGAGCCTCGCCCAATCTGCTGTCCCAACAG
CACCATTCGGCTCACCACCTGACCCACCAGCAACAGCAGCAACAGCTGAGCG
CCCAGGAGGCATTGGGCAAGGGAACGCGACCAAAGAGGGGCAAGTATCGCA
ACTATGACCGCGACAGTTTGGTGGAGGCGGTCAAGGCGGTGCAGAGAGGTG
AAATGTCGGTTCATCGAGCGGGTAGCTACTACGGCGTACCGCATTCCACGCT
GGAGTACAAGGTCAAGGAACGTCACCTGATGCGACCGCGCAAGCGAGAGCC
CAAGCCGCAGCCCGATCTCGTCGGCCTGACCGGACCAGCCAACAAGCTGCAG
CTGGACAAACTGAAGGCGGGACCACATGGTGGCTCCAAGCTGAGCAATGCCC
TCAAGAACCAAAACAATCAGGCGGCTGCGGCGGCGGCGGCGGCAGCAGCAG
CAGCGGCCGCTGCCACGCCCAACGGCCTGAAACTGCCCCTTTTCGAGGCGGG
TCCACAGGCGTTATCCTTTCAGCCGAACATGTTCTGGCCCCAGACGAACGCC
ACGAATGCCTACGGCCTGGACTTCAATCGCATCACGGAGGCGATGCGGAATC
CCCAGGCCTCCAATCACCACGGCCTGATGAAGAGTGCCCAGGACATGGTGGA
GAACGTGTACGATGGCATCATCAGGAAGACGCTGCAGGCGAGCGAGGGCAA
TGGCAGTGCGGCGGGTAATGGCAGCAACGGTAGCAATGGCAACGGGCATGG
GCACGGGCATGGCCATGGACACGCCCTGCTCGATCAGCTGCTGGTGAAGAAG
ACCCCCTTGCCGTTCACCAACCATCGGAACAATGACTACGCCGCCACCTGTTC
GAGTGCCAGCGGGGAGAGCGTAAAGCGGTCGGGCAGTCCCATGGGCAACTA
TGCAGACATCAAGCGGGAGCGCCTGAGCGCCGACAGCGGCGGCAGCAGCGA
TGAGGAGCACTCGGCCAGCCACATCAACAACAACAACAGCGATTTGGCGCAC
AACAAGAACAAGAGCGGCGGCGGCGGCGGCGGCGGCGGCAATGGCCAGACC
AATGGGAACGGCAGGAGCAGCCGGATGACGTCGCGGGATGATTCCGAAACG
GATGCCAGCAGCTTGAAGAGCGGCGAAAGTGGCGGCCAGCAAAACCACAAA
ATGATGGATCTCAATGGCGGCAGCAGCAGCAGCAGTCACATCAAGTGCGAAT
CGGAGGCGGCCACCGGACATCACAGTCCTGGACACCACACCACGTCCATACT
GCACGAGAAGCTGGCCCAGATCAAGGCCGAGCAAGTGGACCAGGCGGATCA
G

E93 ORF MUSII (E93 ORF minus unique start II)
CAGCAGCAGC……GGCGGATCAG (2171-3896=>1726)
CAGCAGCAGCAGCCTGTCGC E93NUSII(F)
AAGTGGACCAGGCGGATCAG>> CTGATCCGCCTGGTCCACTT E93NUSII(B)
CAGCAGCAGCAGCCTGTCGCCGGGCGGACTGGTTGGTGGTCACCACCATCCG
CTGAACAACAACAACAGCCTCAGCATCAGCAACAACAGCAACCACAGCAGC
AACAGCCATCGGAACGGCAGCAATCGCAGCCCGCATTCCGCATCGCCCATGC

TGGCCGCGGCCGTGGCCCAAGGTGGCTACTCCGCCGGCAACAGTTTGCTGAC
CTCATCCTCGTCTAGCATACAGAAGATGATGGCCAGCAATATCCAGCGCCAG
ATCAACGAACAGAGTGGCCAGGAGAGTCTCAGGAACGGAAATGTTAGCGAT
TGCAGCAGCAACAATGGCGGCTCCTCCTCGCTGGGATACAAGAAGCCGAGCA
TTTCGGTGGCCAAGATCATTGGCGGAACGGACACCTCACGGTTCGGAGCCTC
GCCCAATCTGCTGTCCCAACAGCACCATTCGGCTCACCACCTGACCCACCAG
CAACAGCAGCAACAGCTGAGCGCCCAGGAGGCATTGGGCAAGGGAACGCGA
CCAAAGAGGGGCAAGTATCGCAACTATGACCGCGACAGTTTGGTGGAGGCG
GTCAAGGCGGTGCAGAGAGGTGAAATGTCGGTTCATCGAGCGGGTAGCTACT
ACGGCGTACCGCATTCCACGCTGGAGTACAAGGTCAAGGAACGTCACCTGAT
GCGACCGCGCAAGCGAGAGCCCAAGCCGCAGCCCGATCTCGTCGGCCTGACC
GGACCAGCCAACAAGCTGCAGCTGGACAAACTGAAGGCGGGACCACATGGT
GGCTCCAAGCTGAGCAATGCCCTCAAGAACCAAAACAATCAGGCGGCTGCG
GCGGCGGCGGCGGCAGCAGCAGCAGCGGCCGCTGCCACGCCCAACGGCCTG
AAACTGCCCCTTTTCGAGGCGGGTCCACAGGCGTTATCCTTTCAGCCGAACAT
GTTCTGGCCCCAGACGAACGCCACGAATGCCTACGGCCTGGACTTCAATCGC
ATCACGGAGGCGATGCGGAATCCCCAGGCCTCCAATCACCACGGCCTGATGA
AGAGTGCCCAGGACATGGTGGAGAACGTGTACGATGGCATCATCAGGAAGA
CGCTGCAGGCGAGCGAGGGCAATGGCAGTGCGGCGGGTAATGGCAGCAACG
GTAGCAATGGCAACGGGCATGGGCACGGGCATGGCCATGGACACGCCCTGCT
CGATCAGCTGCTGGTGAAGAAGACCCCCTTGCCGTTCACCAACCATCGGAAC
AATGACTACGCCGCCACCTGTTCGAGTGCCAGCGGGGAGAGCGTAAAGCGGT
CGGGCAGTCCCATGGGCAACTATGCAGACATCAAGCGGGAGCGCCTGAGCG
CCGACAGCGGCGGCAGCAGCGATGAGGAGCACTCGGCCAGCCACATCAACA
ACAACAACAGCGATTTGGCGCACAACAAGAACAAGAGCGGCGGCGGCGGCG
GCGGCGGCGGCAATGGCCAGACCAATGGGAACGGCAGGAGCAGCCGGATGA
CGTCGCGGGATGATTCCGAAACGGATGCCAGCAGCTTGAAGAGCGGCGAAA
GTGGCGGCCAGCAAAACCACAAAATGATGGATCTCAATGGCGGCAGCAGCA
GCAGCAGTCACATCAAGTGCGAATCGGAGGCGGCCACCGGACATCACAGTCC
TGGACACCACACCACGTCCATACTGCACGAGAAGCTGGCCCAGATCAAGGCC
GAGCAAGTGGACCAGGCGGATCAG

l(3)S12

a 1376 bp MscI-HindIII fragment extending from -1755 to -3131 of rosy gene (numbering as in Lee et al. 1987,Dutton and Chovnick 1991) is isolated from fly genome DNA. This fragment contains all of the putative ORFs identified for the l(3)S12 gene (Dutton and Chovnick 1991), as well as 1155bp of additional 3' sequence. (Quote from Horowitz and Berg 1995)

AAGCTTTAAC……CTAGACATGG (-3131- -1755 =>1377bp, but the NCBI sequence is 1366bp)

AAGCTTTAACTATGTTTAAT (-3131 - -3112) forward primer

CTCGTAACCCCTAGACATGG (-1774 - -1755) >>> CCATGTCTAGGGGTTACGAG backward primer

AAGCTTTAACTATGTTTAATCTCCCATAGAGCTTCGTTATCTTCGGACGCGAT
GTGGCGCAGATCCCGTGCTTTCGTAACAGTTTTCTCTACGGAATCAGCGGAGG
AATCGGCATCGGCTTGCTGACTTTTCTGGGCACCTCGCGAACCCACCTGTCCA

CCCACGTGGGCTTCGGCTCCTTCTTCTGCGGCACCATCGCCTACTGGATGACC
TGCAGGTAAGTGACTGTGATTAAAACCGTGCGGCGCCCTTATCACTTAATTAT
CAATGTGGCATCCGCAGGTATCAATGGTCCGTCAGGAGATTCGAGCAGCAGC
AATTGCGTGAGGCGATGAGACGACAAGCCCTTTATGAGGGCACCCAAAGGG
AGAGAGACTTAGATCTGAAGTCGGCGTAGCATAAGTCAGATCCTTCATTACT
ACTTCGATGTTTGTTTTCACTCTAGGATACCAAGTAATCCTGGCATTGTTAAA
TCCCTTGTGTTGTAAACTACCTTTTGTTACCAAACTTTATAGTAGCCAATATAT
TATATATATGTTGTAAGTGAAATCTTAGCTTGCCACTCGTAGGGAATTAATTA
TGCCACCATCTTTGATTTGGGATTGACAGAAATCAATCCATCATTTTAATGAT
TGTGGTTCCTAAAACCTGTTGGGCTTTATTGTGCTTTCGATTTGATTTAAGCTG
ATAGAGCTAGTTGTTTACTTTTGAAGAACCTTGAACCCATTTTGACGACCTTT
TAACTTTTTAATAGTTTTTTTTTACCATTTACATAAAAGCAAAATTTACAGTCT
AAAAGTTGTATATTCAATACGTTCTGGTACGTAAAAGGGTTATTTAATGCACC
ATTTTTACAACTTTAGATAGTATACTTTTTACTTGAGTGGGAGCCATATCACA
AAAATGAAATGAAAGGTTAAAAAAGTATCTAAAAGTAAGTTTCAATTAATAT
TCTGAAAAATAATTGCATTTCATGTACGTTTCTCTGAAATACCCCTAATTCGA
TACTATTTATAAATCTGGAAGATATATAGCTAGGAAATAGATTTATATATTTG
CCTAGTTTGAACACCTTGTGAACATTTTGTTTAAAAAAAAAGCGTATCGTGGT
ACTAGAAAACTTTAAGATACAGATAAATAACTTCACTATACCGAGGTTTTGG
TGTTTCTTGAGACTGAAGGACAATCTTCAAAATGGTCTTAATTCTTGAGATAG
AAAAGCTGCAGTTCTCATGCTGAATAGTGATCAGTACGATTTTATCTGACAGA
TTGCTTTCCTAGCTGACCACACCGATCTCGGATGGGTATCTGCTCTAATCCGA
GATAGCAGGTAGTAGAACTGCTGCCTCGTAACCCCTAGACATGG

