Molecular studies of the role of the negative transcription elongation factor *Nelf-E* in *Drosophila* development using RNA interference

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ABSTRACT

The elongation step of transcription is now recognized as a critical target for transcription regulation. An increasing number of elongation factors have been identified, and the regulatory mechanism of elongation seems to be as complex as that of transcription initiation. A multitude of factors interact and regulate each other to mediate the exquisite regulation of transcription in response to biological processes. Promoter proximal pausing of the RNA polymerase II was first discovered on the *hsp70* gene, but has also been documented on estrogen stimulated genes. It is suggested that NELF functions as a control point for proper mRNA capping.

Here we describe the characterization of *Drosophila Nelf-E,* one of the subunits of the Negative transcription elongation factor complex. Functional analyses were performed to assess the role of *Nelf-E* during *Drosophila* development. RT-PCR on *Nelf-E* knock-down flies showed an up-regulation of integrin and integrin-associated proteins.

Further analyses are needed to investigate the functional implications of the NELF complex, and to authenticate the target gene of this transcription elongation repressor.

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1. Introduction

A critical control point for gene expression of various genes and hence diverse biological processes is the elongation step of RNA polymerase II transcription. The involvement of three transcription elongation factors, namely, DRB (5,6-di-chloro-1-β-D-ribofuranosyl-benzimidazole) sensitivity-inducing factor (DSIF), NELF (negative elongation factor), and a positive transcription elongation factor b (P-TEFb) has been demonstrated in humans. DSIF and P-TEF-b have homologues in eukaryotes ranging from yeast to human. Homologous of the four subunits of NELF identified in humans have been recognized in *Drosophila melanogaster*, but so far no homologous are evident in other model organisms such as yeast or *Caenorhabditis elegans* (*C. elegans)*. Thus, the regulatory potential provided by NELF could be restricted to a subset of eukaryotes. In this thesis *Drosophila melanogaster* was used as model organism to investigate one of the four subunits of NELF, called Nelf-E.

1.1 The model organism *Drosophila melanogaster*

Drosophila melanogaster, belonging to the order of *Diptera* (two-winged insects) and the family *Drosophilidae*, is an extraordinarily attractive model organism. It serves as a model system for investigation of many developmental and cellular processes, owing to the combination of an easy to manipulate genetic system, a short life cycle, relatively low cost, and biological complexity comparable to that of a mammal. Compared to general living organisms, model organisms are well-established experimental systems. In addition there are fewer ethical constrains encountered when using them. The common ancestor of flies and vertebrates is traced back 700 million years, at the Protostome-Deuterostome split, but many of the relevant developmental processes are essentially conserved (Adams et al., 2000). Surprisingly many of the genes in *Drosophila melanogaster* have clear homologues in higher eukaryotes, like humans (Friedman and Hughes, 2001).

Being small, growing rapidly, producing many progenies and being readily available are crucial in terms of housing *Drosophila*, given the budget and space limitation of research laboratories. In

addition *Drosophila* has been used as a model organism for about 100 years, and a considerable number of techniques and well established experimental systems have been developed, providing the most important model systems for genetic, epigenetic and developmental studies (Rubin and Lewis, 2000). Another asset of *Drosophila* is that there is no meiotic recombination in males, making it relatively easy to track chromosomes through generations. The *Drosophila* genome is spread across four chromosomes, which can be visualized in the larval salivary glands as the giant polytene chromosomes. These polytene chromosomes begin as normal chromosomes, but through successive rounds of DNA replication without any cell division, called endoreplication, they become large, banded chromosomes. By Feulgen staining the chromosomes, the alternating highly and moderately dense regions on the chromosomes, called band and interbands, can be visualized in the light microscope. The structure of the chromosomes can thus easily be determined making it possible to probe genes and position them on the chromosome, which provides a valuable tool in mapping genes. The *Drosophila* exoskeleton can be affected by mutations, and in particular it is attractive due to all the external features of the fly such as wings, body color, bristles and compound eyes, for which the resulting phenotypes can be identified by investigating the fly in the stereomicroscope. Thus, phenotypic mutants arising from genomic mutations can be identified and linked.

Drosophilists have developed an ever-increasing repertoire of sophisticated techniques that make the fruit fly one of the best model organisms for genetic analysis of almost any process (Rubin and Lewis, 2000). Large genetic screens make it possible to identify genes necessary for a particular process, and is a great potential to dissect a specific gene function (St Johnston, 2002). *Drosophila* provides a model system for studying human diseases, as genes underlying many genetic disorders, including cancer and neurological diseases (Fortini et al., 2000), are conserved throughout evolution.

1.1.1 The life cycle of *Drosophila melanogaster*

Drosophila is a holometabolous insect that undergoes a full metamorphosis with a four-stage life history, consisting of an egg stage followed by a larval stage, which in turn is

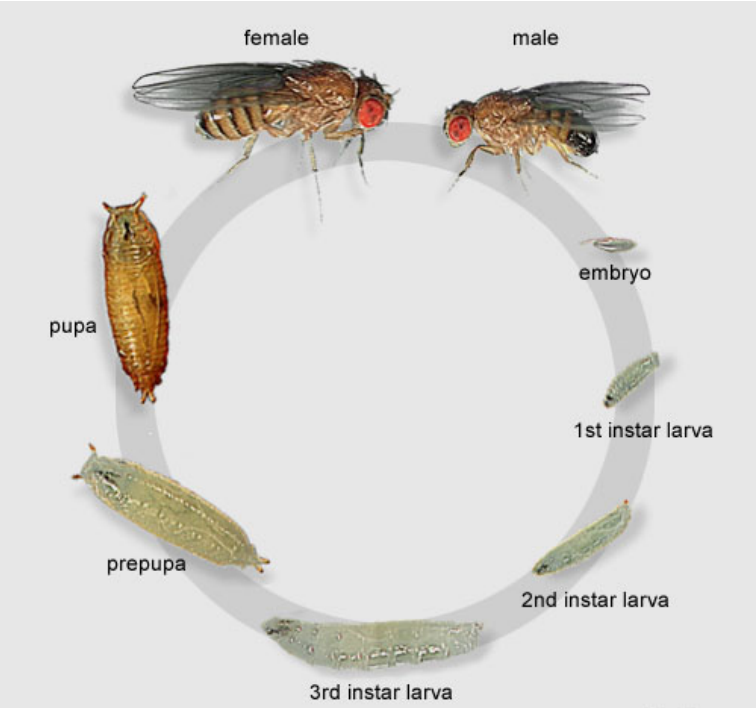


Figure 1.1.1 The life cycle of *Drosophila melanogaster*.
Embryogenesis last for one day before the egg hatches into a larva. The larval stages consist of three instars, where the first and second instars last for one day, and the third lasts for 2 days. During the pupal stage the animal goes through metamorphosis. After five days eclosion occurs, and the adult fly emerges. Image adapted from *FlyMove* (Weigmann et al., 2003).

interposed by a pupal stage before the adult stage. The life cycle starts with a fertilized egg that is laid in nutritious food. The embryonic development lasts for about one day, succeeded by hatching and a larval stage. The larval development is divided in three stages, or *instars*, which are separated by molting, where the larva constantly consumes food and gains size. Approximately 40 hours into the third instar the larva climbs to a dry and clean place where it stops moving, everts its spiracles used for gas exchange, and allows larval cuticle to harden into a puparium (pupal case) that surrounds the organism during the time of its metamorphosis. During the pupal stage, which lasts for five days, an essential remodeling of the body takes place. Most larval tissues are destroyed by programmed cell death during prepupal and early pupal stages (Robertson, 1936; Jiang et al., 1997), organs are histolyzed and adult structures are formed during this metamorphosis. Metamorphosis in *Drosophila* may be divided into two stages: A 12 hour

prepupal period marked by pupariation or the onset of the larval-pupal transition, and a subsequent pupal period lasting 84 hours. The whole process from fertilization to eclosion of the adult fly takes about 10 days at 25°C (Figure 1.1.1.)

1.2 Wing development in *Drosophila melanogaster*

In contrast to embryonic development that occurs in a syncytial environment, limb development is established in a cellular setting. The proteins directing limb development are secreted, signaling molecules instead of transcription factors that are controlling embryonic development. *Drosophila* limbs (legs, wings, halters, antennae, mouth parts) derive from structures called imaginal discs. Each body limb rises from a separate imaginal disc. Imaginal discs begin as small clusters of cells which are set aside during embryogenesis. During larval development these cells proliferate to form folded, single layer, epithelial sacs. The proliferation of cells in the disc ceases just prior to differentiation which begins at the time of pupation. The differentiation is accompanied by an eversion of the discs.

During wing development, the wing is derived from the wing imaginal disc which is subdivided into distinct anteriorposterior (AP), dorsoventral (DV) and wing-notum (limb-body wall) primordial, (figure 1.2.1.) The wing disc primordium is formed from a small cluster of about 40 cells, and proliferates to encompass approximately 50.000 cells when the disc is mature for differentiation. Embryonic ectoderm cells from the posterior compartment of the second thoracic parasegment and the anterior compartment of the third engender the disc by an invagination. The invagination occurs at an intersection of stripes generated from the expression of two genes, *wingless* (*wg*), a segment polarity gene, establishing a DV stripe of Wingless (Wg), and *decapentaplegic* (*dpp*), expressed in a lateral stripe running perpendicular to the cells expressing Wg (Cohen et al., 1993).

The fist differentiation of cells in the wing is established during embryogenesis (Wieschaus and Gehring, 1976; Lawrence and Morata, 1977), and it involves the

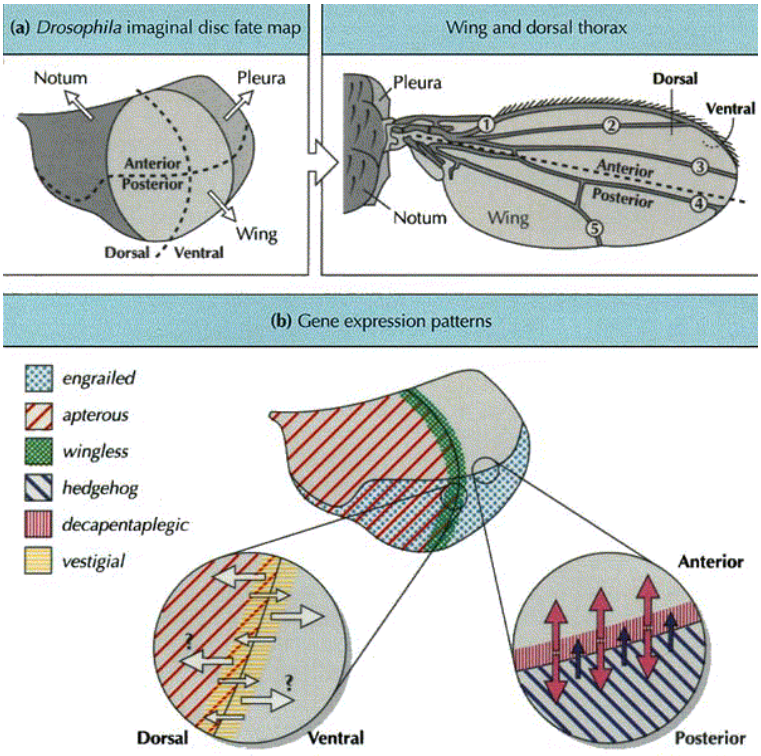


Figure 1.2.1 Wing development

(a) Imaginal discs are specified during embryogenesis and continue to grow during larval stages, and finally differentiate into the adult structure during metamorphosis. The boundaries between the AP and DV compartments are indicated on the larval imaginal disc and the adult structure. (b) Genes controlling the early *Drosophila* wing development. The expression patterns of these genes provide positional information in the disc, guiding the subsequent wing differentiation. Cell-Cell interactions across the boundaries of the disc are important in pattern elaboration. Cartoon adapted from (North and French, 1994)

segregation between anterior cells and posterior cells. The gene *engrailed* (*en*) is specifically expressed in posterior compartments (Kornberg et al., 1985), specifying the identity of posterior cells. Posterior cells lacking *engrailed* function behave as anterior

cells (Lawrence and Morata, 1976). This broad subdivision of the disc provides a framework for cell-cell interactions which elaborates the pattern. Further subdividing of the wing disc happens in the third larval instar and transpires along the DV axis (Garcia-Bellido et al., 1976). Expression of the *vestigal* (*vg*) gene is induced in a stripe centered on the boundary between the dorsal and ventral surfaces of the wing. Where the *apterous* (*ap*) gene is expressed, the cells are

given dorsal identity, nonexpressing cells are given a ventral identity. The expression correlates with the time at which the DV lineage restriction is first observed in the wing disc. Genetic analysis have shown that *ap* function is required to specify dorsal cell fate in the wing (Diaz-Benjumea and Cohen, 1993).

During metamorphosis the imaginal disc evaginates. The central region bulges out and flattens, apposing its dorsal and ventral surfaces and bringing together the notum and pleura. This evagination is dependent on extracellular proteins and transmembrane proteins. One class of these transmembrane proteins is integrins, a major family of cell surface receptors that link the extracellular matrix (ECM) to the actin cytoskeleton. Integrins work as heterodimers, consisting of noncovalently associated α and β subunits, and the combination of specific subunits has been shown to be important in determining the affinity for specific ligands (Zusman et al., 1990). Their intracellular domains interact with the cytoskeleton while their extracellular domains bind to adhesive molecules such as fibronectin, laminin and collagen (Hynes, 1987), as well as activating many intracellular signaling pathways (Hynes, 2002). In *Drosophila* the position-specific (PS) antigens, PS1 and PS2, are α integrin subunits (Leptin, 1987). These subunits bind to a β subunit known as PS3 or PSβ, encoded by the gene *myospheroid* (*mys*) (Leptin, 1987). Different heterodimers of these proteins are concentrated in specific embryonic tissues (Zusman et al., 1990). In the wing imaginal discs, α_{PS1} and α_{PS2} are expressed on the dorsal and ventral surfaces respectively, while PSβ is found throughout the disc. Maintaining the close apposition of the dorsal and ventral surfaces of the wing at metamorphosis is thought to be necessary for proper shaping and organization of the wing as well as the normal patterning of wing crossveins (Zusman et al., 1990).

Several other proteins have been shown to interact in the evagination process and in maintaining the close apposition of the wing surfaces. *wing blister* (*wb*) encoding a α chain of laminin, in *Drosophila* is indispensable in the adhesion between cell layers (Martin et al., 1999). *blistery* (*by*), a *Drosophila* ortholog of the protein tensin is postulated to have a role in integrin adhesion, linking integrins and the cytoskeleton (Torgler et al., 2004).