E93 pipsqk
TGAGCGCCCA…….TTCAGCCGAA (2653-3097 =>445bp)
TGAGCGCCCAGGAGGCATTG (2653-2672) E93 pipsq(F)
GCGTTATCCTTTCAGCCGAA (3078-
3097) >>> TTCGGCTGAAAGGATAACGC E93 pipsq(B)(3097-3078)

TGAGCGCCCAGGAGGCATTGGGCAAGGGAACGCGACCAAAGAGGGGCAAGT
ATCGCAACTATGACCGCGACAGTTTGGTGGAGGCGGTCAAGGCGGTGCAGAG
AGGTGAAATGTCGGTTCATCGAGCGGGTAGCTACTACGGCGTACCGCATTCC
ACGCTGGAGTACAAGGTCAAGGAACGTCACCTGATGCGACCGCGCAAGCGA
GAGCCCAAGCCGCAGCCCGATCTCGTCGGCCTGACCGGACCAGCCAACAAGC
TGCAGCTGGACAAACTGAAGGCGGGACCACATGGTGGCTCCAAGCTGAGCA
ATGCCCTCAAGAACCAAAACAATCAGGCGGCTGCGGCGGCGGCGGCGGCAG
CAGCAGCAGCGGCCGCTGCCACGCCCAACGGCCTGAAACTGCCCCTTTTCGA
GGCGGGTCCACAGGCGTTATCCTTTCAGCCGAA

E93 AccI
ACCCATGTAA……ATCGAAAATC (6569-8284 => 1716 bp)
ACCCATGTAAAGAAATTGTA (6569-6588) E93AccI(F)Forward primer
TACCGTATGGATCGAAAATC (8265-8284) >>> GATTTTCGATCCATACGGTA
E93AccI(B) Backward primer

ACCCATGTAAAGAAATTGTAGACAGATCGGATAAAACGAAACTAAACCAAG
CACAAGCTAATGGCCCAAATGCAGTTGGCCCCGAAAATCGAGCGCTGCATTT
GGCCAAGAGAATTTCTTAAGCTACGGCACACATCACTGAAAACAAAAACTGA
AAACTGAATACTGAATACTGAGAATAGGAAACAGTAAGCAGAAGACAAGAT
CGATGGTACTGTTCAGAACATATATAGTTGTATATATTTTGGAATAATGTTTA
CCAGTTCAAGTCAAAATTAAAAGGAAAAAAAAATGCAGTCTTTTATAATGCA
AAATTATACAAAGAAAAATTACAATTTCGCAACGCTAAAAAATGAAAAACG
AAAATATTGATGTAAAAAGAATGAAAATCAAACTTAAAATAATAAACAAGA
TAAAGTGCAATTATACGGTTTAAATAAGCAAATTTAAGAAACAAACGTTATA
AACAAAAACACAAACATGTTAAAACAATGAAAATATTTCGAAGCAAGTTTAG
CTACAAATTCCAAGGCAACTGATAATGACAAGAACCATTTACAAGAAAACCC
AAGACAGCAAAGTACAGTGCGTTTCATAACCCGCAATACGGGCAATCGAATA
CTACCATTCCATTGCCCATGGAGAACTGAAACGCACTTTGGCCCTACTTCATA
TTGATAGGGTAATCGGATCCAAAATCTGTAAACCAAATTTTGGGATCAGCGA
TTAAAACCTTAACGGAAGTTCATAACTGCAGAAAAAAAAAGTCGAAAGTCGA
AATGTCAACCTAGTGGTAGTTCGAAACACAAAAACAAAATAAACCAATCGAG
TGTAATTGAGTGACAGCTTGAGAATGTTGAATTGTATAGAATTTTTGCTTGTG
CACCTGGTCGAGGGGGGCGTGGTTGCGCCCCCTTTTTGGTTTCTAGGTGAACA
GGCGAAAACGCTCAGGTACGTGTTTTATTTTTCGGAGAGAAACAAGATTGAT
TACCCATACATTACTTATTCTTTGTTTTACTACAACATAATAGTTAATATTTGT
ATAAAAAAAAAAGACGACGATGGCGAGAAGGGAAAACCAGCTAAAAAAATT
GATATATTCATAATATAGAATTGTTTTAAATGGTTTGAGAGCGAAAAATATTG
AGGGTTTCTAGCGTGCTTCATGAAATTGCTCATATTTGTGTATAAAACCTTAT
TTGTTCAATGCGAATCAAAATTTGCTGAATATAAGTGCATATATATATAATTA
GAGTTTATTTTTCGGTTTAGTTAAGCGCATAAAATGATTACGATTTAAATAAT
TATTATTAGTTATGACCTAATAGTAGGCAAATCAAAATTTCTTCACATAAAAC
AATCAACTTCTACTTTCAAATAATTTCTAGACGTATGTAACTATAGTTATTATT
CTATTATTATACGGACTAAAACTATTTAGCGCGTTGTTAGACTCGATTTATGG
TTTGTACATATTAGACAACATTTTTATGGTATTCTCCTCTTTTTTTATTATTACT
AGCATTATTACTCCCTATTTTAATTGACTTCTTAAATGGGCAACATCATTTTGA
ACTATAGTGCAATTGTTTCAAACAAAACCAAGCAACCAACAACAAATATATT
AACATTAAAGATTAATATAATTTACAAGCTTTCTTTGCCGATGCCAAGAAGG
ATGAATAACGCATATGTCTACCGTATGGATCGAAAATC

E93 5exoI

E93exo5-1(f)(1372-1391): CCACGCCGTCGGGAAGAAGG

E93exo5-1(b)(1944-1922): Acugcuaaaucugcuguccgcca >>>>

TGGCGGACAGCAGATTTAGCAGT

CCACGCCGTCGGGAAGAAGGGCGTACAGTGAAGAGGATCTGAGCCGGGCCC
TACAGGATGTGGTGGCCAACAAGCTAGATGCCCGGAAATCGGCTAGCCAGCA
CCATGAGCAGCGCTCCATTCTGGACAACCGGCTGTTCAAGATGAAACACCAT
GACCAGGAGCAGGATCATGATGGCGACGAGCTCGAGGACTCCAACGATGAT
GCTGAGGCGGAAGTGGACAGCAATGCGTCGACACCGGTGTATCCGGCAGAGT
TTGCAAGGGCACAACTGCGCAAACTGAGCCACCTGTCCGAGCACAATGGCAG
CGATCTGGGCGAGGATGTGGATCGTGGATCGCCGAAAATGGGGCGACATCCG
GCCTGTGGCAATGCCAGTGCCAATCAGGGCGCACCGCCATCCATTCCGCTGG

ATGCCAATGTCCTGCTGCACACTCTGATGCTGGCTGCTGGGATTGGTGCAATG
CCGAAGCTGGATGAAACGCAAACGGTGGGCGACTTTATCAAGGGTCTGCTGG
TGGCCAACAGTGGTGGCATAATGAACGAGGGACTGCTAAATCTGCTGTCCGC
CA

E93 5exoII

E93exo5-2(f)(2037-2056):Cagcaacatgtcgccgccta
E93exo5-2(b)(2652-2633):Ccagcaacagcagcaacagc >>>> gctgttgctgctgttgctgg
CAGCAACATGTCGCCGCCTACCGGCATCGCCTGCCCAAGTCGGAGACTCCGG
AAACGAACTCCTCGTTGGATCCGAACGATGCCAGCGAGGATCCCATACTGAA
GATTCCGTCCTTCAAGGTCAGCGGTCCGGCCAGCAGCAGCAGCCTGTCGCCG
GGCGGACTGGTTGGTGGTCACCACCATCCGCTGAACAACAACAACAGCCTCA
GCATCAGCAACAACAGCAACCACAGCAGCAACAGCCATCGGAACGGCAGCA
ATCGCAGCCCGCATTCCGCATCGCCCATGCTGGCCGCGGCCGTGGCCCAAGG
TGGCTACTCCGCCGGCAACAGTTTGCTGACCTCATCCTCGTCTAGCATACAGA
AGATGATGGCCAGCAATATCCAGCGCCAGATCAACGAACAGAGTGGCCAGG
AGAGTCTCAGGAACGGAAATGTTAGCGATTGCAGCAGCAACAATGGCGGCTC
CTCCTCGCTGGGATACAAGAAGCCGAGCATTTCGGTGGCCAAGATCATTGGC
GGAACGGACACCTCACGGTTCGGAGCCTCGCCCAATCTGCTGTCCCAACAGC
ACCATTCGGCTCACCACCTGACCCACCAGCAACAGCAGCAACAGC