1.3 Eclosion in *Drosophila melanogaster*

At the end of the third larval instar, approximately 120 hours after the beginning of embryonic development the metamorphosis begins. As mentioned before metamorphosis in *Drosophila* is divided into two stages: A 12 hour prepupal period marked by pupariation (the onset of the larval-pupal transition), and a subsequent pupal period lasting 84 hours. Ecdysteroid hormone secreted from the ring gland is suddenly released marking pupariation. The puparium is formed from larval cuticle, and it surrounds the metamorphosing fly until it ecloses. Approximately 12 hours from the start of pupariation the process of eversion of the head takes place, marking the beginning of the true pupal stage. This is orchestrated by abdominal muscles contractions that last for 10 minutes. Imaginal disc undergo eversion to form the basic shape of the adult head, thorax and abdomen during the pula stage. The imaginal discs of wings, legs and halters fuse to form the thorax, and eye antennal complex fuses to form head capsule. The head and thorax fuse with the abdomen.

The metamorphosis in insects is controlled by three hormones, namely the steroid ecdysone and the sesquiterpenoid juvenile hormone (JH) (Zhou and Riddiford, 2002). amd the eclosion hormone (eh).These hormones coordinate the switch in gene expression necessary for metamamorphosis, first to the pupa, then to the adult. In the absence of JH, ecdysone triggers gene expression promoting metamorphosis. In *Drosophila* JH has no effect on the differentiation of the head and thorax externally, but it disrupts metamorphosis of the nervous and muscular systems when given during prepupal period (Restifo and Wilson, 1998).

1.4 The transcription machinery in eukaryotes

Transcription is the process where the genetic information from DNA is transferred to RNA. The DNA sequence is enzymatically copied by a multi-subunit DNA-dependent RNA polymerase to produce a complementary RNA. The polymerase is conserved among the tree phylogenetic domains of *Eubacteria, Archaea,* and *Eucarya*. In eubacteria and archaea transcription of the major classes of genes, including rRNA, mRNA, and tRNA, is accomplished by a single multi-subunit RNA polymerase, whereas in eukaryotic species, three highly related enzymes, RNA polymerase I, II and III, are responsible for recognizing nuclear gene promoters and then for

transcription of the genes. Each of these RNA polymerases transcribes a specific set of genes, and each is dependent on accessory factors, known as transcription factors, to recognize its cognate promoter sequences. RNA polymerase I transcribes only ribosomal RNA, while RNA polymerase III transcribes catalytic or structural RNA molecules, some of which are involved in fundamental metabolic processes, specifically components of the protein synthesis apparatus and components of the splicing and tRNA processing apparatus, as well as RNAs of unknown function (Schramm and Hernandez, 2002). The last enzyme is RNA polymerase II (RNAPII), which is responsible for the transcription of protein coding genes and some small nuclear RNAs (snRNAs) genes.

RNA polymerase II transcribes the protein-coding genes (mRNA genes). The RNAPII promoters are divided into a core region, the minimal region capable of directing transcription *in vitro*, and a regulatory region consisting of promoter proximal elements and distal enhancer elements. Interaction between these regulatory elements and transcription factors control initiation of transcription by RNAPII. The regulatory regions are highly varied in structure, reflecting the need for exquisite and complex regulation of the genes to obtain correct synthesis patterns of cellular proteins. The core enzyme of RNAPII holds the active site, but is unable to recognize promoter sequences by itself and to modulate production of the RNA transcripts of individual genes in response to developmental and environmental signals. For these critical biochemical problems, supporting proteins are necessary. Accurate initiation of transcription depends on assembling RNAPII and the transcription factors TFIID, TFIIB, TFIIF, TFIIE, and TFIIH into a preinitiation complex (PIC). Transcriptional activators bind to promoter proximal elements, occurring 50 to 200bp upstream of the start site, in order to regulate transcription. Finally, factors modulating RNAPII activity bind to distal enhancer elements, which can occur in either direction and orientation relative to the transcription initiation site (refer to Figure 1.4.1). Transition to transcription elongation complex (TEC) is associated with disruption of the PIC and new contacts with elongation factors are formed.

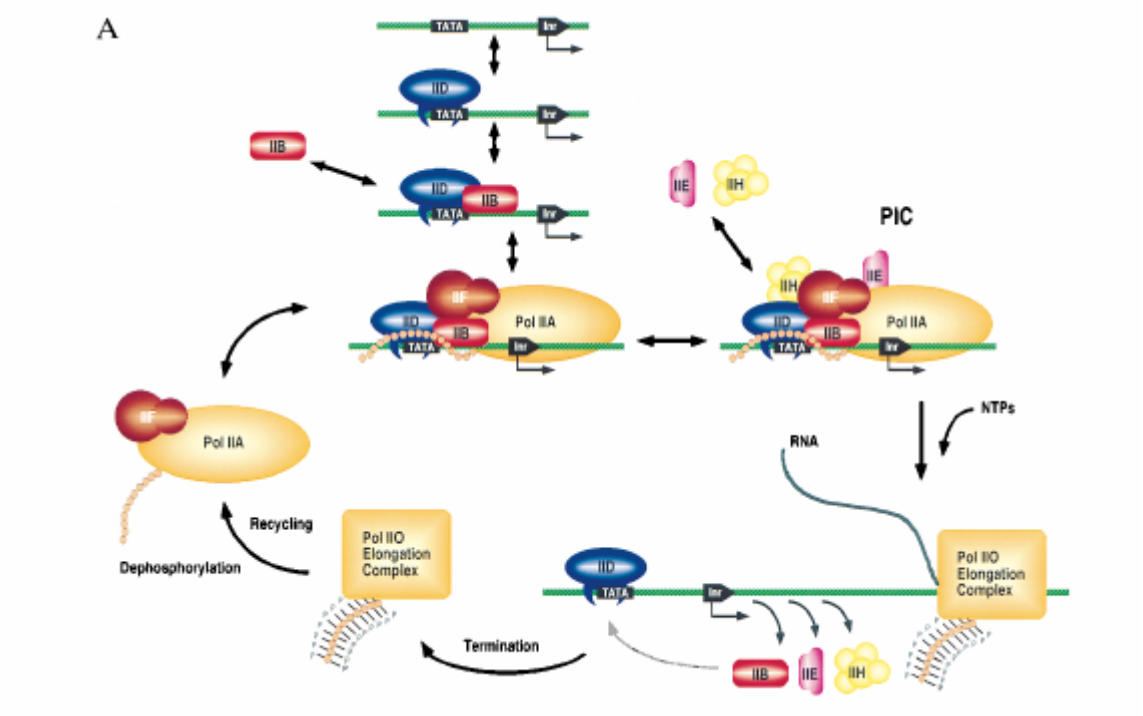


Figure 1.4.1 The RNA polymerase II during initiation and elongation

(A) PIC assembly is a sequential coordinated accretion of general transcription factors. Before elongation RNAPII is phosphorylated. Following termination , phosphatases are responsible for recycling the RNAPII to its nonphosphorylated form. This allows the enzyme to reinitiate transcription *in vitro*. Adapted from (Nikolov and Burley, 1997). Cartoon not to scale.

The largest subunit of eukaryotic RNAPII has a domain at its C-terminus called the carboxy-terminal domain (CTD). It is composed of a heptapeptide tandemly repeated several times and it is a target of kinases and phosphatases. The phosphorylation of CTD works as a regulation mechanism, as it allows proteins that have a function in the transcription process to interact with the domain. *In vivo*, two forms of RNAPII are observed on the basis of weather the CTD is highly phosphorylated (hyperphosphorylated) or nonphosphorylated (hypophosphorylated). The nonphosphorylated form of RNAPII associates with the preinitiation complex (Archambault and Friesen, 1993), while RNAPII phosphorylated on the CTD is associated with the alteration from initiation to elongation (Laybourn and Dahmus, 1989). Proteins regulating this phosphorylation and deposphorylation, and proteins binding to the CTD regulate the elongation process. The

proteins can be divided into negative transcription elongation factors, responsible for abortive elongation, and positive transcription elongation factors, stimulating elongation. DRB (5,6-di-chloro-1-β-D-ribofuranosyl-benzimidazole), inhibits kinases responsible for phosphorylating the CTD, inducing arrest of elongation. DSIF (for *D*RB *s*ensitivity-*i*nducing *f*actor) represses transcription in collaboration with NELF complex in the presence of DRB (Yamaguchi et al., 1999a), while positive transcription elongation factor b (P-TEFb) stimulates elongation in a DRB-sensitive fashion by phosphorylating CTD of RNAPII. NELF and DSIF negatively regulate elongation through interaction with RNAPII containing hypophosphorylated CTD (Yamaguchi et al., 1999b). P-TEFb might promote elongation by blocking interactions of DSIF and NELF with the elongation complex by phosphorylating CTD (Price, 2000).

1.5 The negative transcription elongation factor complex

Negative transcription elongation factor complex (NELF) inhibits transcription elongation *in vitro* and is implicated in causing promoter proximal pausing in collaboration with DSIF on the *hsp70* gene in *Drosophila* (Wu et al., 2005). Biochemical data indicate that NELF and DSIF could provide a checkpoint during early elongation, to ensure proper capping of nascent transcripts. This theory is in accordance with the broad and overlapping distribution of NELF and DSIF observed on the polytene chromosomes, indicating that these proteins affect transcripts of many genes (Wu et al., 2003).

1.5.1 Molecular characterization of *Drosophila* NELF

Drosophila NELF has four subunits similar to subunits of human NELF. The subunits NELF-B and NELF-D are highly conserved throughout their amino acid sequences, whereas NELF-A and NELF-E contain non-conserved regions inserted between conserved N- and C-terminal regions. Wu et al. (2003) identified single candidates for NELF-D and NELF-E in *Drosophila* by using BLAST (Basic Local Alignment Search Tool), and later NELF-A and NELF-B. The interaction between NELF subunits have been analyzed by Narita *et al.* and they have proposed that NELF-B and NELF-D form a central core that brings together NELF-A, associating with NELF-D, and NELF-E, associating with NELF-B (Wu et al., 2003). The NELF-A subunit binds to RNAPII, and the subunit NELF-E has a RNA binding domain. Both interactions are critical for NELF

function in transcriptional pausing in vitro (Yamaguchi et al., 2002; Narita et al., 2003). Co-immunoprecipitation analysis have also showed that NELF-D and NELF-E associate with each other (Wu et al., 2003). The complex can inhibit transcription elongation *in vitro* when DISF is present. Both NELF and DISF have been identified at the promoters of *hsp70* and *β1-tubulin* genes, where pausing of the RNAPII has previously been detected (Wu et al., 2005). In addition NELF was found to be recruited to estrogen-stimulated genes (Aiyar et al., 2004).

At the *hsp70* gene, NELF but not DSIF appears to dissociate from the elongation complex during heat shock induction (Wu et al., 2003). Hyperphosphorylation of CTD by P-TEFb and other kinases has been thought to overcome the inhibition by NELF and DSIF, dissociating them from the TEC, but more recent results indicates that phosphorylation of NELF and DSIF may also be involved (Fujinaga et al., 2004).

1.6 Genetic tools for investigating gene function

Forward genetic screens in *Drosophila melanogaster* have been and will continue to be an important method to identify genes that are involved in a biological process. Mutations represent an essential tool for analyzing gene function. Breeding experiments in the beginning of the 20th century performed by Thomas Hunt Morgan and coworkers, led to the discovery of a mutant fly with white eyes, resulting from a spontaneous mutation. These mutations occur infrequently, and thus new ways of generating mutations had to be developed in order to perform genetic screens. Mutations can be made in various genes with the use of a mutagenic agent. The most commonly used mutagen in *Drosophila* is ethyl methane sulphonate (EMS), its assets being that it is easy to administer and causes the highest frequency of mutations. These mutations are point mutations which can have drawbacks when used for screening purposes. Firstly, the mapping of point mutations to specific genes was very difficult and laborious. A second drawback is that males mutagenized with EMS often yields mosaic progeny. Other chemical mutagens can be used, as well as X-ray or gamma irradiation, which induce mainly double-stranded DNA breaks, that do not cause mosaicism (St Johnston, 2002).

New additional and popular strategies to generate mutations are based on the use of insertional mutagenesis. Using engineered transposable elements (P-elements) containing independently scorable genetic markers such as eye color, body color, drug resistance, or dominant visible characters, multiple insertion can easily be manipulated (Bellen et al., 2004). A mutated gene by P-element insertion can easily be identified and mapped by sequencing (St Johnston, 2002). One drawback of P-elements is that they favor insertion into 5′-noncoding regions, making it impossible to mutate every gene in the genome (Spradling et al., 1995). The Berkely *Drosophila* Genome Project (BDGP) gene-disruption project generated a large collection of *Drosophila* strains that each contain a single, genetically engineered P-element inserted in a defined genomic region. P-elements in these lines carry enhancer traps that can be used to acquire information about the expression pattern of disrupted genes through enhancer trap screens (Spradling et al., 1999). This library of P-element insertions were supplied to the Bloomington *Drosophila* stock center (IN, USA) (Spradling et al., 1995), which is available for the public.

1.7 GAL4/UAS expression system

The GAL4/UAS system is designed for targeted gene expression in *Drosophila*. The system allows for activation of any cloned gene in a broad range of tissue- and cell- specific patterns (Brand and Perrimon, 1993). GAL4, a yeast transcriptional activator, triggers transcription in flies from promoters with GAL4 binding sites. In yeast the GAL4 protein regulates the transcription of the genes Gal1 and GAL10 through direct binding to four essential and related 17 base pair sequences, called Upstream Activating Sequences (UAS) (Giniger et al., 1985). In *Drosophila*, GAL4 protein does not activate native *Drosophila* genes and has no deleterious phenotypic effects.

In this system the target gene is separated from its transcriptional activator. The target gene is in one transgenic line and the transcriptional activator in a different line. The target gene remains silent in the absence of its activator in one line, and in the other line the activator protein is present but has no target gene to activate, ensuring that parental lines are viable. Only by crossing the two lines is the target gene turned on in the progeny, making it possible to study phenotypic effects of misexpression (Brand and Perrimon, 1993). The target gene is placed under upstream

activating sequence (UAS) control in *Drosophila*, and is positively transcriptionally stimulated by GAL4 expression (Fischer et al., 1988).