E93 5exoIII

E93exo5-3(f)(3098-3117):Catgttctggccccagacga
E93exo5-3(b)(3899-3880):Tggaccaggcggatcagtta >>> taactgatccgcctggtcca
CATGTTCTGGCCCCAGACGAACGCCACGAATGCCTACGGCCTGGACTTCAAT
CGCATCACGGAGGCGATGCGGAATCCCCAGGCCTCCAATCACCACGGCCTGA
TGAAGAGTGCCCAGGACATGGTGGAGAACGTGTACGATGGCATCATCAGGA
AGACGCTGCAGGCGAGCGAGGGCAATGGCAGTGCGGCGGGTAATGGCAGCA
ACGGTAGCAATGGCAACGGGCATGGGCACGGGCATGGCCATGGACACGCCC
TGCTCGATCAGCTGCTGGTGAAGAAGACCCCCTTGCCGTTCACCAACCATCG
GAACAATGACTACGCCGCCACCTGTTCGAGTGCCAGCGGGGAGAGCGTAAA
GCGGTCGGGCAGTCCCATGGGCAACTATGCAGACATCAAGCGGGAGCGCCTG
AGCGCCGACAGCGGCGGCAGCAGCGATGAGGAGCACTCGGCCAGCCACATC
AACAACAACAACAGCGATTTGGCGCACAACAAGAACAAGAGCGGCGGCGGC
GGCGGCGGCGGCGGCAATGGCCAGACCAATGGGAACGGCAGGAGCAGCCGG
ATGACGTCGCGGGATGATTCCGAAACGGATGCCAGCAGCTTGAAGAGCGGCG
AAAGTGGCGGCCAGCAAAACCACAAAATGATGGATCTCAATGGCGGCAGCA
GCAGCAGCAGTCACATCAAGTGCGAATCGGAGGCGGCCACCGGACATCACA
GTCCTGGACACCACACCACGTCCATACTGCACGAGAAGCTGGCCCAGATCAA
GGCCGAGCAAGTGGACCAGGCGGATCAGTTA

E93 234exo

CCCTAGAACGCGTTGCTGAA E93_234exo(F)(430-449)
GGCGATGTGAAGTCCGTCAG>>>> CTGACGGACTTCACATCGCC
E93_234exo(B)(1357-1338)

CCCTAGAACGCGTTGCTGAAGAATGTATGGGTCGCAGGCAATGGAAACATTA
TCAAGACAAACTGACGTGCAGCCACTTGAATATCGAGGAGCAACAGCCCATA
GCAATAGCCGGTTCCGAGGACGAGCCATCGCAATACAACCACAGCAGCAAG
GAGATCAGCCAGAGCAATCCCAACCACTGTAAGACAGAGAACCACCGTCTG
GAGCAGCAACACAACGGCAGCCAGCTATTGGAAGAAGAAGATTCTGAGAAC
AACCAAACATCACACGATTCATCACGTACACCAACACCGGGAGCCACCAGTA
CACCATCACCACCGCCAGAACCCATCGATTGGAGACCGTCGGCCAAGTGCAA
CTTCTGTGTTAACGGTCGCCTGCTAACGGTTAACGCCCAGGGCAAGTTGGTGG
CCGAGTCAGCAGCAACTGCCACTAGTAGTAGCACTAGTAATAGTCACATTCA
TCAGCACGACAGTGACAGCAACTCGAGTGCATCACTGCCCCACCACATCAGC
AGCAGCAGCAGCAGCAACAACAATAGCAGTGGCAACAGGGCACGCCACATT
GCTGCTGCAAGTGCAAGAGCAACACCAGCAGCGGCCACACCCGCCAACTCCC
TTGAACTCTACAAGCTGCTGACCCAGCGGGCAGCCAAAATGACATCGATGGA
CTCGATGGCCGCCCAGCTGGCGCAATTCTCACTGCTGGCCGACTTCAATCTGA
TCAACTCGCTGGCCAGCCAACAGCAGCAGCAGCAGCAGCAACAGATCGCTA
GTGCGGTAACGCCAACTACCTCAGAAGTATCTGCAGCCGCAATCAGTCCCGC
ACTCAAAGATACACCCAGTCCCAGTGTGGATGCACCGCTCGATCTTAGCAGC
AAACCATCGCCGAACTCATCGATTAGCGGCGATGTGAAGTCCGTCAG

E93 5exo boundary

tatattttaggggaaatccg E93 5exo intron (f) (17829412-17829431)(successful)
GCTGAGGCGGAAGTGGACAG (1578-1597)>>> CTGTCCACTTCCGCCTCAGC
E93 5exo exon II(b)(1597-1578)

tatattttag gggaaatccg aaacgaattg acagaaatat acaatcttat cacaacacct gagtgagtgg
TTGTAAAGCA GAATATGACT TTTACGAACT CGCAATAAAC CAATCATCAT
GAGCTTTAAC AGATATTACG AAATATAACC AGTGTAGGTT TATTATTTAC
CTATATATAC AGGTATACAT TAGACTTTTA GAAGTTTGTG CTCCTTAAAA
ACTTTTGGAT TGTCACTATA AACTTAATTT GTTTCCGTTA TCCCTTGCAG
AGCCTGTGCC ACGCCCACGC CGTCGGGAAG AAGGGCGTAC AGTGAAGAGG
aTCTGAGCCG GGCCCTACAG GATGTGGTGG CCAACAAGCT AGATGCCCGG
AAATCGGCTA GCCAGCACCA TGAGCAGCGC TCCATTCTGG ACAACCGGCT
GTTCAAGATG AAACACCATG ACCAGGAGCA GGATCATGAT GGCGACGAGC
TCGAGGACTC CAACGATGAT GCTGAGGCGG AAGTGGACAG

clone2-unique start

Clone_2(f) (1-20) forward primer to make clone_2 unique start: gca gaa ata caa cgc atc cg
Clone_2(b) (592-573) reverse primer to make clone_2 unique start: ctt tcg ggt ttt ttt tta tt
GCAGAAATACAACGCATCCGATATGCAACGGAACGCATTTTCGGACGCGGCC
CGAGTCGTTCCGAGGTGTAGCGTCCGATTCCGATTCAGATTCAGATTCCGACG
ATCGCGTATATCAATCaTATTAAACAAAAACAACAACTGCAACAACAACGGC
TACACTTTTGTTAGTAAATTTCTGTTGAATTAAAATACGAAACAAAACATATA
AAAAAGTGCAATCCAAAAAAAACAAATATTAACGTTAAACGAACTATAAAG
CAAAATAATTAAAAAAGTAGCCATTACTTTTGAATTGTTTTCAAATGCAAAA
AAGAAAAAGGTTCGTAAGCAACAAAAAAAAAGTTAAATGCTAAAGCATTTT

GATAATGCAACATATCAAAGAGCCAGAATTTAAACACAGCAAAAAATAAAT
AAAATAATTAATATACAAACACGCGGGGCAACTGTCAAAAAATCTACAACAA
CACAAAGTGCAACTTTCAACAACAACAAAATAAGTGCAGCAGTACAACAAC
AAAAAGCATTACACAATTGAACAAACAACATACAAAAAAAAAATATTGCCA
CCAAATAAAAAAAAACCCGAAAG

1st half pipsq (P1)
TGAGCGCCCAGGAGGCATTG E93 pipsq(F) (2653-2672)
Gagcccaagccgcagcccga >>> tcgggctgcggcttgggctC E93 pipsq_II(b)(2878-2859)
TGAGCGCCCAGGAGGCATTGGGCAAGGGAACGCGACCAAAGAGGGGCAAGT
ATCGCAACTATGACCGCGACAGTTTGGTGGAGGCGGTCAAGGCGGTGCAGAG
AGGTGAAATGTCGGTTCATCGAGCGGGTAGCTACTACGGCGTACCGCATTCC
ACGCTGGAGTACAAGGTCAAGGAACGTCACCTGATGCGACCGCGCAAGCGA
GAGCCCAAGCCGCAGCCCGA

2nd half pipsq (P2)
Tctcgtcggcctgaccggac E93 pipsq_II(f)(2879-2898)
GCGTTATCCTTTCAGCCGAA (3078-
3097) >>> TTCGGCTGAAAGGATAACGC E93 pipsq(B)(3097-3078)
TCTCGTCGGCCTGACCGGACCAGCCAACAAGCTGCAGCTGGACAAACTGAAG
GCGGGACCACATGGTGGCTCCAAGCTGAGCAATGCCCTCAAGAACCAAAAC
AATCAGGCGGCTGCGGCGGCGGCGGCGGCAGCAGCAGCAGCGGCCGCTGCC
ACGCCCAACGGCCTGAAACTGCCCCTTTTCGAGGCGGGTCCACAGGCGTTAT
CCTTTCAGCCGAA

Primers for RTPCR

Primer #1: E93 3rd common exon (E93_3_com_exo(b)) (1357-1338) first strand primer for RT-PCR
GGCGATGTGAAGTCCGTCAG >>>> CTGACGGACTTCACATCGCC

Primer #2: E93 3rd common exon internal (E93_3_com_exo_int(b)) (1323-1304) backward PCR primer for RT-PCR
TAGCAGCAAACCATCGCCGA >>>> TCGGCGATGGTTTGCTGCTA

Primer #3: LP0 unique start primer (LP0_US(f)) (576-596) forward PCR primer or RT-PCR
GCCGATTGTTCATATGTGAG