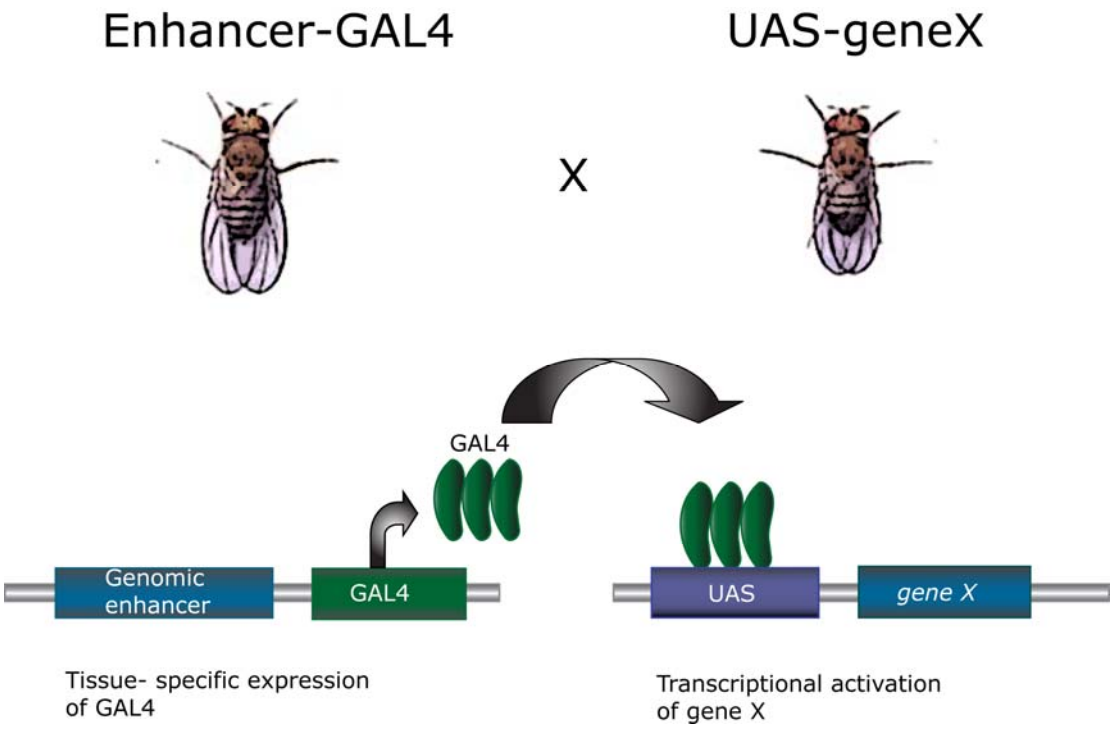


Figure 1.7.1 The GAL4/UAS expression system.

The yeast transcriptional activator Gal4 can regulate gene expression in *Drosophila* by inserting the upstream activating sequence (UAS), to which Gal4 binds, next to a gene of interest (gene X). Expression of the *GAL4* gene is controlled by a nearby genomic enhancer relative to where the *GAL4* gene was inserted in the *Drosophila* genome. Several enhancer-trap lines have been created, making it possible to express *GAL4* in a huge variety of cell-type and tissue-specific patterns. By crossing the two lines containing the UAS and the *GAL4* gene, the progeny will express *GAL4*, and Gal4 will stimulate expression of *gene X* in a pattern reflecting the genomic enhancer.

To acquire GAL4 expression in different tissues and at different times, the gene encoding GAL4 is inserted in the genome under control of diverse *Drosophila* promoters, advancing the generation of fly strains with ectopic expression of the target gene.

1.8 RNA induced gene silencing.

RNA interference (RNAi) is a mechanism in which the presence of small fragments of double-stranded RNA (dsRNA) whose sequence matches a given gene interferes with the expression of that gene at a post-transcriptional stage. Initially observed in the nematode worm, where dsRNA resulted in sequence-specific gene silencing (Fire et al., 1998), this phenomenon has been demonstrated to be effective in virtually any organism, from protozoa to plants and animals. The RNAi mechanism can be used to investigate the role of a gene by preventing gene function and observe what effect, if any, this has on the organism's phenotype.

The present understanding of the mechanisms underlying dsRNA-induced gene silencing is derived from genetic studies in *C. elegans* and plants, and from biochemical studies of *Drosophila* extracts. In both plants and animals, the RNAi process is characterized by the presence of RNAs of about 22 nucleotides in length, called guide sequences, that are homologous to the gene that is being suppressed (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). These guide sequences instruct a multicomponent nuclease, known as the RNA-induced silencing complex (RISC), to destroy specific messenger RNAs (Hammond et al., 2000). An enzyme called Dicer is a member of the RNase III family of nucleases that specifically cleave dsRNA. Dicer produces putative guide RNAs and it is therefore proposed to initiate RNAi process. The enzyme is evolutionarily conserved in worm, flies, plants, fungi and mammals (Bernstein et al., 2001).

1.8.1 Current model of the RNAi mechanism

Biochemical and genetic approaches have led to the current model of the RNAi mechanism which includes both an initiation and effector step (Hutvagner and Zamore, 2002). RNAi is initiated when the enzyme Dicer digests input dsRNA into 21-23 nucleotide guide sequences, also called small interfering RNAs (siRNA) (Hammond et al., 2001; Nykanen et al., 2001; Sharp, 2001; Hutvagner and Zamore, 2002) The process is adenosintriphosphate (ATP) dependent, and successive cleavage events degrade the RNA to 19-21 base pairs duplexes (siRNA), each with 2-nucleotide 3′ overhangs (Bernstein et al., 2001; Hutvagner and Zamore, 2002), a configuration that is functionally important for incorporation into RISC complexes (Elbashir et al., 2001b; Nykanen et al., 2001).

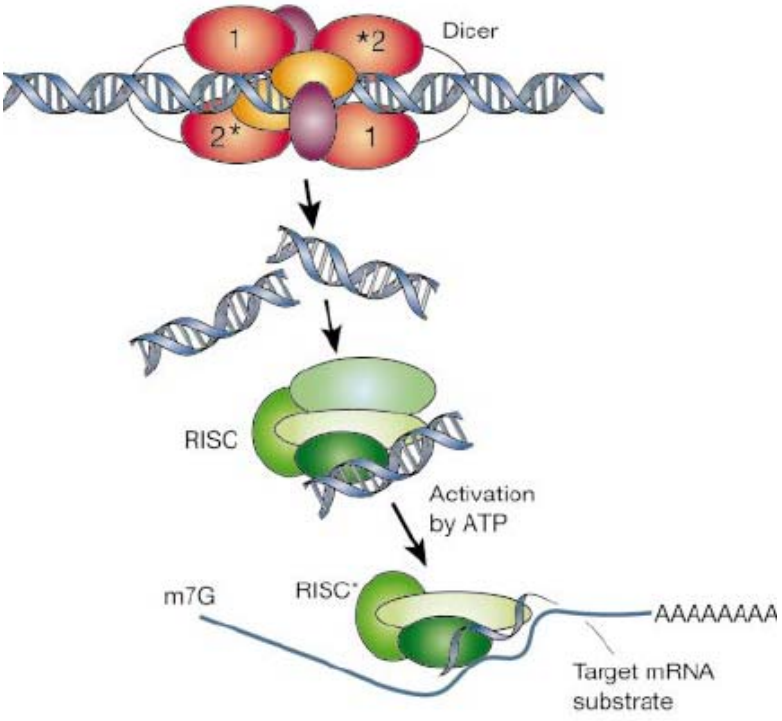


Figure 1.8.1.1 Dicer and RISC (RNA-induced silencing complex).

Two Dicer molecules, consisting of five domains each, cleave double-stranded RNA into small interfering RNAs of about 22 nucleotides in length. The enzyme is thought to work as a dimeric enzyme, based on knowledge about the RNase III family of enzymes (Bernstein et al., 2001). The RISC complex incorporates the small interfering RNAs, which seemingly identifies substrates through Watson-Crick base-pairing (Hammond et al., 2000). Cleavage is apparently endonucleolytic and happens only in the region homologous to the siRNA (Zamore et al., 2000). Cartoon adapted from (Hannon, 2002)

In the effector step, the siRNAs are incorporated into a multicomponent nuclease complex to form RISC. RISC needs to be activated from a latent form, containing a double stranded siRNA, to an active form, by unwinding the siRNAs (Nykanen et al., 2001). The active RISC targets and destroys the homologous transcript by base pairing interactions and cleaves the messenger RNA at approximately 12 nucleotides from the 3′ terminus of the siRNA (Hammond et al., 2001; Nykanen et al., 2001; Sharp, 2001; Hutvagner and Zamore, 2002).

Methods of gene silencing provide valuable approaches to the genome functional analysis. Double-strand RNA is a powerful signal capable of inducing gene-specific silencing, representing a tool for obtaining targeted disruption of a given gene function, overcoming either the need for mutants, or the knowledge of a complete and detailed gene structure to determine the gene function. For efficient induction of RNAi in *Drosophila*, the initiating RNA must be double-stranded and must also be several hundred nucleotides in length (Sharp, 1999). The introduction of dsRNA can be accomplished by injection of dsRNA corresponding to a single gene into an organism, but this injection only interferes with gene expression transiently and is not stably inherited (Montgomery et al., 1998; Li et al., 2000; Wianny and Zernicka-Goetz, 2000). Therefore, use of RNAi to study gene function in the late stages of development has been limited. In *Drosophila*, this problem has been circumvented by developing a method to express dsRNA as an extended hairpin-loop RNA (hpRNA). The hpRNA is expressed from a transgene exhibiting dyad symmetry in a controlled temporal and spatial pattern, thus enabling study of late-acting gene function in *Drosophila* (Kennerdell and Carthew, 2000).

1.9 Aim of this project

Previous study on *Nelf-E* using reverse genetics identified several knock-down phenotypes, shading some light on the potential role of this gene in *Drosophila* development. The gene codes for a negative transcription elongation factor, but not much is known about which gene it may regulate.

The aim of this thesis was to use genetic tools available to perform functional analysis of the gene *Nelf-E* in *Drosophila melanogaster*. The main objectives have been to:

-use RNA interference to study the role of *Nelf-E* during *Drosophila* development
-investigate the expression level in different tissues during development
-identify putative genes regulated by *Nelf-E* function

2. Materials and methods

2.1 DNA and RNA methods

2.1.1 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify desired segments of DNA (Mullins and Faloona, 1987; Sambrook and Russel, 2001). Following three steps the target sequence is amplified. First, denaturation of the template occurs be heating, second, primers flanking the target sequence anneal to their complementary sequences, and during the last step the annealed primers are extended by DNA polymerase. The cycle is repeated and leads to an exponential amplification of the DNA segment. General PCR reactions were used for several purposes; to amplify genes of interest, or to amplify desired DNA fragments for cloning and sequencing, and in screening for positive bacterial colonies after transformation into TOP10 cells.

DyNAzyme™ II DNA Polymerase (Finnzymes, Espoo, Finland) is a standard thermo stable polymerase for amplification of desired DNA fragments and for screening. The polymerase generates a 3' dATP overhang which facilitates ligation into a TOPO vector (section 2.1.9).

To create constructs a proofreading enzyme *Pfu* (Fermentas Life Sciences, Ontario, Canada) was used in the PCR to obtain higher accuracy of amplification. The *Pfu* polymerase is a highly thermo stable DNA polymerase from the hyperthermophilic archaeum *Pyrococcus furiosus.* In addition to its template dependent polymerization of nucleotides into duplex DNA in the 5′ => 3′ direction, it also exhibits 3′ => 5′ exonuclease activity, to correct nucleotide misincorporation errors.

The standard setup for the PCR reactions was 1x reaction buffer, 200µM dNTP, 0.2µM primers, 0.5-1U DNA polymerase and in addition a desired amount of template. Milli-Q water was used to dilute primers and to adjust to desired volume.

All programs used were variations of the general program:
(denaturation) 94°C 5 minutes, 94°C 30 seconds, (annealing) 60°C (temperature depending on primers Tm) 30 seconds, (elongation) 72°C 1-3 minutes depending on length of DNA, 72°C 5 minutes.

All the reactions were run in accordance with the manufacturer's recommendations, and hot starts were used to increase sensitivity, specificity and yield. Primers were ordered from Invitrogen Life Technologies (CA, USA). PCR products were analyzed by agarose gel electrophoresis, and a negative control was always included.

2.1.2 Agarose gel electrophoresis

DNA was separated according to size, identified and purified on 1% agarose gels (SeaKem®Leagarose, Cambrex Biosciences) by electrophoresis. Before electrophoresis, appropriate volume of loading buffer was added to each sample (Sambrook and Russel, 2001).

The size standard GeneRuler™ 1kb DNA ladder (Fermentas Life Sciences, Ontario, Canada) was used to determine the size of the migrated DNA fragments. The gel was run in 1x TAE buffer (40mM Tris-acetate, 1mM EDTA), for 40-60 minutes and the electric voltage applied was 80-100 Volts. For visualization of the DNA fragments, 0.6µg/ml ethidium bromide (EtBr) was added to the agarose gel.

2.1.3 Reverse transcriptase PCR (RT-PCR)

RT-PCR was used to document the expression of the genes *Negative elongation factor E (Nelf-E*), *inflated* (*if*), *blistery* (*by*), *multiple edematous wings* (*mew*), *myospheroid* (*mys*), *eclosion hormone* (*eh*), in specific tissues of the RNAi transgenic lines and in wild type. To investigate the expression level of a gene, RT-PCR was used as a semi-quantitative method, as all reactions were run on equal amount of RNA, and primers for *L14* were used as control. First strand cDNA was synthesized from isolated total RNA from a tissue using a reverse transcriptase enzyme,

SuperScript™III (Invitrogen Life Technologies, CA, USA). Gene specific primers ordered from Invotrogen Life Technologies (CA, USA) were then used in a PCR reaction.

2.1.3.1 Isolation of total RNA from *Drosophila melanogaster*

Tissue was harvested from larva, pupae, or adult flies and frozen at -80ºC. Total RNA was extracted from wild type adult flies, pupae, or wings, and transgenic flies, pupae, or wings. This was either done using the Trizol® reagent (Invitrogen Life Technologies, CA, USA) or by using the SV Total RNA Isolation System Kit (Promega, WI, USA) in accordance with the manufacturer's recommendations and in an RNase-free environment. The RNA was eluted in 100µl nuclease free water. All isolations were treated with DNase (Invitrogen Life Technologies, CA, USA), and stored at -80ºC.

2.1.3.2 Quantification of RNA

RNA was quantified on a Lambda 25 UV/Vis spectrophotometer (Perkin Elmer), or on NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA).

2.1.3.3 Checking the RNA integrity

Isolated RNA was analyzed by agarose gel electrophoresis to check the integrity of the RNA. The 28S and rRNA (ribosomal RNA) bands should appear as strong bands and mRNA should appear as a smear.

2.1.3.4 First strand cDNA synthesis

First strand cDNA was synthesized from the isolated total RNA using SuperScript™III First-strand Synthesis System for RT-PCR (Invitrogen Life Technologies, CA, USA). The RT-reaction was done in accordance with manufacturer's recommendations, and Oligo-dT primers were used. The same amount of RNA was used for the RT-reaction, making the RT-PCR a semi-quantitative method for checking the expression level of the genes of interest in different tissues.

2.1.3.5 RT-PCR reactions

In the PCR 2µl-13µl of the RT-reaction was used as template and DyNAzyme II DNA Polymerase (Finnzymes, Espoo, Finland) was used for amplification. As a control to the quality and amount of cDNA, *L14* primers were used, since *L14* is highly expressed at approximately the same level in all tissues. Genomic DNA was used to control that the bands obtained on the gel resulted from amplification with cDNA as template and not genomic DNA as template.

2.1.4 Purification of DNA fragments

Purifications of separated DNA fragments on an agarose gel were purified by first cutting the fragments out of the gel, and then using Wizard SV GEL and PCR Clean-Up System (Promega, WI, USA). Supplied protocol from the manufacturer was followed. Gel slices containing DNA was melted and applied on columns containing a DNA binding silica membrane. Post washing, the DNA was eluted in 50µl nuclease free water.