Primer #3: E93 unique start primer (E93_US(f)) (408-428) forward PCR primer for RT-PCR
CACATCAGCAGCTATGAAAT

Clones from cDNA screening

Clone1 (LP library F111 pBluescript SK-)

AATGAAAAGTCCGCATCAACAATAAAAATCTGCCTGCATTTTTGCCTTTTGTG
TGAGCTGCCCACCAGAACGAGAGAAGCACTTTTATTGTATATAAAAATTATA
TACATCGCCGGGAGGAGCTGCAGCAACCCACTCCAAGCCAGGGTTGCCACGT
CCTGAGCTGCTGTAAGCTCTCCGCAGCAGCTGCAGCAGCATCAGCATCGCAG
CAGCATCAGCAGCAGCGCAGCAACCGCCGCAGCATCAATTTGGCTTTTGGGC
AGAGATAATTTAAGACAAATATATGTGATGCTATGCACATCAGCAGCTATGA
AATATCCCTAGAACGCGTTGCTGAAGAATGTATGGGTCGCAGGCAATGGAAA
CATTATCAAGACAAACTGACGTGCAGCCACTTGAATATCGAGGAGCAACAGC
CCATAGCAATAGCCGGTTCCGAGGACGAGCCATCGCAATACAACCACAGCAG
CAAGGAGATCAGCCAGAGCAATCCCAACCACTGTAAGACAGAGAACCACCG
TCTGGAGCAGCAACACAACGGCAGCCAGCTATTGGAAGAAGAAGATTCTGA
GAACAACCAAACATCACACGATTCATCACGTACACCAACACCGGGAGCCACC
AGTACACCATCACCACCGCCAGAACCCATCGATTGGAGACCGTCGGCCAAGT
GCAACTTCTGTGTTAACGGTCGCCTGCTAACGGTTAACGCCCAGGGCAAGTT
GGTGGCCGAGTCAGCAGCAACTGCCACTAGTAGTAGCACTAGTAATAGTCAC
ATTCATCAGCACGACAGTGACAGCAACTCGAGTGCATCACTGCCCCACCACA
TCAGCAGCAGCAGCAGCAGCAACAACAATAGCAGTGGCAACAGGGCACGCC
ACATTGCTGCTGCAAGTGCAAGAGCAACACCAGCAGCGGCCACACCCGCCAA
CTCCCTTGAACTCTACAAGCTGCTGACCCAGCGGGCAGCCAAAATGACATCG
ATGGACTCGATGGCCGCCCAGCTGGCGCAATTCTCACTGCTGGCCGACTTCA
ATCTGATCAACTCGCTGGCCAGCCAACAGCAGCAGCAGCAGCAGCAACAGAT
CGCTAGTGCGGTAACGCCAACTACCTCAGAAGTATCTGCAGCCGCAATCAGT
CCCGCACTCAAAGATACACCCAGTCCCAGTGTGGATGCACCGCTCGATCTTA
GCAGCAAACCATCGCCGAACTCATCGATTAGCGGCGATGTGAAGTCCGTCAG
AGCCTGTGCCACGCCCACGCCGTCGGGAAGAAGGGCGTACAGTGAAGAGGA
TCTGAGCCGGGCCCTACAGGATGTGGTGGCCAACAAGCTAGATGCCCGGAAA
TCGGCTAGCCAGCACCATGAGCAGCGCTCCATTCTGGACAACCGGCTGTTCA
AGATGAAACACCATGACCAGGAGCAGGATCATGATGGCGACGAGCTCGAGG
ACTCCAACGATGATGCTGAGGCGGAAGTGGACAGCAATGCGTCGACACCGGT
GTATCCGGCAGAGTTTGCAAGGGCACAACTGCGCAAACTGAGCCACCTGTCC
GAGCACAATGGCAGCGATCTGGGCGAGGATGTGGATCGTGGATCGCCGAAA
ATGGGGCGACATCCGGCCTGTGGCAATGCCAGTGCCAATCAGGGCGCACCGC
CATCCATTCCGCTGGATGCCAATGTCCTGCTGCACACTCTGATGCTGGCTGCT
GGGATTGGTGCAATGCCGAAGCTGGATGAAACGCAAACGGTGGGCGACTTTA
TCAAGGGTCTGCTGGTGGCCAACAGTGGTGGCATAATGAACGAGGGACTGCT
AAATCTGCTGTCCGCCAGTCAGGAGAACAGCAATGGCAATGCCTCGCTGCTG
CTGCAACAGCAACAGCATCAGCAACACCATCAGCAACACCATCAGCAGCAG
CAGCAGCAGCAACATGTCGCCGCCTACCGGCATCGCCTGCCCAAGTCGGAGA
CTCCGGAAACGAACTCCTCGTTGGATCCGAACGATGCCAGCGAGGATCCCAT
ACTGAAGATTCCGTCCTTCAAGGTCAGCGGTCCGGCCAGCAGCAGCAGCCTG
TCGCCGGGCGGACTGGTTGGTGGTCACCACCATCCGCTGAACAACAACAACA
GCCTCAGCATCAGCAACAACAGCAACCACAGCAGCAACAGCCATCGGAACG
GCAGCAATCGCAGCCCGCATTCCGCATCGCCCATGCTGGCCGCGGCCGTGGC
CCAAGGTGGCTACTCCGCCGGCAACAGTTTGCTGACCTCATCCTCGTCTAGCA
TACAGAAGATGATGGCCAGCAATATCCAGCGCCAGATCAACGAACAGAGTG

GCCAGGAGAGTCTCAGGAACGGAAATGTTAGCGATTGCAGCAGCAACAATG
GCGGCTCCTCCTCGCTGGGATACAAGAAGCCGAGCATTTCGGTGGCCAAGAT
CATTGGCGGAACGGACACCTCACGGTTCGGAGCCTCGCCCAATCTGCTGTCC
CAACAGCACCATTCGGCTCACCACCTGACCCACCAGCAACAGCAGCAACAGC
TGAGCGCCCAGGAGGCATTGGGCAAGGGAACGCGACCAAAGAGGGGCAAGT
ATCGCAACTATGACCGCGACAGTTTGGTGGAGGCGGTCAAGGCGGTGCAGAG
AGGTGAAATGTCGGTTCATCGAGCGGGTAGCTACTACGGCGTACCGCATTCC
ACGCTGGAGTACAAGGTCAAGGAACGTCACCTGATGCGACCGCGCAAGCGA
GAGCCCAAGCCGCAGCCCGATCTCGTCGGCCTGACCGGACCAGCCAACAAGC
TGCAGCTGGACAAACTGAAGGCGGGACCACATGGTGGCTCCAAGCTGAGCA
ATGCCCTCAAGAACCAAAACAATCAGGCGGCTGCGGCGGCGGCGGCGGCAG
CAGCAGCAGCGGCCGCTGCCACGCCCAACGGCCTGAAACTGCCCCTTTTCGA
GGCGGGTCCACAGGCGTTATCCTTTCAGCCGAACATGTTCTGGCCCCAGACG
AACGCCACGAATGCCTACGGCCTGGACTTCAATCGCATCACGGAGGCGATGC
GGAATCCCCAGGCCTCCAATCACCACGGCCTGATGAAGAGTGCCCAGGACAT
GGTGGAGAACGTGTACGATGGCATCATCAGGAAGACGCTGCAGGCGAGCGA
GGGCAATGGCAGTGCGGCGGGTAATGGCAGCAACGGTAGCAATGGCAACGG
GCATGGGCACGGGCATGGCCATGGACACGCCCTGCTCGATCAGCTGCTGGTG
AAGAAGACCCCCTTGCCGTTCACCAACCATCGGAACAATGACTACGCCGCCA
CCTGTTCGAGTGCCAGCGGGGAGAGCGTAAAGCGGTCGGGCAGTCCCATGGG
CAACTATGCAGACATCAAGCGGGAGCGCCTGAGCGCCGACAGCGGCGGCAG
CAGCGATGAGGAGCACTCGGCCAGCCACATCAACAACAACAACAGCGATTT
GGCGCACAACAAGAACAAGAGCGGCGGCGGCGGCGGCGGCGGCGGCAATGG
CCAGACCAATGGGAACGGCAGGAGCAGCCGGATGACGTCGCGGGATGATTC
CGAAACGGATGCCAGCAGCTTGAAGAGCGGCGAAAGTGGCGGCCAGCAAAA
CCACAAAATGATGGATCTCAATGGCGGCAGCAGCAGCAGCAGTCACATCAA
GTGCGAATCGGAGGCGGCCACCGGACATCACAGTCCTGGACACCACACCACG
TCCATACTGCACGAGAAGCTGGCCCAGATCAAGGCCGAGCAAGTGGACCAG
GCGGATCAGTTATAGGAGCAGCCGATGGCCGCGAATCCAGCGTTCGCCTGGC
CACCGCTGGCCGCCCACTACTACAGCTTCTAGGCGGAGGGAGGGGGAACACC
AAATTAAGCCACGTTTTTTGATAGTACCATACAAATCACTAAATAGAATTATA
TATATATATATATATATATATATACATATATAATAATTTATGCCAGCCAGCTG
ACCGATGTGCGTGGTAAATGTGCGCTAGCTCTTAGTTAAATGTGTAATCAACT
GCATAGGGGAAAAACAAAACCACAGGAAATCATAAACAACAACAAACAAAC
AAAAAAAACAAAAA

Clone2 (LP library F211 pBluescript SK-)
GCAGAAATACAACGCATCCGATATGCAACGGAACGCATTTTCGGACGCGGCC
CGAGTCGTTCCGAGGTGTAGCGTCCGATTCCGATTCAGATTCAGATTCCGACG
ATCGCGTATATCAATCATATTAAACAAAAACAACAACTGCAACAACAACGGC
TACACTTTTGTTAGTAAATTTCTGTTGAATTAAAATACGAAACAAAACATATA
AAAAAGTGCAATCCAAAAAAAACAAATATTAACGTTAAACGAACTATAAAG
CAAAATAATTAAAAAAGTAGCCATTACTTTTGAATTGTTTTCAAATGCAAAA
AAGAAAAAGGTTCGTAAGCAACAAAAAAAAAGTTAAATGCTAAAGCATTTT
GATAATGCAACATATCAAAGAGCCAGAATTTAAACACAGCAAAAAATAAAT
AAAATAATTAATATACAAACACGCGGGGCAACTGTCAAAAAATCTACAACAA

CACAAAGTGCAACTTTCAACAACAACAAAATAAGTGCAGCAGTACAACAAC
AAAAAGCATTACACAATTGAACAAACAACATACAAAAAAAAAATATTGCCA
CCAAATAAAAAAAAACCCGAAAGAAAATTCGCGCTTCATTTTCACGCGCTGC
ATATAATTTACTCTGTGCTCCACATATATATATATATATATATATAATAAATA
AAAACCAACCAATAGCCCAAAAAAAAAGAAATGAAAAGTCCGCATCAACAA
TAAAAATCTGCCTGCATTTTTGCCTTTTGTGTGAGCTGCCCACCAGAACGAGA
GAAGCACTTTTATTGTATATAAAAATTATATACATCGCCGGGAGGAGCTGCA
GCAACCCACTCCAAGCCAGGGTTGCCACGTCCTGAGCTGCTGTAAGCTCTCC
GCAGCAGCTGCAGCAGCATCAGCATCGCAGCAGCATCAGCAGCAGCGCAGC
AACCGCCGCAGCATCAATTTGGCTTTTGGGCAGAGATAATTTAAGACAAATA
TATGTGATGCTATGCACATCAGCAGCTATGAAATATCCCTAGAACGCGTTGCT
GAAGAATGTATGGGTCGCAGGCAATGGAAACATTATCAAGACAAACTGACGT
GCAGCCACTTGAATATCGAGGAGCAACAGCCCATAGCAATAGCCGGTTCCGA
GGACGAGCCATCGCAATACAACCACAGCAGCAAGGAGATCAGCCAGAGCAA
TCCCAACCACTGTAAGACAGAGAACCACCGTCTGGAGCAGCAACACAACGG
CAGCCAGCTATTGGAAGAAGAAGATTCTGAGAACAACCAAACATCACACGAT
TCATCACGTACACCAACACCGGGAGCCACCAGTACACCATCACCACCGCCAG
AACCCATCGATTGGAGACCGTCGGCCAAGTGCAACTTCTGTGTTAACGGTCG
CCTGCTAACGGTTAACGCCCAGGGCAAGTTGGTGGCCGAGTCAGCAGCAACT
GCCACTAGTAGTAGCACTAGTAATAGTCACATTCATCAGCACGACAGTGACA
GCAACTCGAGTGCATCACTGCCCCACCACATCAGCAGCAGCAGCAGCAGCAA
CAACAATAGCAGTGGCAACAGGGCACGCCACATTGCTGCTGCAAGTGCAAG
AGCAACACCAGCAGCGGCCACACCCGCCAACTCCCTTGAACTCTACAAGCTG
CTGACCCAGCGGGCAGCCAAAATGACATCGATGGACTCGATGGCCGCCCAGC
TGGCGCAATTCTCACTGCTGGCCGACTTCAATCTGATCAACTCGCTGGCCAGC
CAACAGCAGCAGCAGCAGCAGCAACAGATCGCTAGTGCGGTAACGCCAACT
ACCTCAGAAGTATCTGCAGCCGCAATCAGTCCCGCACTCAAAGATACACCCA
GTCCCAGTGTGGATGCACCGCTCGATCTTAGCAGCAAACCATCGCCGAACTC
ATCGATTAGCGGCGATGTGAAGTCCGTCAGAGCCTGTGCCACGCCCACGCCG
TCGGGAAGAAGGGCGTACAGTGAAGAGGATCTGAGCCGGGCCCTACAGGAT
GTGGTGGCCAACAAGCTAGATGCCCGGAAATCGGCTAGCCAGCACCATGAGC
AGCGCTCCATTCTGGACAACCGGCTGTTCAAGATGAAACACCATGACCAGGA
GCAGGATCATGATGGCGACGAGCTCGAGGACTCCAACGATGATGCTGAGGCG
GAAGTGGACAGCAATGCGTCGACACCGGTGTATCCGGCAGAGTTTGCAAGGG
CACAACTGCGCAAACTGAGCCACCTGTCCGAGCACAATGGCAGCGATCTGGG
CGAGGATGTGGATCGTGGATCGCCGAAAATGGGGCGACATCCGGCCTGTGGC
AATGCCAGTGCCAATCAGGGCGCACCGCCATCCATTCCGCTGGATGCCAATG
TCCTGCTGCACACTCTGATGCTGGCTGCTGGGATTGGTGCAATGCCGAAGCTG
GATGAAACGCAAACGGTGGGCGACTTTATCAAGGGTCTGCTGGTGGCCAACA
GTGGTGGCATAATGAACGAGGGACTGCTAAATCTGCTGTCCGCCAGTCAGGA
GAACAGCAATGGCAATGCCTCGCTGCTGCTGCAACAGCAACAGCATCAGCAA
CACCATCAGCAACACCATCAGCAGCAGCAGCAGCAGCAACATGTCGCCGCCT
ACCGGCATCGCCTGCCCAAGTCGGAGACTCCGGAAACGAACTCCTCGTTGGA
TCCGAACGATGCCAGCGAGGATCCCATACTGAAGATTCCGTCCTTCAAGGTC
AGCGGTCCGGCCAGCAGCAGCAGCCTGTCGCCGGGCGGACTGGTTGGTGGTC
ACCACCATCCGCTGAACAACAACAACAGCCTCAGCATCAGCAACAACAGCA

ACCACAGCAGCAACAGCCATCGGAACGGCAGCAATCGCAGCCCGCATTCCGC
ATCGCCCATGCTGGCCGCGGCCGTGGCCCAAGGTGGCTACTCCGCCGGCAAC
AGTTTGCTGACCTCATCCTCGTCTAGCATACAGAAGATGATGGCCAGCAATAT
CCAGCGCCAGATCAACGAACAGAGTGGCCAGGAGAGTCTCAGGAACGGAAA
TGTTAGCGATTGCAGCAGCAACAATGGCGGCTCCTCCTCGCTGGGATACAAG
AAGCCGAGCATTTCGGTGGCCAAGATCATTGGCGGAACGGACACCTCACGGT
TCGGAGCCTCGCCCAATCTGCTGTCCCAACAGCACCATTCGGCTCACCACCTG
ACCCACCAGCAACAGCAGCAACAGCTGAGCGCCCAGGAGGCATTGGGCAAG
GGAACGCGACCAAAGAGGGGCAAGTATCGCAACTATGACCGCGACAGTTTG
GTGGAGGCGGTCAAGGCGGTGCAGAGAGGTGAAATGTCGGTTCATCGAGCG
GGTAGCTACTACGGCGTACCGCATTCCACGCTGGAGTACAAGGTCAAGGAAC
GTCACCTGATGCGACCGCGCAAGCGAGAGCCCAAGCCGCAGCCCGATCTCGT
CGGCCTGACCGGACCAGCCAACAAGCTGCAGCTGGACAAACTGAAGGCGGG
ACCACATGGTGGCTCCAAGCTGAGCAATGCCCTCAAGAACCAAAACAATCAG
GCGGCTGCGGCGGCGGCGGCGGCAGCAGCAGCAGCGGCCGCTGCCACGCCC
AACGGCCTGAAACTGCCCCTTTTCGAGGCGGGTCCACAGGCGTTATCCTTTCA
GCCGAACATGTTCTGGCCCCAGACGAACGCCACGAATGCCTACGGCCTGGAC
TTCAATCGCATCACGGAGGCGATGCGGAATCCCCAGGCCTCCAATCACCACG
GCCTGATGAAGAGTGCCCAGGACATGGTGGAGAACGTGTACGATGGCATCAT
CAGGAAGACGCTGCAGGCGAGCGAGGGCAATGGCAGTGCGGCGGGTAATGG
CAGCAACGGTAGCAATGGCAACGGGCATGGGCACGGGCATGGCCATGGACA
CGCCCTGCTCGATCAGCTGCTGGTGAAGAAGACCCCCTTGCCGTTCACCAAC
CATCGGAACAATGACTACGCCGCCACCTGTTCGAGTGCCAGCGGGGAGAGCG
TAAAGCGGTCGGGCAGTCCCATGGGCAACTATGCAGACATCAAGCGGGAGC
GCCTGAGCGCCGACAGCGGCGGCAGCAGCGATGAGGAGCACTCGGCCAGCC
ACATCAACAACAACAACAGCGATTTGGCGCACAACAAGAACAAGAGCGGCG
GCGGCGGCGGCGGCGGCGGCAATGGCCAGACCAATGGGAACGGCAGGAGCA
GCCGGATGACGTCGCGGGATGATTCCGAAACGGATGCCAGCAGCTTGAAGAG
CGGCGAAAGTGGCGGCCAGCAAAACCACAAAATGATGGATCTCAATGGCGG
CAGCAGCAGCAGCAGTCACATCAAGTGCGAATCGGAGGCGGCCACCGGACA
TCACAGTCCTGGACACCACACCACGTCCATACTGCACGAGAAGCTGGCCCAG
ATCAAGGCCGAGCAAGTGGACCAGGCGGATCAGTTATAGGAGCAGCCGATG
GCCGCGAATCCAGCGTTCGCCTGGCCACCGCTGGCCGCCCACTACTACAGCT
TCTAGGCGGAGGGAGGGGGAACACCAAATTAAGCCACGTTTTTTGATAGTAC
CATACAAA