2.1.5 Quantification of DNA

Quantification of DNA samples was done using the Hoefer DyNAQuant 200 fluorometer (Hoefer® Scientific Instruments, CA, USA) using the fluorescent dye Hoechst 33258 (Turner BioSystems, Inc, CA, USA) as described by the manufacturer, or by using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA).

2.1.6 Restriction cutting of DNA with endonucleases

PCR products and the vector pUASp were digested with the restriction endonucleases BamHI and KpnI (Promega, WI, USA) in accordance with manufacturer's recommendations.

2.1.7 Dephosphorylation of digested DNA

Before ligating desired DNA fragment into a vector, a dephosphorylation was performed on the digested vector to prevent religation of the vector. The enzyme prevents religation by removing PO_4^{3-} from the 5' end of the vector DNA sequence. Digested vector was incubated with 1 unit of

Shrimp Alkaline Phosphatase, SAP (Fermentas Life Science, Ontario, Canada) per 1x 10^{-9} of 5'-termini in accordance with manufacturer's recommendations.

2.1.8 Ligation of DNA with T4- Ligase

All ligation reactions were performed as recommended by the manufacturer and incubated over night at 4-18ºC. T4-Ligase from Promega (WI, USA) or Invitrogen Life
Technologies (CA, USA) were used.

2.1.9 Cloning of PCR products using the TOPO cloning system

To clone *Negative transcription elongation factor E* (*Nelf-E*), the TOPO cloning system (Invitrogen Life Technologies, CA, USA) was used. PCR products were isolated and purified from gels, and cloned into the vectors pCR®2.1-TOPO and pCR-Blunt II-TOPO (Invitrogen Life Technologies, CA, USA). The pCR®2.1-TOPO vector is a linearized vector containing single 3' thymide (T) overhangs with Topoisomerase I covalently bound. Polymerases such as DyNAzyme™ (Finnzymes, Espoo, Finland) used in the PCR add a single deoxyadenosine (A) to the 3' end of the PCR product. This A will anneal to the 3' T overhang in the linearized vector. The ligation of the PCR product and vector is facilitated through the energy-rich bond between the Topoisomerase and the vector DNA which is attacked by the 5'–hydroxy group of the PCR product.

The *Pfu* Polymerase (Fermentas Life Science, Ontario, Canada) gives PCR products with blunt ends. pCR®-Blunt II-TOPO plasmid vector was used to sub-clone fragments with blunt ends. It is also supplied in linearized state with Topoisomerase I from *Vaccina* Virus covalently bound to the 3' end of each DNA strand.
The TOPO Cloning System allows direct selection of desired recombinants through the ccdB gene which will inhibit growth of the *E. coli* cells if it is not disrupted by the insertion of the PCR product.

2.1.10 Cloning of DNA fragments using the Gateway Cloning Technology

The Gateway® Cloning System (Invitrogen Life Technologies, CA, USA) was used to create a vector for over expression assay. The cloning method is a recombinational cloning method, based on in vitro site specific recombination properties of bacteriophage lambda (Landy, 1989). The cloning system is used to accomplish directional cloning of PCR products and sub-cloning of the DNA sequence into new vector backbones at high efficiency (Hartley et al., 2000). In this recombinational cloning process, DNA segments flanked by recombination sites (*att*-sites) are mixed in vitro with a vector also containing recombination sites, and incubated with bacteriophage λ integrase recombination proteins, resulting in transfer of the DNA segment into the vector. The Gateway® system carries out two reactions, a BP reaction to create an entry clone mediated by the integrase (Int) and integration host factor (IHF) proteins, and a LR reaction to get the final expression clone mediated by Int, IHF, and excisionase (Xis). Recombination occurs between the site specific attachment (*att*) sites: in the BP reaction the *att*B and *att*P are recombined, resulting in *att*L sites in the entry clone, and in the LR reaction the sites *att*L, from the entry clone, and *att*R, from the destination vector, are recombined, creating an expression clone containing *att*B sites. The orientation of the DNA segment is maintained during recombination because *att*B1 will recombine with *att*P1, but not *att*P2. Also the directionality of the reaction is controlled by the use of λ system, because different combinations of proteins and binding sites mediate the BP reaction and the LR reaction.

The Gateway® Cloning System has dual selection systems. By imposing antibiotic resistance selection for the desired construct and a selection (encoded by the ccdB gene) against starting molecules and intermediates, the desired clone is obtained.

2.1.10.1 Over expression construct

Over expression construct was made using the Gateway Cloning Technology. An entry clone was made by recombining *nelfattB1* and *nelfattB2* flanked PCR products into the donor vector pDONR™/Zeo (Invitrogen Life Technologies, CA, USA). The reaction was set up as described in the protocol from manufacturer, and incubated over night at room temperature. To verify the entry clones, sequencing using the *M13 forward* and *M13 reverse* primers were performed. To make expression clones, entry clones were recombined with the destination vector, pPWG, which

has a strong UAS promoter (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). The expression constructs were confirmed by sequencing.

2.1.11 Rapid DNA extraction for PCR amplification

To differentiate between PCR products produced with cDNA as template and genomic DNA as template, a reaction containing genomic DNA as template was run with all primers used in RT-PCR experiments. The method to obtain the DNA needed for these reactions is based on extracting DNA from a single fly. First, a single fly is homogenized in Squishing buffer, SB (10mM TrisCl pH 8.2, 1mM EDTA, 25mM NaCl and, 200µg/ml Proteinase K) for 20 seconds. Then, after incubation at room temperature for 30 minutes, the Proteinase K is inactivated at 95ºC for 2 minutes. The DNA was stored at 4ºC.

2.1.12 Isolation of plasmid DNA from bacterial culture

Bacterial cultures treated with SDS (sodium dodecyl sulphate) and alkali will lyse, and genomic DNA and proteins will become denaturized while the plasmids are released in the supernatant.

2.1.12.1 Miniprep

The Wizard Plus SV Miniprep DNA Purification System (Promega, WI, USA) was used to isolate and purify plasmid DNA in accordance with the manufacturer. Cells from 1.5ml bacterial culture were harvested and the plasmid DNA was eluted in 50µl of nuclease free water.

2.1.12.2 Midiprep

Plasmid DNA isolation and purification from 50ml cultures were done with the Pure Yield™ plasmid Midiprep System (Promega, WI, USA) according to the manual.

2.2 Sequencing

Sequencing was performed at two different facilities in Norway and Germany; with a MegaBACE ™ 1000 instrument using DyEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, NJ, USA) provided by the sequencing facility, ABI sequencing laboratory, at the Department of Molecular Biosciences (IMBV), and at the MWG-Biotech AG. (Ebersberg, Germany) facility.

2.3 Bacterial methods

2.3.1 Growth and storage of bacteria

E. coli cultures were grown over night in LB-medium (10g/l Bacto Tryptone, 5g/l Bacto Yeast Extract, 0.17M NaCl) at 37ºC with shaking. To obtain single colonies, *E. coli* cells were plated on to LA-plates (LB medium with 15g agar per liter containing appropriate antibiotic selection marker (100µl/ml)) and incubated at 37ºC over night (ON). For permanent storage of all cultures glycerol was added to the cultures. 1ml culture containing 8% glycerol was made and stored at -80ºC.

2.3.1.1 One Shot® TOP 10 chemically competent cells

For cloning of PCR products the pCR® 2.1-TOPO and pCR-Blunt II-TOPO (Invitrogen Life Technologies, CA, USA), pPWG (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html) and pUASp (Rorth, 1998) vectors were transformed into One Shot® TOP 10 cells (Invitrogen Life Technologies, CA, USA).

2.3.2Transformation of *E.coli*

All transformations for *E. coli* were done by heat shock of the cells in accordance with the manufacturer. The *E. coli* cells are incubated with the plasmid at 42ºC for 30 seconds and immediately transferred back on ice. After the heat shock the cells were added SOC medium (2% Bacto Trypton, 0.5% Bacto Yeast Extract, 100mM NaCl, 2.5M KCl, 10mM $MgSO_4$, and 20% glucose), and incubated at 37ºC with horizontal shaking (180-200rpm) for one hour in order to grow. To select for transformed cells, 10-200µl of cells were spread on to pre-warmed (37ºC) LA-plates containing appropriate antibiotic and incubated overnight at 37ºC.

2.4 Fly Stocks

2.4.1 Wild type

Reference stock used was MS^3 (personal communication). The stock was caught in the wild by Marianne Stabel.

2.4.2 Balancer stocks

y w; Sp/CyO; D/TM3, Sb

y w; D/TM3, Sb

y w; Sp/Cyo; Dr/Ser

2.4.3 Stocks used for the over expression assay

#4414: *y[1] w[*]; P{w[+mC]=Act5C-GAL4}25FO1/CyO, y[+]*

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBst0004414&resultlist=fbstock27650.data)

GAL4 expression driven by the promoter of the ubiquitously expressed gene *Actin 5C*.

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0000042)

#5460: *w[*]: P{w[+mW.hs]=GAL4-da.G32}UH1*

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBst0005460&resultlist=fbstock27960.data)

GAL4 expression driven by the promoter of the ubiquitously expressed gene *daughterless*.

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0000413)

2.4.4 Stocks used in the RNA interference assay

#6788: *y[1] w[*]; P{w[+mC]=UAS-Nelf-E.IR}17A10*

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBst0006788&resultlist=/tmp-shared/stockquery_129.240.90.246-17798.tmp)

UAS-RNAi construct for inhibiting *Nelf-E* expression

#5460: *w[*]: P{w[+mW.hs]=GAL4-da.G32}UH1*

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBst0005460&resultlist=fbstock27960.data)

#4414: *y[1] w[*]; P{w[+mC]=Act5C-GAL4}25FO1/CyO, y[+]*

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBst0004414&resultlist=fbstock27650.data)

#1553: *y[1] w[1]; P{w[+mC]=lacW}Mbs[S095304]/TM3, Sb[1] Ser[1]*

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBst0001553&resultlist=fbstockt27650.data)

GAL4 expression driven by the promoter of the gene *decapentaplegic*.

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0000490)

#3041: *y[1] w[1118]; P{w[+mW.hs]=GawB}ap[md544]/CyO*

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBst0003041&resultlist=fbstockt27650.data)

The flies express GAL4 in an *apterous* pattern.

(http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0000099)

2.5 Handling flies

2.5.1 Food.

Flies were raised on potato mash-yeast-agar medium at 20ºC or 25ºC.

2.5.2 Anaesthetizers

Ether was used to anaesthetize flies for examination.

2.5.3 Collecting virgins

Virgin females were collected in order to set up crosses between female and male flies of known genotype. The female flies store sperm in the ventral receptacle and spermatheca after courtship and mating, and the stored sperm is sufficient to allow females to lay eggs for many days. Virgins were collected and identified by the presence of the dark meconium in the gut, visible through the ventral abdominal wall.

2.5.4 Collecting and synchronizing pupae

In order to obtain precisely aged pupae from wild type lines and transgenic lines three approaches were used; white prepupae were collected and timed, or the observation that 4 hours after puparium formation an air bubble forms in the abdomen which eventually leads to the organism becoming buoyant, and finally a third method was to maintain third instar larvae on food containing 0.1% bromophenol blue which makes it possible to differentiate between larvae that are ready to begin pupariation, white intestine, and larva that are still eating, blue intestine. Staged prepupae were synchronized at the white prepupal stage (0 hours prepupae) or at 4 hours after puparium formation (APF) when they become buoyant and allowed to age at 20°C and 25°C for the appropriate time.

2.5.5 Collecting wings

Wings were collected from wild type flies and transgenic flies, immediately after eclosion.

2.6 Genetics

2.6.1Over expression of the gene *Nelf-E* using the vector pUASp

The gene *Nelf-E* was amplified through PCR with *Nelf-E* cDNA as template and the primers *NERcA* and *NEFcA* containing restriction sites for the endonucleases BamHI and KpnI respectively using the *Pfu* Polymerase (Fermentas Life Sciences, Ontario, Canada). The PCR product was cloned using TOPO Cloning System (Invitrogen Life Technologies) into the plasmid vector pCR®-Blunt II-TOPO. After sequencing the vector, pCR®-Blunt II-TOPO containing the *Nelf-E* fragment, and the expression vector pUASp (Rorth, 1998) were cut with the restriction endonucleases BamHI and KpnI (Promega, WI, USA) as recommended by manufacturer, and a subsequent dephosphorylation reaction was performed on the expression vector with Shrimp Alkaline Phospatase (Fermentas Life Sciences, Ontario, Canada). After ligation, the expression vector pUASp was transformed into competent *E. coli* TOP 10 cells (Invitrogen Life Technologies, CA, USA) and purified before it was injected into w^{1118} *Drosophila* embryos. The pUASp vector contains the *mini-white* gene and a UAS promoter. The transgenic flies were crossed to different balancer stocks to map the P-element insertion.

2.6.2Over expression of the gene *Nelf-E* using Gateway Technology

A construct was made for over expression of the gene *Nelf-E* to investigate the function of the gene. cDNA of *Nelf-E* was used as template in PCR using the primers *nelfattB1* and *nelfattB2*. Using Gateway Cloning Technology (Invitrogen Life Technologies, CA, USA) the PCR product was cloned into the vector pPWG (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html), via the vector pDONR/Zeo (Invitrogen Life Technologies, CA, USA). The pPWG vector contains a Gateway cassette, a strong UAS promoter, and a copy of the *mini-white* gene. After sequencing the vector was

injected into w^{1118} embryos (Ann Mari Voie) using P-element transformation. In order to map what chromosome the P-element was inserted, these transgenic flies were crossed to different balancer stocks. To overexpress the gene, these resulting stocks can be crossed to different GAL4 drivers, like the constitutively expressed drivers #4414 or #5460 flies.

2.6.3 Preparation of DNA for injection

To crate transgenic flies it is necessary to inject DNA into Drosophila embryos. 6µg of cloned vector DNA and 2µg of helper DNA (Δ2-3) were mixed together with 1/10 volume of 3M NaAc and 1.5 volumes of 96% ethanol. Post centrifugation (2min, 13000rpm) the DNA was washed in 70% ethanol, before it was air-dried, and dissolved in 20µl injection buffer (5mM KCl, 0.1mM phospate buffer pH 6.8).

2.7 Bioinformatics

The Vector NTI v 9.0.0 (Informax Invitrogen Life Technologies, CA, USA) was used for designing all the primers, to find restriction sites for endonucleases in various DNA sequences and for analyzing sequencing results.

National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) was used as a platform to retrieve annotated gene sequences from the GeneBank database with the search engine Entrez.

3. Results

Nelf-E has recently been characterized molecularly (Wu et al., 2005), but still not much is known about which genes the Nelf-E protein, together with the other proteins in the NELF complex, is involved in regulating. In this thesis the genes *Nelf-E* has been investigated. The expression pattern of *Nelf-E* was manipulated in a spatiotemporal manner, to examine the biological consequences.

3.1 Functional analysis of *Nelf-E*

In order to investigate the function of Nelf-E, we wanted to use heritable RNA interference (RNAi) (Kennerdell and Carthew, 2000) as a process to create knock down lines for the gene *Nelf-E* and to study if the knock down effect would show any mutant phenotype. Previous attempts to induce mutations in *Nelf-E* by P-element excision mutagenesis have failed (A. Lambertsson, unpublished results). RNAi lines were created by Espen Enerly (Enerly et al., 2002). The coding sequence of *Nelf-E* from position 51 to 861 was amplified twice introducing unique sequences at the product ends. The products were simultaneously cloned into the pUAST vector (via the pGEM-T- vector (Promega, WI, USA). The resulting construct *P{w*$^{+mC}$*Nelf-E*$^{IR.dsRNA.Scer\UAS}$*=UAS-Nelf-E.IR}* referred to as *P{UAS-Nelf-E.IR}* was transformed into the strain *y Df(1)w67c23*. Ten different transformant lines were obtained and the insertion was mapped in all the lines to autosomal insertions. These RNAi lines contain the P element, with an inverted repeat (IR) of *Nelf-E* (figure 3.1.1.) No phenotypic effects of the insertion was detected, except for three lines that were homozygous lethal (Enerly et al., 2002; Espen Enerly, 2002).

When the construct is expressed it creates double stranded RNA (dsRNA) of the gene *Nelf-E*. *In vivo* dsRNA is cleaved by the cell's defense system into short 21-23 nucleotide fragments, that guide sequence- specific mRNA degradation, or translational repression (Yang et al., 2000; Zamore et al., 2000; Elbashir et al., 2001a). One of these transformant lines, 17A10, homozygote for the P {*UAS-Nelf-E.IR}* construct, was crossed to fly strains expressing GAL4 protein in defined tissues and at specific times during development. This would lead to expression of the *P{Nelf-E.IR}* construct, and result in sequence specific *Nelf-E* endogenous mRNA degradation.

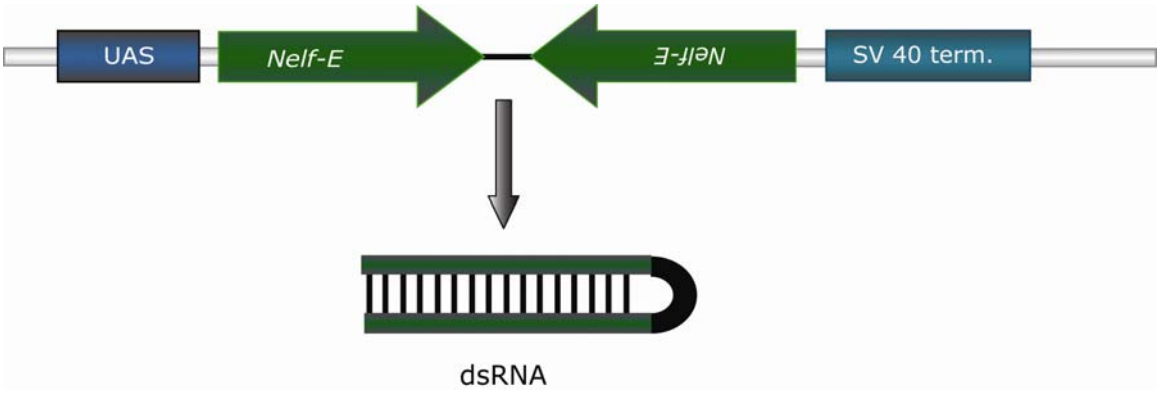


Figure 3.1.1 The transgene construct P{UAS*Nelf-E*.IR}

The coding sequence of *Nelf-E* followed by the same sequence inverted is cloned in between the UAS sequence and the SV 40 terminator. The resulting transcript will fold into dsRNA that will direct sequence specific mRNA degradation, depleting the cells for *Nelf-E* transcripts. Cartoon is adapted from Espen Enerly, and not to scale.

Crosses were performed with one of the RNAi lines. Some of the crosses have previously been reported (Enerly et al., 2002). We wanted to do some of the crosses over again, in order to further characterize the knock-down phenotype and to isolate tissues for expressional analyses. Overview of the crosses performed with the RNAi lines and different GAL4 drivers, and the observed phenotypes of progeny is presented in table 3.1.2

UAS-Nelf-E.IR	**GAL4 driver**	**Phenotype observed**
17A10	#3041 (GAL4-*ap*)	Wing blister
17A10	#1553 (GAL4-*dpp*)	Wing blisters
17A10	#4414 (GAL4- *Act 5C*)	Embryonic lethal
17A10	#5460 (GAL4- *da*)	Pupal lethal

Table 3.1.2 Summary of the crosses performed with the *UAS-NELF-E.IR* transgenic lines

An overview of the crosses performed with *UAS-Nelf-E.IR*

3.1.1 Knock-down of *Nelf-E* expression cause a wing blister phenotype

The strain #3041 contains the enhancer detection vector *P{GawB}* (P{GawB}ap^{md544}; Bloomington stock Center), (figure 3.1.1.1), that expresses GAL4 in a manner that reflects the expression pattern of the gene where it inserted (Brand and Perrimon, 1993), namely *apterous* (*ap)*. *ap* is expressed in the dorsal cells of the developing wing.

Figure 3.1.1.1 p{GawB} vector

The P-element P{GawB} is inserted in the *Drosophila* genome in the vicinity of the regulatory element of the *apterous* gene. This element drives *GAL4* expression in the same pattern as *ap*. The P-element contains the gene *White*, making it possible to track the element in the appropriate genetic background. Cartoon is not to scale.

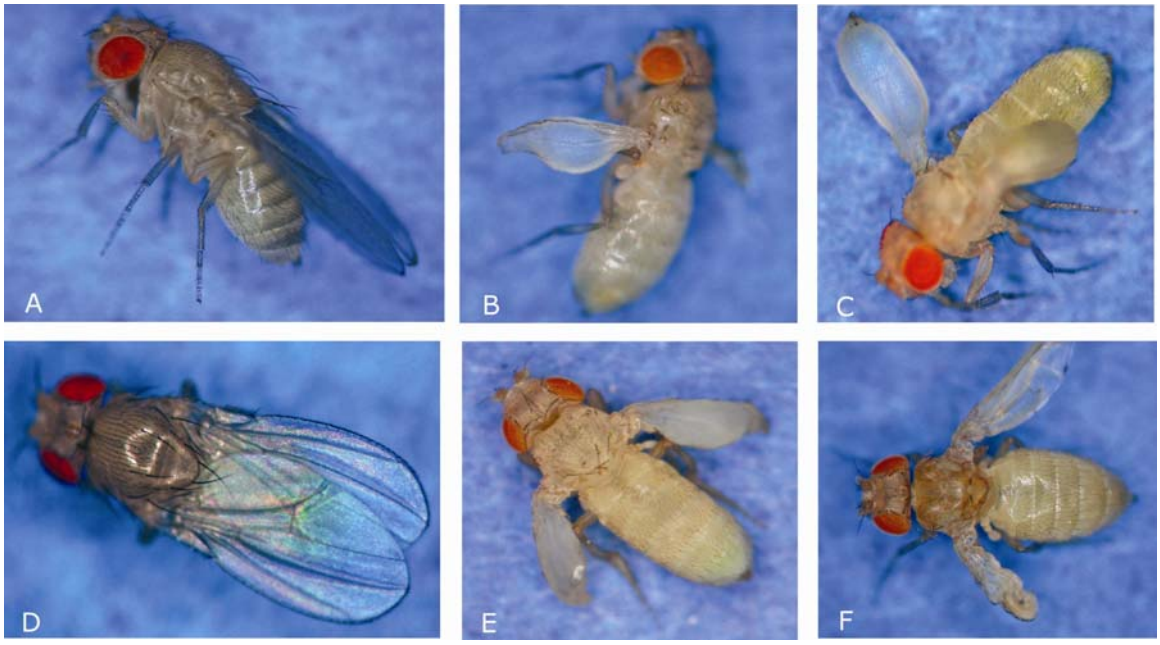


Figure 3.1.1.2 Phenotype produced by localized RNA interference of *Nelf-E* using the GAL4- *ap* driver.

Flies containing the RNAi construct for targeted *Nelf-E* gene silencing, in a Gal4 background show a prominent blistering of the wing compared to wild type flies seen in picture A and D. The wing surfaces are not connected, giving a prominent wing phenotype where the two wing surface layers are completely separated. (F) Several of the transgenic animals had wings that did not unfold properly, as the left wing on this fly. In addition many flies had problems exiting the pupal case.

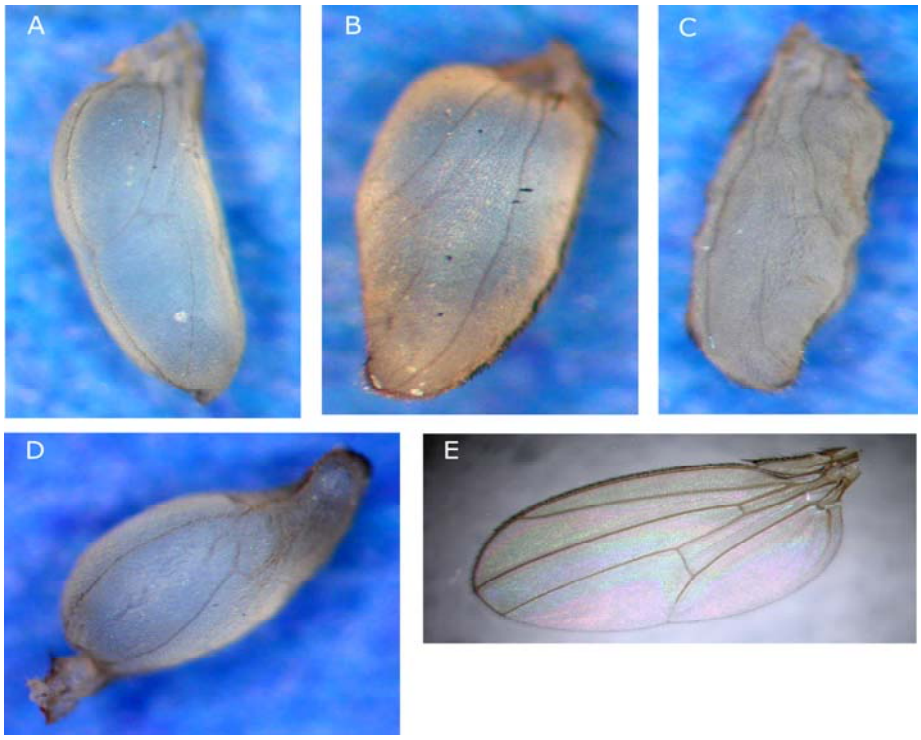


Figure 3.1.1.3 Detailed pictures of wings from flies expressing the RNAi construct in an *ap* pattern.
Pictures A-D show the phenotype manifested in flies lacking *Nelf-E* function in an *ap* pattern. All the wings are filled with a liquid matter, completely separating the two wing cell layers as compared to the wild type wing in (E).

The progeny from this cross all had severely inflated wings, with the two layers of the wing completely separated (figure 3.1.1.2 and figure 3.1.1.3). The intercellular space between the two wing-surfaces was filled with a liquid matter. In addition, quite a few flies carrying both constructs only managed to get halfway out of the pupal case.

3.1.2 Nelf-E RNA interference driven by the *dpp* promoter causes wing blisters

To confirm results published by Enerly (Enerly et al., 2002), a cross between the transgenic line 17A10 and #1553 was done. The strain #1553 contains the *P{GaTB}* vector with the gene specific promoter of *decapentaplegic* (*dpp*) subcloned in front of the GAL4 gene (P{GAL4-dpp.blk1}40C.6; Bloomington stock Center), see Figure 3.1.2.1.

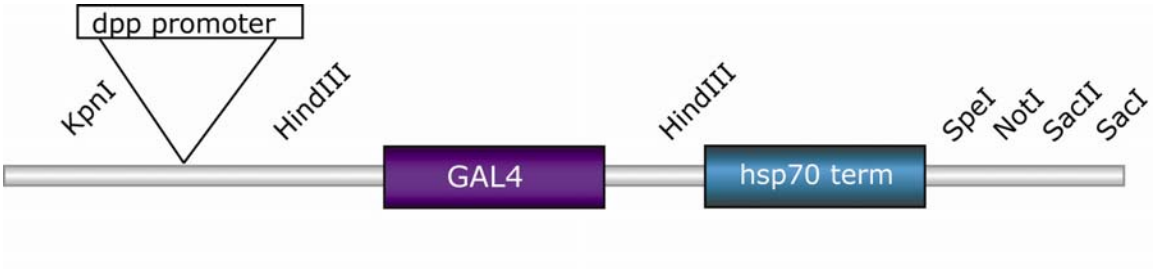


Figure 3.1.2.1 The P{GaTB} construct with the gene specific promoter of *decapentaplegic* subcloned.
The strain #1553 contains the *P{GaTB}* element with the gene specific promoter of *decapentaplegic* subcloned in front of the *GAL4* gene. The promoter will drive the expression of *GAL4* in cells that express *dpp*. Cartoon is not to scale.

Dpp protein is a secreted morphogen, and the *dpp* gene product is involved in several developmental processes from embryo to adult in *Drosophila*. The gene is expressed in the imaginal disks (Staehling-Hampton et al., 1994), in a narrow stripe of cells, along the anterior-posterior compartment boundary to ensure proper growth and patterning of the *Drosophila* appendages. When crossed to the transgenic line carrying the *P{UAS- Nelf-E.IR}* construct the progeny have a wing blistering phenotype. The wing corresponds to one of the regions where *dpp* is normaly expressed. The wings have blisters that deflate after a while, leaving a clear disfigurement on the wing (Figure 3.1.2.2). These results were consistent with those of Enerly (2002).

Figure 3.1.2.2 Phenotype resulting from targeted down regulation of *Nelf-E* in a *dpp* pattern.
Transgenic lines carrying the *P{UAS- Nelf-E.IR}* construct were crossed to GAL4-*dpp* flies, and the progeny carrying both the construct and the driver show prominent blistering of the wing blade of newly hatced flies. As the flies get older the blisters collapse, leaving scares on the wings. .Picture from (Enerly et al., 2002).

3.1.3 Depletion of *Nelf-E* function during development causes pupal lethal phenotype

Flies expressing GAL4, #5460, under the ubiquitous promoter *daughterless (P{Gal4-da.G32} UH1*; Bloomington stock Center) were crossed with the transgenic strain 17A10. The 17A10 carries the coding sequence for the gene *Nelf-E* followed by an inverted repeat of the same sequence, under the control of UAS, resulting in targeted gene knock-down of *Nelf-E*..

We observed a pupal lethal phenotype in progeny from this cross. The pupae developed and adult appendages became visible, but eclosion did not occur at 25ºC. At 20ºC eclosion instigated and the flies survived.