Clone3 (LP library F311 pBluescript SK-)

TCCGAACGATGCCAGCGAGGATCCCATACTGAAGATTCCGTCCTTCAAGGTC
AGCGGTCCGGCCAGCAGCAGCAGCCTGTCGCCGGGCGGACTGGTTGGTGGTC
ACCACCATCCGCTGAACAACAACAACAGCCTCAGCATCAGCAACAACAGCA
ACCACAGCAGCAACAGCCATCGGAACGGCAGCAATCGCAGCCCGCATTCCGC
ATCGCCCATGCTGGCCGCGGCCGTGGCCCAAGGTGGCTACTCCGCCGGCAAC
AGTTTGCTGACCTCATCCTCGTCTAGCATACAGAAGATGATGGCCAGCAATAT
CCAGCGCCAGATCAACGAACAGAGTGGCCAGGAGAGTCTCAGGAACGGAAA
TGTTAGCGATTGCAGCAGCAACAATGGCGGCTCCTCCTCGCTGGGATACAAG
AAGCCGAGCATTTCGGTGGCCAAGATCATTGGCGGAACGGACACCTCACGGT

TCGGAGCCTCGCCCAATCTGCTGTCCCAACAGCACCATTCGGCTCACCACCTG
ACCCACCAGCAACAGCAGCAACAGCTGAGCGCCCAGGAGGCATTGGGCAAG
GGAACGCGACCAAAGAGGGGCAAGTATCGCAACTATGACCGCGACAGTTTG
GTGGAGGCGGTCAAGGCGGTGCAGAGAGGTGAAATGTCGGTTCATCGAGCG
GGTAGCTACTACGGCGTACCGCATTCCACGCTGGAGTACAAGGTCAAGGAAC
GTCACCTGATGCGACCGCGCAAGCGAGAGCCCAAGCCGCAGCCCGATCTCGT
CGGCCTGACCGGACCAGCCAACAAGCTGCAGCTGGACAAACTGAAGGCGGG
ACCACATGGTGGCTCCAAGCTGAGCAATGCCCTCAAGAACCAAAACAATCAG
GCGGCTGCGGCGGCGGCGGCGGCAGCAGCAGCAGCGGCCGCTGCCACGCCC
AACGGCCTGAAACTGCCCCTTTTCGAGGCGGGTCCACAGGCGTTATCCTTTCA
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GCCTGATGAAGAGTGCCCAGGACATGGTGGAGAACGTGTACGATGGCATCAT
CAGGAAGACGCTGCAGGCGAGCGAGGGCAATGGCAGTGCGGCGGGTAATGG
CAGCAACGGTAGCAATGGCAACGGGCATGGGCACGGGCATGGCCATGGACA
CGCCCTGCTCGATCAGCTGCTGGTGAAGAAGACCCCCTTGCCGTTCACCAAC
CATCGGAACAATGACTACGCCGCCACCTGTTCGAGTGCCAGCGGGGAGAGCG
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ACATCAACAACAACAACAGCGATTTGGCGCACAACAAGAACAAGAGCGGCG
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GCCGGATGACGTCGCGGGATGATTCCGAAACGGATGCCAGCAGCTTGAAGAG
CGGCGAAAGTGGCGGCCAGCAAAACCACAAAATGATGGATCTCAATGGCGG
CAGCAGCAGCAGCAGTCACATCAAGTGCGAATCGGAGGCGGCCACCGGACA
TCACAGTCCTGGACACCACACCACGTCCATACTGCACGAGAAGCTGGCCCAG
ATCAAGGCCGAGCAAGTGGACCAGGCGGATCAGTTATAGGAGCAGCCGATG
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CATACAAATCACTAAATAGAATTATATATATATATATATATATATATATACAT
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AGCTCTTAGTTAAATGTGTAATCAACTGCATAGGGGAAAAACAAAACCACAG
GAAATCATAAACAACAACAAACAAACAAAAAAAACAAAAA

Clone5 (LP library F511 pBluescript SK-)

CCCGCACTCAAAGATACACCCAGTCCCAGTGTGGATGCACCGCTCGATCTTA
GCAGCAAACCATCGCCGAACTCATCGATTAGCGGCGATGTGAAGTCCGTCAG
AGCCTGTGCCACGCCCACGCCGTCGGGAAGAAGGGCGTACAGTGAAGAGGA
TCTGAGCCGGGCCCTACAGGATGTGGTGGCCAACAAGCTAGATGCCCGGAAA
TCGGCTAGCCAGCACCATGAGCAGCGCTCCATTCTGGACAACCGGCTGTTCA
AGATGAAACACCATGACCAGGAGCAGGATCATGATGGCGACGAGCTCGAGG
ACTCCAACGATGATGCTGAGGCGGAAGTGGACAGCAATGCGTCGACACCGGT
GTATCCGGCAGAGTTTGCAAGGGCACAACTGCGCAAACTGAGCCACCTGTCC
GAGCACAATGGCAGCGATCTGGGCGAGGATGTGGATCGTGGATCGCCGAAA
ATGGGGCGACATCCGGCCTGTGGCAATGCCAGTGCCAATCAGGGCGCACCGC
CATCCATTCCGCTGGATGCCAATGTCCTGCTGCACACTCTGATGCTGGCTGCT
GGGATTGGTGCAATGCCGAAGCTGGATGAAACGCAAACGGTGGGCGACTTTA

TCAAGGGTCTGCTGGTGGCCAACAGTGGTGGCATAATGAACGAGGGACTGCT
AAATCTGCTGTCCGCCAGTCAGGAGAACAGCAATGGCAATGCCTCGCTGCTG
CTGCAACAGCAACAGCATCAGCAACACCATCAGCAACACCATCAGCAGCAG
CAGCAGCAGCAACATGTCGCCGCCTACCGGCATCGCCTGCCCAAGTCGGAGA
CTCCGGAAACGAACTCCTCGTTGGATCCGAACGATGCCAGCGAGGATCCCAT
ACTGAAGATTCCGTCCTTCAAGGTCAGCGGTCCGGCCAGCAGCAGCAGCCTG
TCGCCGGGCGGACTGGTTGGTGGTCACCACCATCCGCTGAACAACAACAACA
GCCTCAGCATCAGCAACAACAGCAACCACAGCAGCAACAGCCATCGGAACG
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TACAGAAGATGATGGCCAGCAATATCCAGCGCCAGATCAACGAACAGAGTG
GCCAGGAGAGTCTCAGGAACGGAAATGTTAGCGATTGCAGCAGCAACAATG
GCGGCTCCTCCTCGCTGGGATACAAGAAGCCGAGCATTTCGGTGGCCAAGAT
CATTGGCGGAACGGACACCTCACGGTTCGGAGCCTCGCCCAATCTGCTGTCC
CAACAGCACCATTCGGCTCACCACCTGACCCACCAGCAACAGCAGCAACAGC
TGAGCGCCCAGGAGGCATTGGGCAAGGGAACGCGACCAAAGAGGGGCAAGT
ATCGCAACTATGACCGCGACAGTTTGGTGGAGGCGGTCAAGGCGGTGCAGAG
AGGTGAAATGTCGGTTCATCGAGCGGGTAGCTACTACGGCGTACCGCATTCC
ACGCTGGAGTACAAGGTCAAGGAACGTCACCTGATGCGACCGCGCAAGCGA
GAGCCCAAGCCGCAGCCCGATCTCGTCGGCCTGACCGGACCAGCCAACAAGC
TGCAGCTGGACAAACTGAAGGCGGGACCACATGGTGGCTCCAAGCTGAGCA
ATGCCCTCAAGAACCAAAACAATCAGGCGGCTGCGGCGGCGGCGGCGGCAG
CAGCAGCAGCGGCCGCTGCCACGCCCAACGGCCTGAAACTGCCCCTTTTCGA
GGCGGGTCCACAGGCGTTATCCTTTCAGCCGAACATGTTCTGGCCCCAGACG
AACGCCACGAATGCCTACGGCCTGGACTTCAATCGCATCACGGAGGCGATGC
GGAATCCCCAGGCCTCCAATCACCACGGCCTGATGAAGAGTGCCCAGGACAT
GGTGGAGAACGTGTACGATGGCATCATCAGGAAGACGCTGCAGGCGAGCGA
GGGCAATGGCAGTGCGGCGGGTAATGGCAGCAACGGTAGCAATGGCAACGG
GCATGGGCACGGGCATGGCCATGGACACGCCCTGCTCGATCAGCTGCTGGTG
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TCCATACTGCACGAGAAGCTGGCCCAGATCAAGGCCGAGCAAGTGGACCAG
GCGGATCAGTTATAGGAGCAGCCGATGGCCGCGAATCCAGCGTTCGCCTGGC
CACCGCTGGCCGCCCACTACTACAGCTTCTAGGCGGAGGGAGGGGGAACACC
AAATTAAGCCACGTTTTTTGATAGTACCATACAAATCACTAAATAGAATTATA
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GCATAGGGGAAAAACAAAACCACAGGAAATCATAAACAACAACAAACAAAC
AAAAAAAACAAAA

Clone6 (LP library F611 pBluescript SK-)