We dissected several pupae to look at the developing fly inside, and compared them to wild type flies also dissected out of pupae. No visible morphological differences between the transgenic progeny and wild type flies were observed (figure 3.1.3.1). We also encountered a living animal dissecting a pupa raised on 25ºC for 1oo hours APF. The fly was alive, slightly moving its head and legs when stimulated. During a six hour observation the legs and wing remained unfolded.

In addition, embryos from #5460 females crossed to 17A10 males did not hatch, but embryos from 17A10 females crossed to #5460 males did hatch, and developed as described previously at the different temperatures.

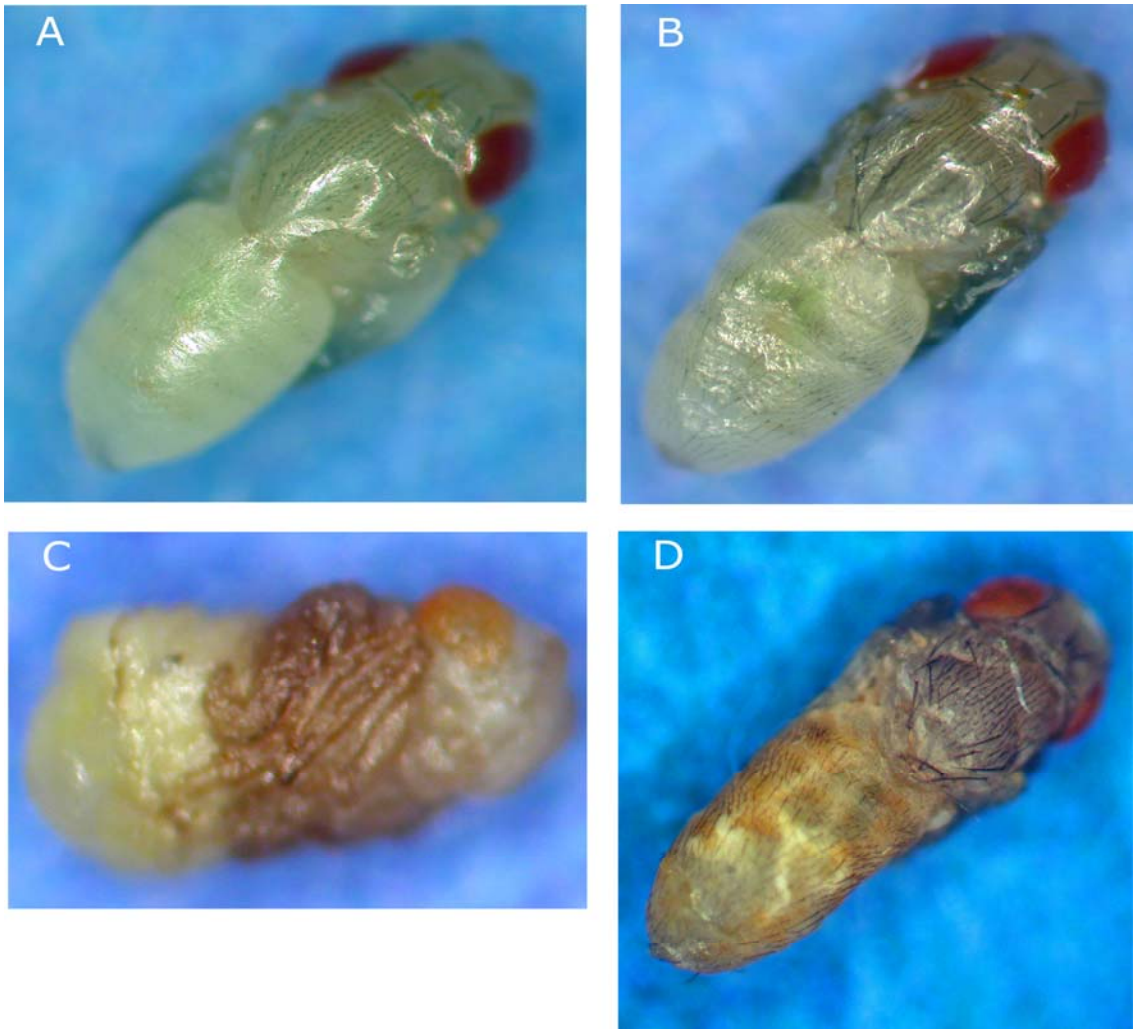


Figure 3.1.3.1 Pictures of pupae from transgenic lines and wildtype pupae.

(A) Dissected wild type pupa to reveal the developing animal inside, 72 hours after puparium formation. (B) Wild type fly dissected out of pupal case 96 hours after puparium formation. (C) Progeny from the cross between 17A10 and # 5460. The developing animal was dissected out of the pupal case after 72 hours relative to puparium formation. Comparing the animal to the wild type animal in picture A, all adult appendages are equally developed in both animals. Picture (D) shows a 96 hours APF old progeny from the cross between 17A10 and #5460. No phenotypic treat was found when compared to wild type animal in picture (B), and adult appendages were equally developed on both animals.

3.1.4 Nelf-E is essential for embryo development.

The strain #4414 (Bloomington stock Center) contains the P element *P{w[+mC]=Act5C-GAL4}25FO1*, expressing GAL4 protein under the control of the constitutive promoter of *Actin 5C*. The progeny carrying both the GAL4 driver and the *P{UAS-Nelf-E.IR}* construct die as embryos, indicating that *Nelf-E* is an essential gene for normal embryonic development.

3.2 Abolishing the RNAi effect with the *P{EPgy2}* element

The strain 17A10 was crossed to the stock #15989 (Bloomington Stock Center) containing the P-element *P{EPgy2}EY07065*. This element consists of both an enhancer, GAL4 binding sites, and the promoter that are used to direct transcription of flanking sequences. The P-element is homozygote lethal, and the transposition is endowed with the cis-acting sequences 5′ and 3′ P-element ends. The element is inserted 11 nucleotides 5′ of *RpL14* inn opposite orientation and 0,1kb 5′ of *Nelf-E* in the same orientation (http://flypush.imgen.bcm.tmc.edu/pscreen/plqlsearch2.cgi), (figure 3.2.1.) The transgenes *mini-white* and *intronless-yellow* are functional and makes it possible to follow the transposon in the appropriate genetic background. The P-element promoter will regulate ectopic expression of immediately adjacent downstream gene, in this case the *Nelf-E* gene, when combined with a source of GAL4.

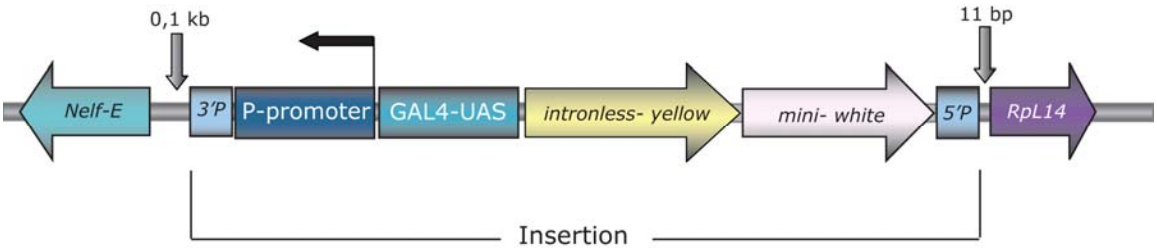


Figure 3.2.1 The *P{EPgy2}EY07065*

The element expresses the *Nelf-E* gene in a GAL4 background. The expression level depends on the driver used to activate the GAL-UAS, and can therefore be specifically expressed in only a subset of cells or at a specific developmental time.

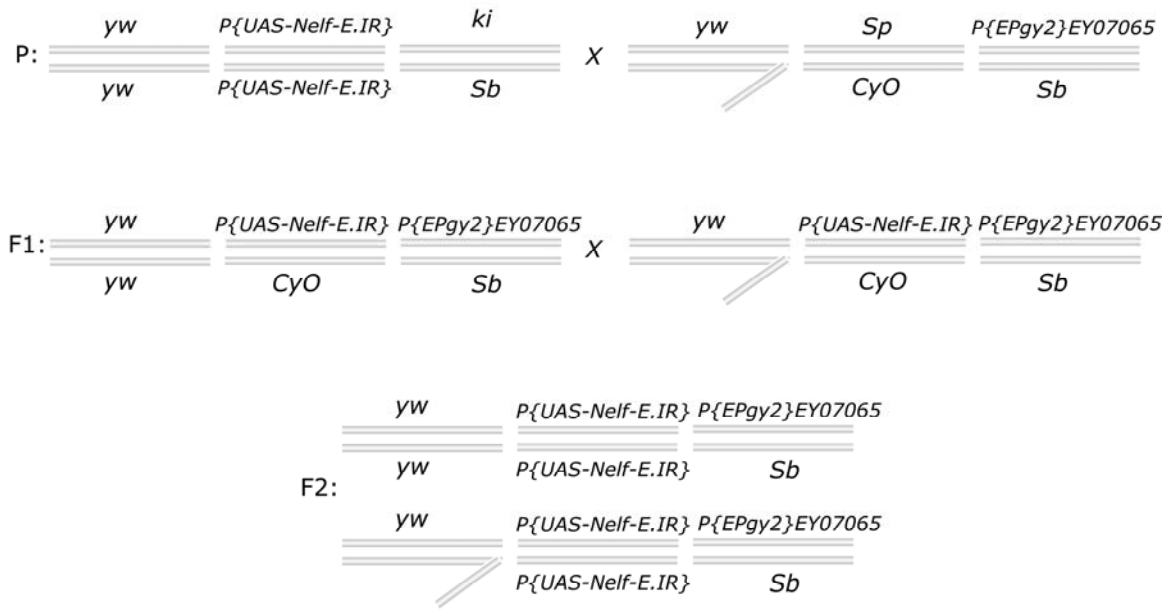


Figure 3.2.2 The cross to obtain F2 generation, LA2004, showing the genotype of the flies

The parental lines 17A10, homozygous for the *P{UAS-Nelf-E.IR}* element on the second chromosome, and #15989, containing the *P{EPgy2}EY07065* on the third chromosome, were crossed. The offspring, F1 generation, were selected for phenotypes of the genes *CyO* and *Sb*. These flies contain the *P{UAS-Nelf-E.IR}* on the second chromosome, and the *P{EPgy2}EY07065* on the third. By crossing these flies with one another, the F2 generation becomes homozygous for the *P{UAS-Nelf-E.IR}* element and heterozygous for the element *P{EPgy2}EY07065* on the third chromosomes. *CyO* , *Sb* and the element *P{EPgy2}EY07065* are homozygous lethal. Cartoon not to scale.

Off the cross the progeny was collected and divided according to manifested phenotypes. Flies in F1 generation expressing the appropriate phenotypes, were crossed to each other to obtain the desired combination of both P-elements in F2 generation, (figure 3.2.2.) Progeny from this second cross, F2, was named LA2004. The flies are homozygote for *P{w[+mC]=UAS-Nelf-E.IR}* on the second chromosome and have the *P{EPgy2}EY07065* on the third chromosome balanced against *Stubble* (*Sb)*.

The *P{w[+mC]=UAS-Nelf-E.IR}* will decrease the *Nelf-E* transcript level in the flies in a GAL4 background, while the *P{EPgy2}EY07065* will increase *Nelf-E* transcript level in the presence of GAL4. We wanted to investigate if the ectopic expression of *Nelf-E* from the *P{EPgy2}EY07065* element would abolish the effect of expressed *P{w[+mC]=UAS-Nelf-E.IR}*. We examined if flies

containing both P-elements in a defined GAL4 background lacked the phenotypes observed in crosses between 17A10 and the same corresponding GAL4 lines.

The LA2004 flies were crossed to several GAL4 lines, including #1553, #4414, #3041, and #5460. We did not observe a change in phenotype between flies containing only *P{w[+mC]=UAS-Nelf-E.IR}* and flies containing both P-elements in a GAL4 background.

3.3 Expression analyses of genes involved in wing morphogenesis in knock-down mutants

We wanted to further investigate the wing blistery phenotype observed in progeny from the cross between 17A10 and #3041, and explore which genes were affected by the lack of Nelf-E protein and thus NELF, and causing the inflated wing phenotype. In addition we wanted to investigate weather expression of *Nelf-E* was reduced in the *Nelf-E* RNAi transformant flies. RT-PCR was done on wings from transgenic flies containing *P{w[+mC]=UAS-Nelf-E.IR}* in a GAL4 background, and on wild type wings, as a semi quantitative method to establish differences in the transcription level of the potential target genes. There are several genes, that when mutated, are observed to give similar adult phenotype, as the phenotype manifested in progeny from the cross between 17A10 and #3041. These mutations, causing viable wing blister phenotypes, have already been identified as *vesiculated*, *wing blister*, *blistery* and *bloated (Gelbart, 1998)* including the genes for integrin and laminin, and in addition *blistered* (Walsh and Brown, 1998). We used gene specific primers for the genes *inflated* (*if*)*, blistery* (*by*)*, multiple edematous wings* (*mew*)*,* and *myospheroid* (*mys)* (appendix). The gene products of *mys*, *mew* and *if* are the position specific (PS) integrins. The *mys* gene codes for the β_{PS} integrin subunit (MacKrell et al., 1988; Leptin et al., 1989), *mew* encodes the α_{PS1} integrin subunit (Wehrli et al., 1993; Brower et al., 1995), and the gene *if* encodes the α_{PS2} subunit (Brower and Jaffe, 1989; Brabant and Brower, 1993). The gene *by* encodes the *Drosophila* tensin protein (Lee et al., 2003). In addition primers for *Nelf-E* were used to manifest that there was in fact a down regulation of *Nelf-E* expression in the wings. To control the amount and quality of total RNA in the first strand cDNA reaction, primers for the gene *L14* were used.

Wing tissue was collected right after eclosion, while the wings were still unfolded from wild type flies, and as soon as it was possible to identify appropriate phenotype from the transgenic flies. The same amount of total RNA from wild type sample and mutant sample was always used, making the reaction semi quantitative, although the amount of total RNA varied from 1ng to 13ng for each RT reaction. The RT-PCR was run a various number of cycles, ranging from 35 to 50 cycles. None of the genes investigated showed expression in the transformant RNAi wings and wild type wings at 35 cycles. At 50 cycles expression of *L14* was confirmed by visible PCR products of appropriate size, as a faint band in the gel, in wild type flies, but no PCR products were detected with the rest of the primer sets (figure3.3.1). In samples of transgenic flies, no expressions of any of the genes were detectable (data not shown). In order to investigate the expression level of these genes in the wing more precisely, quantitative methods, like northern or Real Time PCR must be used.

Figure 3.3.1 RT-PCR used for semi-quantify the expression of *Nelf-E, by, mys, if and mew* in wild type wings. The primers for *Nelf-E* amplify a fragment of 800 bp, but no such band is visible at 50 cycles. *L14* primers amplified a fragment of about 500 bp, indicating that the RT-PCR was successful. Genomic contamination would give a 1000 bp band.