ATAGCAATAGCCGGTTCCGAGGACGAGCCATCGCAATACAACCACAGCAGC
AAGGAGATCAGCCAGAGCAATCCCAACCACTGTAAGACAGAGAACCACCGT
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AACAACCAAACATCACACGATTCATCACGTACACCAACACCGGGAGCCACCA
GTACACCATCACCACCGCCAGAACCCATCGATTGGAGACCGTCGGCCAAGTG
CAACTTCTGTGTTAACGGTCGCCTGCTAACGGTTAACGCCCAGGGCAAGTTG
GTGGCCGAGTCAGCAGCAACTGCCACTAGTAGTAGCACTAGTAATAGTCACA
TTCATCAGCACGACAGTGACAGCAACTCGAGTGCATCACTGCCCCACCACAT
CAGCAGCAGCAGCAGCAGCAACAACAATAGCAGTGGCAACAGGGCACGCCA
CATTGCTGCTGCAAGTGCAAGAGCAACACCAGCAGCGGCCACACCCGCCAAC
TCCCTTGAACTCTACAAGCTGCTGACCCAGCGGGCAGCCAAAATGACATCGA
TGGACTCGATGGCCGCCCAGCTGGCGCAATTCTCACTGCTGGCCGACTTCAAT
CTGATCAACTCGCTGGCCAGCCAACAGCAGCAGCAGCAGCAGCAACAGATC
GCTAGTGCGGTAACGCCAACTACCTCAGAAGTATCTGCAGCCGCAATCAGTC
CCGCACTCAAAGATACACCCAGTCCCAGTGTGGATGCACCGCTCGATCTTAG
CAGCAAACCATCGCCGAACTCATCGATTAGCGGCGATGTGAAGTCCGTCAGA
GCCTGTGCCACGCCCACGCCGTCGGGAAGAAGGGCGTACAGTGAAGAGGAT
CTGAGCCGGGCCCTACAGGATGTGGTGGCCAACAAGCTAGATGCCCGGAAAT
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CTCCAACGATGATGCTGAGGCGGAAGTGGACAGCAATGCGTCGACACCGGTG
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AGCACAATGGCAGCGATCTGGGCGAGGATGTGGATCGTGGATCGCCGAAAAT
GGGGCGACATCCGGCCTGTGGCAATGCCAGTGCCAATCAGGGCGCACCGCCA
TCCATTCCGCTGGATGCCAATGTCCTGCTGCACACTCTGATGCTGGCTGCTGG
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GCAACAGCAACAGCATCAGCAACACCATCAGCAACACCATCAGCAGCAGCA
GCAGCAGCAACATGTCGCCGCCTACCGGCATCGCCTGCCCAAGTCGGAGACT
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GCCGGGCGGACTGGTTGGTGGTCACCACCATCCGCTGAACAACAACAACAGC
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CGCAACTATGACCGCGACAGTTTGGTGGAGGCGGTCAAGGCGGTGCAGAGA
GGTGAAATGTCGGTTCATCGAGCGGGTAGCTACTACGGCGTACCGCATTCCA

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AGCCCAAGCCGCAGCCCGATCTCGTCGGCCTGACCGGACCAGCCAACAAGCT
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TGCCCTCAAGAACCAAAACAATCAGGCGGCTGCGGCGGCGGCGGCGGCAGC
AGCAGCAGCGGCCGCTGCCACGCCCAACGGCCTGAAACTGCCCCTTTTCGAG
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GAATCCCCAGGCCTCCAATCACCACGGCCTGATGAAGAGTGCCCAGGACATG
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AGCGATGAGGAGCACTCGGCCAGCCACATCAACAACAACAACAGCGATTTG
GCGCACAACAAGAACAAGAGCGGCGGCGGCGGCGGCGGCGGCGGCAATGGC
CAGACCAATGGGAACGGCAGGAGCAGCCGGATGACGTCGCGGGATGATTCC
GAAACGGATGCCAGCAGCTTGAAGAGCGGCGAAAGTGGCGGCCAGCAAAAC
CACAAAATGATGGATCTCAATGGCGGCAGCAGCAGCAGCAGTCACATCAAGT
GCGAATCGGAGGCGGCCACCGGACATCACAGTCCTGGACACCACACCACGTC
CATACTGCACGAGAAGCTGGCCCAGATCAAGGCCGAGCAAGTGGACCAGGC
GGATCAGTTATAGGAGCAGCCGATGGCCGCGAATCCAGCGTTCGCCTGGCCA
CCGCTGGCCGCCCACTACTACAGCTTCTAGGCGGAGGGAGGGGGAACACCAA
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CGATGTGCGTGGTAAATGTGCGCTAGCTCTTAGTTAAATGTGTAATCAACTGC
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AAAAAACAAAAA

Clone7 (LP library F711 pBluescript SK-)

TGCAGCAGCATCAGCATCGCAGCAGCATCAGCAGCAGCGCAGCAACCGCCG
CAGCATCAATTTGGCTTTTGGGCAGAGATAATTTAAGACAAATATATGTGAT
GCTATGCACATCAGCAGCTATGAAATATCCCTAGAACGCGTTGCTGAAGAAT
GTATGGGTCGCAGGCAATGGAAACATTATCAAGACAAACTGACGTGCAGCCA
CTTGAATATCGAGGAGCAACAGCCCATAGCAATAGCCGGTTCCGAGGACGAG
CCATCGCAATACAACCACAGCAGCAAGGAGATCAGCCAGAGCAATCCCAAC
CACTGTAAGACAGAGAACCACCGTCTGGAGCAGCAACACAACGGCAGCCAG
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GAGTGCATCACTGCCCCACCACATCAGCAGCAGCAGCAGCAGCAACAACAAT
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AGCGGGCAGCCAAAATGACATCGATGGACTCGATGGCCGCCCAGCTGGCGC

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AGCGGCGATGTGAAGTCCGTCAGAGCCTGTGCCACGCCCACGCCGTCGGGAA
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GCAATGGCAATGCCTCGCTGCTGCTGCAACAGCAACAGCATCAGCAACACCA
TCAGCAACACCATCAGCAGCAGCAGCAGCAGCAACATGTCGCCGCCTACCGG
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ACGATGCCAGCGAGGATCCCATACTGAAGATTCCGTCCTTCAAGGTCAGCGG
TCCGGCCAGCAGCAGCAGCCTGTCGCCGGGCGGACTGGTTGGTGGTCACCAC
CATCCGCTGAACAACAACAACAGCCTCAGCATCAGCAACAACAGCAACCAC
AGCAGCAACAGCCATCGGAACGGCAGCAATCGCAGCCCGCATTCCGCATCGC
CCATGCTGGCCGCGGCCGTGGCCCAAGGTGGCTACTCCGCCGGCAACAGTTT
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CGCCAGATCAACGAACAGAGTGGCCAGGAGAGTCTCAGGAACGGAAATGTT
AGCGATTGCAGCAGCAACAATGGCGGCTCCTCCTCGCTGGGATACAAGAAGC
CGAGCATTTCGGTGGCCAAGATCATTGGCGGAACGGACACCTCACGGTTCGG
AGCCTCGCCCAATCTGCTGTCCCAACAGCACCATTCGGCTCACCACCTGACCC
ACCAGCAACAGCAGCAACAGCTGAGCGCCCAGGAGGCATTGGGCAAGGGAA
CGCGACCAAAGAGGGGCAAGTATCGCAACTATGACCGCGACAGTTTGGTGGA
GGCGGTCAAGGCGGTGCAGAGAGGTGAAATGTCGGTTCATCGAGCGGGTAG
CTACTACGGCGTACCGCATTCCACGCTGGAGTACAAGGTCAAGGAACGTCAC
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TGACCGGACCAGCCAACAAGCTGCAGCTGGACAAACTGAAGGCGGGACCAC
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ACATGTTCTGGCCCCAGACGAACGCCACGAATGCCTACGGCCTGGACTTCAA
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AGTCCTGGACACCACACCACGTCCATACTGCACGAGAAGCTGGCCCAGATCA
AGGCCGAGCAAGTGGACCAGGCGGATCAGTTATAGGAGCAGCCGATGGCCG
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ATAAACAACAACAAACAAACAAAAAAAACAAAAA

Bacterial stocks

Box #	Slot #	Exp#	Name	Date	Antibiotics	Origin
8	6E	1	E18set1 clone1.3 pSTBlue1	9/8/2004	Amp, Kan	Joanna
8	7E	1	E47aset clone2.2 pSTBlue1	9/8/2004	Amp, Kan	Joanna
8	8E	1	E18set2 clone4.2 pSTBlue1	9/8/2004	Amp, Kan	Joanna
8	1F	1	E47aset2 clone5.5 pSTBlue1	9/8/2004	Amp, Kan	Joanna
8	2F	1	E31set2 clone6.5 pSTBlue1	9/8/2004	Amp, Kan	Joanna
8	3F	2	E18set3 clone1.4 pSTBlue1 / E93 unique start	9/22/2004	Amp, Kan	Joanna
8	4F	2	E47aset3 clone2.2 pSTBlue1	9/22/2004	Amp, Kan	Joanna
8	5F	2	E31set3 clone3.4 pSTBlue1	9/22/2004	Amp, Kan	Joanna
8	6F	2	E18set4 clone4.1 pSTBlue1	9/22/2004	Amp, Kan	Joanna
8	7F	2	E47aset4 clone5.2 pSTBlue1	9/22/2004	Amp, Kan	Joanna
8	8F	5	E31set1 clone7 pSTBlue1	9/29/2004	Amp, Kan	Joanna
8	1G	5	E18cg31353 clone2 pSTBlue1	9/29/2004	Amp, Kan	Joanna
8	2G	5	E47 cg31353 clone4 pSTBlue1	9/29/2004	Amp, Kan	Joanna
8	3G	5	E31 cg31353 clone5 pSTBlue1	9/29/2004	Amp, Kan	Joanna
8	4G	7	E31set4 clone31.4.1 pSTBlue1	10/19/2004	Amp, Kan	Joanna
8	7G		LP08695 pOT2		Cmp	Flybase
8	8G		LP08695 unique F pSTBlue	1/6/2005		DMD
8	2H		LP0-UAST in DH5a bacteria strain		Amp	DMD
8	3H		LP0-UAST in XL1Blue bacteria strain		Amp	DMD
9	1A	11	E93 2/18-4 pBS	8/6/2005	Amp	Eric
9	2A	11	E93 rp-2 pBS	8/6/2005	Amp	Eric
9	3A	11	E93 2/1 rp5 pBS	8/6/2005	Amp	Eric

9	4A	11	E93 ORF pBluescript II SK+	8/7/2005	Amp	Eric
9	5A	11	pUAST E93	8/6/2005	Amp	Eric
Joanna	1A	31	E93 ORF NUS-pSTBlue1	3/4/2006	Amp, Kan	Joanna
Joanna	1B	31	E93 ORF NUSII-pSTBlue1	3/4/2006	Amp, Kan	Joanna
Joanna	1C	37	l(3)S12 pSTBlue1	4/20/2006	Amp, Kan	Joanna
Joanna	1D	37	E93 pipsqk pSTBlue1	4/20/2006	Amp, Kan	Joanna
Joanna	1E	29	E93 AccI 6569-8284 pSTBlue1	2/17/2006	Amp, Kan	Joanna
Joanna	1F	67	E93 5exoII pSTBlue1	7/14/2006	Amp, Kan	Joanna
Joanna	1G	67	E93 5exoIII forward pSTBlue1	7/14/2006	Amp, Kan	Joanna
Joanna	1H	67	E93 234exo pSTBlue1	7/14/2006	Amp, Kan	Joanna
Joanna	2A	67	E93 5exoI pSTBlue1	7/14/2006	Amp, Kan	Joanna
Joanna	2B	81	E93 5exoIII backward pSTBlue1	10/2/2006	Amp, Kan	Joanna
Joanna	2C	85	rp49 pBR322(?)	10/5/2006	Amp, Tet	Karmella Haynes
Joanna	2D	82	LP library F111 pBluescript SK- in SOLR bacteria strain	10/6/2006	Amp	Joanna
Joanna	2E	82	LP library F211 pBluescript SK- in SOLR bacteria strain	10/6/2006	Amp	Joanna
Joanna	2F	82	LP library F311 pBluescript SK- in SOLR bacteria strain	10/6/2006	Amp	Joanna
Joanna	2G	82	LP library F511 pBluescript SK- in SOLR bacteria strain	10/6/2006	Amp	Joanna
Joanna	2H	82	LP library F611 pBluescript SK- in SOLR bacteria strain	10/6/2006	Amp	Joanna
Joanna	3A	82	LP library F711 pBluescript SK- in SOLR bacteria strain	10/6/2006	Amp	Joanna
Joanna	3B	88	E93 5exo boundary pSTBlue1	11/1/2006	Amp, Kan	Joanna
Joanna	3C	122	clone2-unique start pSTBlue1 forward	6/7/2007	Amp, Kan	Joanna
Joanna	3D	136	LP0_XhoI-pUAST	6/7/2007	Amp	Joanna
Joanna	3E	143	E93 clone2-pCR8/GW/TOPO	9/1/2007	Spec	Joanna
Joanna	3F	145-42	E93-ORF-pCR8/GW/TOPO	10/18/2007	Spec	Joanna
Joanna	3G	144-34	E93-ORF-e47-pCR8/GW/TOPO	10/23/2007	Spec	Joanna
Joanna	3H	144-86	E93-ORF-CD1-pCR8/GW/TOPO	11/11/2007	Spec	Joanna
Joanna	4A	150-6	E93-ORF-pTW	12/9/2007	Amp	Joanna
Joanna	4B	151-10	E93-ORF-e47-pTW	12/9/2007	Amp	Joanna
Joanna	4C	152-7	E93-ORF-CD1-pTW	12/9/2007	Amp	Joanna
Joanna	4D	144-131	E93-ORF-Δpip-pCR8/GW/TOPO	12/16/2007	Spec	Joanna
Joanna	4E	156-7	E93-ORF-Δpip-pTW	1/1/2008	Amp	Joanna
Joanna	4F	143-71	E93-clone2-pCR8/GW/TOPO	1/1/2008	Spec	Joanna
Joanna	4G	165-7	E93-clone2-pTW	1/3/2008	Amp	Joanna
Joanna	4H	187-8	E93-no-e47-ORF-pCR8/GW/TOPO	Apr-08	Spec	Joanna

Joanna	5A	186-24	E93-antigen-I-pET28a #B	5/25/2008	Kan	Joanna
Joanna	5B	186-24	E93-antigen-I-pET28a #C	5/25/2008	Kan	Joanna
Joanna	5C	186-25	E93-antigen-II-pET28a #B	5/25/2008	Kan	Joanna
Joanna	5D	186-25	E93-antigen-II-pET28a #C	5/25/2008	Kan	Joanna
Joanna	5E	186-22	E93-antigen-III-pET28a #A	5/25/2008	Kan	Joanna
Joanna	5F	188-35	E93-no-e47-ORF-pTW	5/28/2008	Amp	Joanna

The Rabbit and Mouse antisera harvested from each bleeding:

	Rabbit A		Rabbit B	
	volume	box #	volume	box #
pre-immune	4.5ml (900ul*5)	Rab 2nd	5.4ml (900ul*6)	Rab 2nd
	0.9ml (100ul*9)	Rab small	0.9ml (100ul*9)	Rab small
1st bleeding	2.45ml (1.2ml*2+50ul)	Rab small	3.9ml (1.2ml*2+0.5ml+ 0.4ml+0.2ml+100ul*4)	Rab small
2nd bleeding (1/16/2009)	45ml (1ml*45)	Rab 2nd	33ml (1ml*33)	Rab 2nd
	1.8ml (100ul*18)	Rab small	0.9ml (100ul*9)	Rab small
3rd bleeding (2/18/2009)	44.1ml (900ul*49)	Rab 3rd	39.6ml (900ul*44)	Rab 3rd
	0.9ml (100ul*9)	Rab small	0.8ml (100ul*8)	Rab small
4th bleeding (3/17/2009)	45ml (900ul*50)	Rab 4th	45ml (900ul*50)	Rab 4th
	3.6ml (900ul*4)	Rab small	500ul	Rab small
5th bleeding (4/16/2009)	50ml (1ml*50)	Rab 5th	50ml (1ml*50)	Rab 5th
	4ml (1ml*4)	Rab small	5ml (1ml*5)	Rab small
6th bleeding (5/18/2009)	50.4ml (900ul*56)	Rab 6th	39.6ml (900ul*44)	Rab 6th
	0.9ml (900ul*1)	Rab small	0.9ml (900ul*1)	Rab small

Box: Mou small	Mouse #1	Mouse #2	Mouse #3	Mouse #4	Mouse #5	Mouse #6
pre-immune	200ul	200ul	400ul	400ul	300ul	500ul
1st bleeding	100ul	120ul	100ul	20ul	20ul	100ul
2nd bleeding	40ul	200ul	200ul	200ul	400ul	150ul
3rd bleeding	400ul	400ul	400ul	400ul	300ul + 10ul*4	250ul + 10ul*5
4th bleeding	300ul	300ul	300ul	200ul	200ul	300ul
5th bleeding	260ul	220ul	240ul	500ul	240ul	900ul
6th bleeding	500ul	320ul	320ul	660ul	720ul	720ul

The bleeding tests from Rabbit and Mouse antisera:

Bleedings		preim	1st	2nd	3rd	4th	5th	6th
Rabbit A	Exp. 248	-	-	+				
	Exp. 272			+				
	Exp. 284	-	-	+ weak	+ weak			
	Exp. 301		+ weak					+ weak
Rabbit B	Exp. 248	-	+	-				
	Exp. 272			+ weak				
	Exp. 284	-	+	-	-			
	Exp. 301		+ strong					+ weak
Mouse 1	Exp. 235		+					
	Exp. 248	-	+	+				
Mouse 2	Exp. 235		+					
	Exp. 248	-		+				
Mouse 3	Exp. 235		+					
	Exp. 248	-	+	+				
	Exp. 282	-	+	+	+			
Mouse 4	Exp. 235		+					
	Exp. 248	-		-				
	Exp. 282	-	-	-	-			
Mouse 5	Exp. 235		+					
	Exp. 248	-		+				
	Exp. 282	-	+	+	+			
Mouse 6	Exp. 235		+					
	Exp. 248	-		+				
	Exp. 282	-	+	+	+			

Exp. 235: surf western blot
Exp. 248: 2hr AP midgut tissue staining
Exp. 272: en-Gal4>UAS-LP0 embryo stripes
Exp. 282: en-Gal4>UAS-LP0 embryo stripes
Exp. 284: en-Gal4>UAS-LP0 embryo stripes and WT anterior gut cell
Exp. 301: en-Gal4>UAS-LP0 embryo stripes

Note: The Rabbit B 1st bleeding generated the strongest immune response in all rabbit sera, and I used it for most of my experiments. However, I found the 1st bleeding of Rabbit B was mislabeled, and it probably came from Rabbit A. But it was too late to change all my notes and labels on the eppendorf tubes. To be consistent, I call Rabbit B as the rabbit who produced the strongest immune response, and Rabbit A as the rabbit who didn't respond well. Although the Rabbit got a little bit better in its 6th bleeding, it is still weaker than the weakest bleeding from Rabbit B (Rabbit 6th bleeding).