3.4 *Nelf-E* function is essential during pupal stage

We wanted to further investigate the lethal pupal phenotype observed in flies expressing the P{UAS-Nelf-E.IR} in a daughterless pattern. Given the fact that the flies survive through eclosion when kept on 20ºC, we employed temperature shift-up and shift-down experiments through the developmental process of Drosophila, in order to establish the boundaries for the critical time period where Nelf-E function is essential. The GAL4/UAS system is temperature sensitive, At 25°C the expression of the RNAi construct is higher than the expression at obtained

at 20°C. We exploited this asset of the system in order to define this critical time period. A series of timed cultures need to be set up, half at permissive temperature and half at restrictive temperature. The cultures are shifted from one temperature to the other at regular intervals during the life cycle. The percentages of individuals with mutant or wild-type phenotypes are scored. Embryos, larvae and pupae grown at 20ºC were placed at 25ºC after a definite time. The experiment was also done with embryos, larvae and pupae grown at 25ºC that were moved to 20ºC after a distinct time. Female flies were allowed to lay eggs for a period of 1 hour. The embryos were counted and 100 embryos were placed in new vials. The animals were then allowed to develop, and each vial was shifted after a defined time to a higher or lower temperature. The animals survive at 25ºC until approximately 30 hours APF. Individuals kept longer on 25ºC do not eclose and die. The results are presented in Figure 3.4.1.

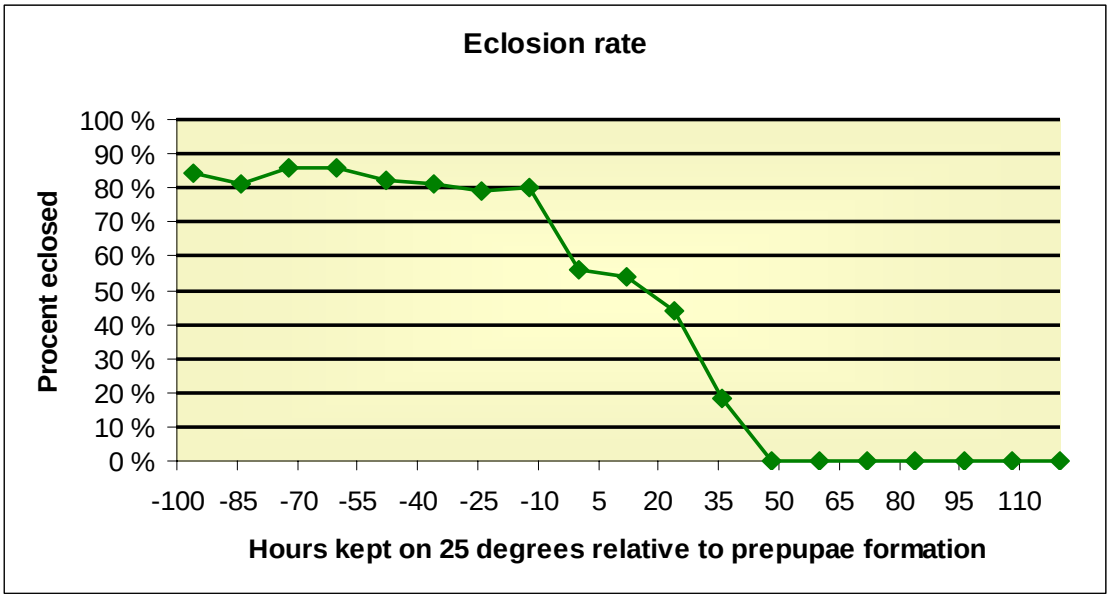


Figure 3.4.1 Eclosion rate for individuals kept at 25°C for a defined period and then moved to 20°C.

Bottles with flies were timed and kept at 25°C for a specific time and then moved to 20°C and allowed to develop for appropriate time. Individuals kept at 25°C for less than 30 hours APF have a normal development and eclose when moved to 20°C. Individuals kept at 25°C for longer periods develop normally, adult appendages become visible, but they fail to eclose after 5 days.

Individuals kept on 20°C developed normally and eclosed at the right time. We wanted to investigate the time required at 20°C for normal development. The GAL4 driver is less active at this temperature, resulting in a lower transcription rate of the RNAi construct, and consequently a lighter knock-down effect.

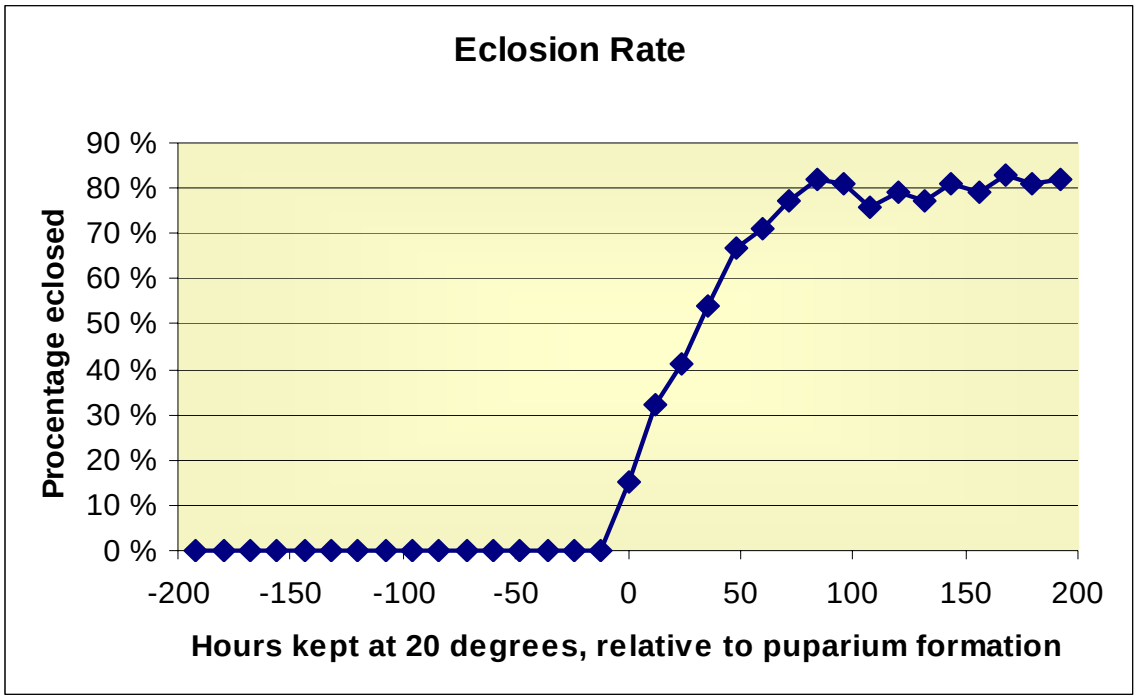
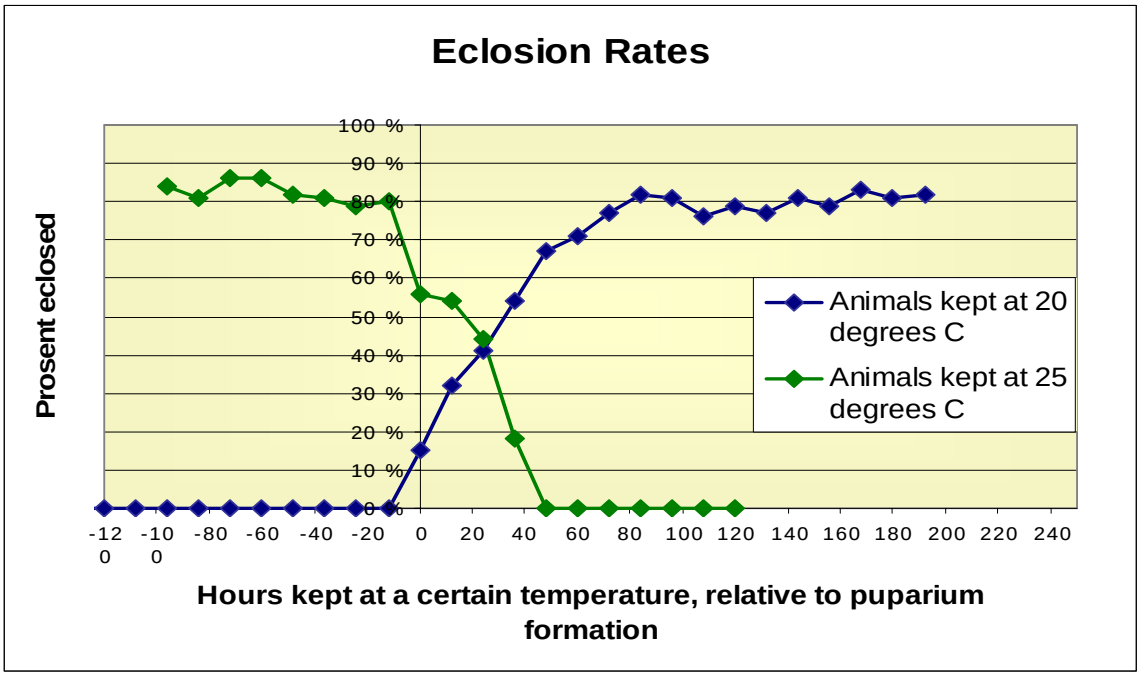


Figure 3.4.2 Eclosion rate for individuals kept at 20°C for a specific time before moved on 25°C.
Individuals grown on 20°C develop normally and eclose at estimated time. Pupae were collected and timed and then allowed to develop at 20°C for a specific time before they were moved to 25°C. The pupae need to develop for approximately 50 hours APF at 20°C to develop and eclose at correct time. Pupae kept at 20°C for less time developed adult structures but eclosion did not start.

To define the critical time period, the generated reciprocal curves are compared. The point at which they move off their plateaux will define the boundaries of the critical time period, and the point of intersection is taken as its midpoint (Figure 3.4.3). The boundaries of critical time period for this mutant are -20 hours relative to pupatrium formation and 50 hours APF. The midpoint is 20 hours APF.



Figur 3.4.3 Defining the critical time periode for the progeny of 17A10 and #5460.

The progeny of 17A10 and #5460 all suffer from lethal pupal phenotype when grown at 25°C, opposed to progeny developing at 20°C. The eclosion rates for the permissive and restrictive temperatures are compared to critical time. The boundaries for this period are from -20 hours relative to puparium formation and 50 hours APF. The mid point is 30 hours APF.

3.5 Decreasing expression level of *Nelf-E* affects expression of PS integrins genes and laminin

RT-PCR was performed on staged pupae in order to investigate if genes thought to be regulated by NELF complex would show a difference in expression level between wild type pupae and pupae where the *Nelf-E* gene product is removed. In addition, to confirm down regulation of *Nelf-E* in the RNAi transformant pupae, *Nelf-E* primers were used in the RT-PCR experiment.

Pupae were staged and collected as described in materials and methods, section 2.5.4. Wild type pupae were staged and collected at the times: 0, 1, 12, 48, 71 and 96 hours APF, while

transformant pupae were collected at the times: 0, 24, 48, 71, 80 and 96 APF. Three samples were made for each time.

To assure that equal amounts of cDNA was being used PCR amplification of first strand cDNA using *L14* primers was performed. The *L14* primers span an intron of the *L14 gene*, giving larger PCR products (1000bp) from genomic DNA than PCR product obtained from cDNA (500bp). The genes investigated in this study were chosen based on the assumptions that Nelf-E regulates the expression of integrins genes or genes associated with integrin function and on the phenotypes observed in the transgenic flies. Gene specific primers for the genes *inflated, blistery, multiple edematous wings, myospheroid and eclosion hormone* (*eh*) were used to amplify the PCR products that quantified the expression level of these genes. The RT reaction was performed with 1 µg of total RNA for both the wild type pupae samples and transformant pupae samples. PCR was run for 40 cycles. A PCR was run with genomic DNA as template for all the gene specific primers to exclude that bands obtained in PCR with cDNA as template were PCR products produced with genomic DNA as template (data not shown). In addition all gene specific primers span introns, giving larger PCR products when genomic DNA serves as template for the reaction compared to PCR products obtained from cDNA.

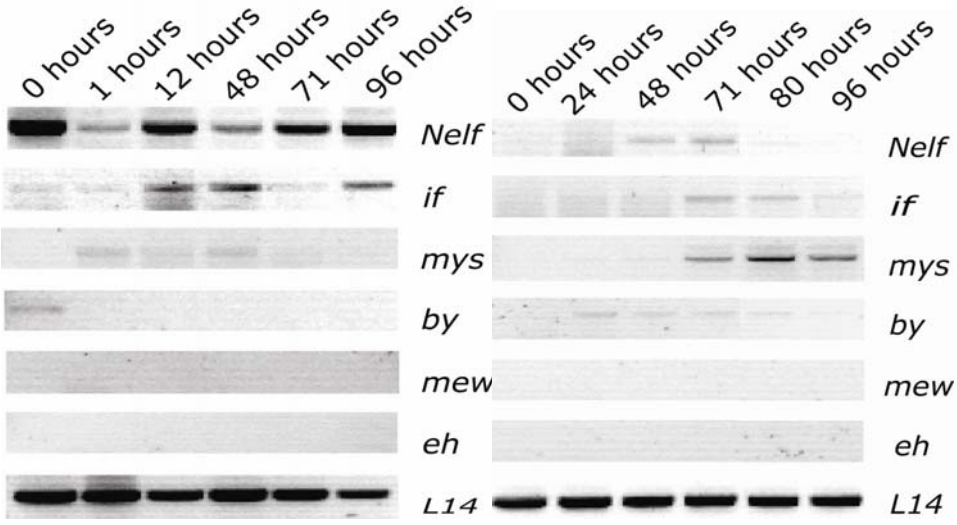


Figure 3.5.1 Semi quantitative RT-PCR on mutant tissue (left) and wild type tissue (right)
The gene specific primers for the genes *Nelf-E, if, mys, by, mew, eh* and *L1*4 amplified fragments of 800 bp, 650 bp 700 bp, 2000 bp, 1800bp and 500 bp respectively. The *L14* control amplified PCR products of 500 bp at similar levels from transgenic pupae and the wild type control

The level of PCR products obtained with the *Nelf-E* gene specific primer is significantly lower in the samples from mutant pupae, expressing the *P{Nelf-E*.IR*}* construct that inhibits expression of endogenous *Nelf-E* at a post-transcriptional level, compared to samples from wild type pupae. This corroborates that the *P{Nelf-E*.IR*}* is expressed and represses *Nelf-E* expression.

The *if* expression in the transgenic RNAi pupae was visibly weaker compared to wild type expression and absent until 71 hours APF where it becomes stronger between 71 hours APF and 80 hours APF. 96 hours APF the expression becomes weaker again in the RNAi pupae. In the wild type pupae the expression is weak but visible until 12 hours APF, but becomes stronger later during the pupal stage.

The gene *mys* is weakly expressed in the wild type pupae until 71 hours APF, but not at 96 hours APF. In the mutant pupae there is a faint band 48 hours APF, and a moderately strong band at 71, 80 and 96 hours APF are visible.
Expression of the gene *by* is visible as a faint band at 0 hours APF in the wild type pupae. Older pupae show no expression of the gene. In the mutant pupae on the other hand, no expression is visible at 0 hours APF, but a weak band is visible throughout the rest of the pupal stage. The genes *mew* and *eh* did not show any expression in wild type pupae as well as mutant pupae.

3.6 Over expression of the *Nelf-E* gene

In order to know more about the biological functions of the *Nelf-E* gene, constructs over-expressing *Nelf-E* were made. The coding sequence of *Nelf-E* was amplified in a PCR reaction using the primers *NERcA* and *NEFcA* with cDNA of *Nelf-E*. The fragment was cloned into the pUASp vector via the TOPO cloning system (see materials and methods, section 2.1.9), by digesting the pUASp vector and the TOPO vector containing the fragment, and then use T4 ligase to ligate the fragment into the pUASp vector (see materials and methods, section 2.1.8 and 2.6.2). After several attempts on cloning the fragment into the pUASp vector, with different ratios of vector and fragment, and different conditions for the ligation reaction, this method was abandoned and we decided to use the gateway cloning system instead (see materials and methods, section 2.1.10.1).

A PCR was run with the gene specific primers *nelfattB1* and *nelfattB2* and *Nelf-E* cDNA. The PCR product was cloned into the vector pPWG (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html), via the vector pDONR/Zeo (Invitrogen Life Technologies, CA, USA). The PCR fragment was cloned behind the UAS promoter (Figure 3.6.1). The UAS promoter is stimulated in a GAL4 background, and should give a high expression of the gene in different tissues and stages according to the GAL4 driver that is being used. The overexpression construct was transformed into w^{1118} flies. The pPWG vector also contains the gene for Green fluorescent protein (GFP) inserted in the same reading frame as the PCR product, making it possible to identify tissues where the construct is being expressed by examining the animals in blue light with a wave lengt of 396nm. We obtained seven different stocks after crossing primary transformants to balancer stocks (see material and methods, section 2.4.7).

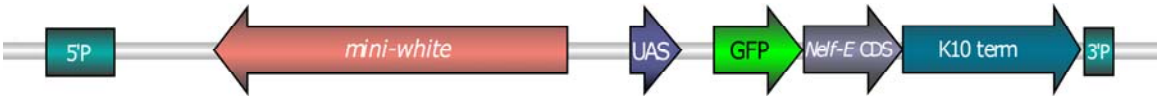


Figure 3.6.1. The over expression construct *pPWG-Nelf-E*

The construct expresses the GFP-Nelf-E transcript in a GAL4 background. The miniwhite makes it possible to select for the element in the appropriate genetic background. Cartoon is to scale.

3.6.1. Investigation of the progeny expressing pPWG-Nelf-E

In order to investigate the consequence of over expressing *Nelf-E* in a specific tissues and developmental stages, the two of the seven strains were further crossed to the GAL4 drivers #4414 and #5460 (see material and methods, section 2.4.3). The progeny from this cross were investigated for abnormal phenotypes. They were also inspected for GFP expression. The GAL4 drivers used are constitutively expressing GAL4 protein, but no GFP was detectable in any of the progeny at any developmental stages. No abnormal phenotypes in any of these progeny. To corroborate that these strains in fact contain the insertion element *pPWG-Nelf-E*, PCRs need to be run using specific primers for the insertion element , and isolated DNA from the strains as template. Unfortunately there was not enough time to complete this experiment.

4. Discussion

The genes coding for subunits of *Drosophila* NELF complex were identified based on similarities to their human counterparts (Wu et al., 2003). Molecular characterization of *Drosophila* NELF has shed light on the function of this complex as a repressor of transcription elongation by RNAPII. The complex is implicated in causing promoter proximal pausing on the *hsp70* gene. In this thesis the gene coding for one of the four subunits of this complex, NELF-E, was analyzed closer in an attempt to determine new genes regulated by the NELF complex.

To improve our understanding of the function of the *Nelf-E* gene, functional studies were performed. Mutations can provide important knowledge about the biological function of a gene. Previous attempts to induce mutations in *Nelf-E* by P-element excision mutagenesis have failed (A. Lambertsson, unpublished results). RNAi-induced gene silencing is a valuable method to study loss-of-function mutations. Therefore, an RNAi construct, abolishing *Nelf-E* function was expressed in a spatial and temporal manner. Flies expressing the construct in wing cells have clearly visible phenotypes while expression in embryos leads to a lethal phenotype (Enerly et al., 2002). In order to study gain-of-function of *Nelf-E*, transgenic lines over expressing this gene were constructed.

4.1 Tissue specific knock-down analysis of *Nelf-E*

To identify loss-of-function mutants for *Nelf-E* in *Drosophila* we used heritable RNAi (Kennerdell and Carthew, 2000). An RNAi line homozygous for the construct *P{UAS-Nelf-E.IR}* was constructed by Enerly (Enerly et al., 2002) using the pUASp vector. We crossed this line to several GAL4 drivers in order to get expression of the construct. The progeny were inspected for mutant phenotypes. Progeny expressing the construct, and thus lacking the *Nelf-E* gene product in an *ap* pattern, develop severe wing blisters, affecting the whole wing surface and completely separating the two wing layers. A similar phenotype was observed in flies lacking *Nelf-E* product in a *dpp* pattern, although in this mutant line the flies developed smaller blisters on the wings, not affecting the entire wing.

Transformants expressing the RNAi construct constitutively by the *Act5C-GAL4* driver died during the embryonic stage, while expression in a *da* pattern results in a pupal lethal phenotype. Both the *Act5C-GAL4* and *da-GAL4* drivers express GAL4 in a constitutive manner. It is reasonable to believe that abolishing *Nelf-E* function in all cells throughout development should give a comparable phenotype. Yet constitutive GAL4 drivers supposed to be expressed in the same pattern give different phenotypes. The expression of GAL4 is dependent on the construct used when creating the line. Some construct are more weakly induced. In our case we observed two phenotypes when crossing the RNAi strain to constitutively Gal4 driver. It is thought that the actin driver is turned on earlier in the embryonic development compared to the *da-GAL4* driver, resulting in two phenotypes.

4.1.1 Loss-of-function mutants affecting wing development

The wing phenotypes manifested in the progeny has previously been characterized in flies containing mutations in the genes coding for the PS integrins, and in the gene *by* (Glass, 1934) and *wg* encoding tensin and laminin respectively (figure 4.1.1.1). Integrins are heterodimers consisting of noncovalently associated α and β subunits responsible for adhesion between different tissues. Their extracellular domains bind to adhesive molecules such as fibronectin, laminin and collagen, and their intracellular domains interact with the cytoskeleton (Hynes, 1987). In the wing imaginal discs the PS antigens show nonuniform and nonhomologous distributions, and during development the expression of the antigens in a particular disc region can change (Brower et al., 1985). The integrin subunits PS1α and PS2α are found in complementary patterns, suggesting cooperation between the subunits in their function. Both these subunits are expressed in the wing but on different surfaces, PS1α on the dorsal surface and PS2α on the ventral surface. The PSβ subunits are found throughout the disc (Brower et al., 1985). In general, PS antigen expression appears to correlate with morphogenetic events in the disc epithelia, suggesting that the antigens are involved in cell-cell recognition and adhesion processes. Laminins are large ECM molecules associated with basement membranes and they play important roles during development, adhesion and cell migration (Timpl and Brown, 1996). The protein Wg is associated with basement membranes of the digestive system and muscle attachment sites during embryonic development. In later developmental stages of *Drosophila,*

during the larval stage, it is found in a specific pattern in wing and eye discs. Adult phenotypes show blisters on the wings, similar to phenotypes observed in integrin genes (Martin et al., 1999).

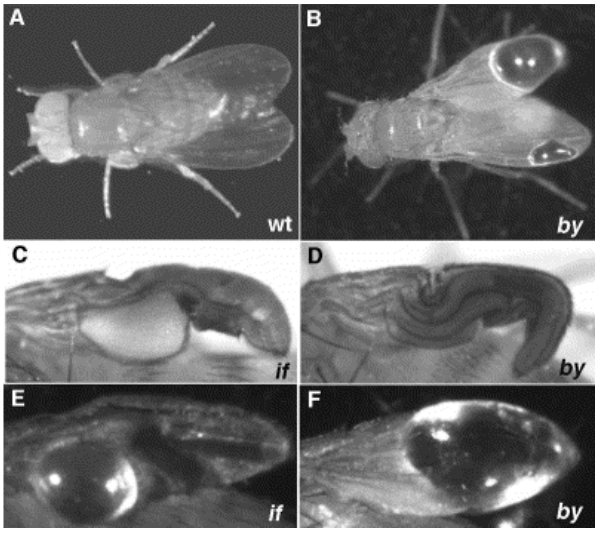


Figure 4.1.1.1 Wing blisters caused by *if* and *by* mutants.
(A) Wild type and (B) blistery mutant (by^{33c}). Comparing blisters from *by* mutants (D and F) and *if* mutants (C and E), the blisters appear later in absence of tensin. The pictures C and D show wings immediately after eclosion, while E and F are taken 5 minutes later. (Torgler et al., 2004)

Adhesion between cell layers and their ECM is crucial to the formation of complex tissues during morphogenesis (Devenport and Brown, 2004). Mutations in genes encoding for proteins mediating adhesion between cell layers in the wing, are manifested by lack of apposition of the two cell layers, resulting in blisters, ranging from lightly affected wings with small blisters, to severely affected wings with the two epithelial sheets completely separated depending on the expression pattern of the affected gene. Figure 4.1.1.1 show phenotypes caused by mutations in *if* and *by*. Comparing them to the phenotypes observed in flies lacking *Nelf-E* function in wing cells (refer to results, figures 3.1.1.2, 3.1.1.3 and 3.1.2.2), an evident similarity is observed. Because of this similarity it was inherent to investigate if changes in expression pattern of *Nelf-E* in wing tissues would influence the expression level of integrin and laminin genes.

4.1.2 Loss-of-function mutant in the pupal stage

By abolishing the function of *Nelf-E* in a *da* expression pattern, the affected individuals fail to exit the pupal case. We observed that individuals grown at 25°C developed a pupal lethal phenotype, while individuals grown at 20°C developed as normal and eclosed at the appropriate time. The GAL4/UAS expression system is a temperature sensitive system. The expression of the

Gal4 gene is much higher at 25°C compared to the expression at 20°C, making the knock down effect much stronger at 25°C compared to the effect at 20°C. By using this system, we were able to regulate the knock-down effect of the RNAi construct, and thus deciding the time frame when the *Nelf-E* function is essential for correct development.

The boundaries for this critical time period when *Nelf-E* product is essential were defined as -20 hours relative to puparium formation and 50 hours APF, with a mid point at 30 hours APF. Metamorphosis is initiated during this period of time and dramatic transformations are taking place in the developing organism. A crawling larva becomes a reproductively active fly. The onset of metamorphosis is signaled by the hormone ecdysone, resulting in the destruction of obsolete tissues used by the larva for survival and growth, and replaced by adult tissues and structures that differentiate from small clusters of diploid progenitor cells (Yin and Thummel, 2005). A number of primary-response target genes, encoding early transcription factors, are induced when ecdysone interact with two nuclear receptors. The early transcription factors, in turn, regulate large sets of downstream secondary-response genes that direct the appropriate stage- and tissue-specific biological responses to the hormone signal.

Puparium formation is triggered by an ecdysone pulse at the end of larval development. Approximately 10 hours later a new pulse signals prepupal-pupal transition. A large flow of ecdysone during pupal development, from 24 to 72 hours APF, controls adult differentiation (D'Avino and Thummel, 2000). Genetic screens performed by D'Avino *et al.* (2002) identified genes required for ecdysone-mediated tissue morphogenesis and a subset of ecdysone-regulated genes. In addition they discovered that ecdysone regulates α-integrin transcription (D'Avino and Thummel, 2000). RT-PCR performed on mutant pupae and wild type pupae, detected changes in expression levels of both the *Nelf-E* gene and the genes for integrins. It is therefore tempting to believe that NELF and ecdysone interact in regulating the expression of integrins, or that they influence each other. NELF complex has been found to be recruited to estrogen-stimulated genes, where it demolishes the level of induction achieved by the addition of estrogen. We therefore hypothesize that the NELF complex might play a similar role at ecdysone stimulated genes (Aiyar et al., 2004). A third model is that the NELF complex acts upon other transcription factors that in turn regulate their target genes.

4.1.3 *Nelf-E* might be essential for embryonic development

Embryos manipulated to express an RNAi construct for targeted *Nelf-E* silencing die during embryonic development when the construct is constitutively expressed. This indicates that *Nelf-E* might be essential for embryonic development. We have shown that down regulation of *Nelf-E* in pupae influence the expression level of integrins and laminin in pupal tissues. It might be prudent to speculate that the observed embryonic lethal phenotype is also caused by altered expression of the integrin genes. PS integrins are present during most of embryonic development, but the integrins are concentrated in specific embryonic tissues. In the three germ layers, α_{PS1} is found in ectodermal and endodermal derivatives, while α_{PS2} is mainly found in mesodermal derivatives and β_{PS} is found in all three germ layers (Leptin et al., 1989). *mys* mutant embryos develop abnormalities resulting in embryonic death (Wright, 1960; Newman and Wright, 1981). However, further analysis needs to be done to examine the observed embryonic mutant and assess if integrins could play a role in the observed phenotype. A method to confirm if integrin expressions are influenced in the mutant is to perform RT-PCR, to manifest differences in expression levels between wild type embryos and mutant embryos. On the other hand, larger screens to detect affected genes would shed light on to which extent NELF regulates transcription elongation. To measure gene expressions of several genes at the same time, a microarray assay is a suitable method. This method makes it possible to detect alterations in the expression level when comparing mutant and wild type individuals.

4.2 RT-PCR on wing tissue from *Nelf-E* RNAi mutants and wild type

The RT-PCR experiments on wing tissue from wild type and *Nelf-E* RNAi mutants were hampered by the low total RNA concentrations obtained in the isolation step. We used maximum amounts of total RNA isolation in all the RT reactions, and experimented with the amount of cDNA solution used in the PCR. In addition the number of cycles was adjusted to 50 cycles to investigate if we were able to get PCR products. We were able to detect a PCR product with the *L14* primers, but the band in the agarose gel was very faint. The gene *L14* is a constitutively expressed gene, and it would be expected that the total RNA isolation would be populated with many transcripts. The other genes that we wanted to detect are not constitutively expressed,

resulting in fewer transcripts in the total RNA, making it more difficult to obtain a visible band. A PCR product using the *L14* primers was only obtained in wild type tissues.

To investigate this phenotype further, it is necessary to establish a protocol for RNA isolation that yields enough RNA. Other techniques for gene expression measurements also rely on isolation of large enough quantities of total RNA, making the RNA isolation the critical step in the investigation.

4.3 The regulation of *Nelf-E* expression during the pupal stage in *Drosophila melanogaster*

RT-PCR results obtained with gene specific primers of *Nelf-E* on wild type pupae show that Nelf-E is expressed throughout the pupal period. However, the expression level is not uniform. At the times 0 hours, 12 hours and 96 hours APF there is a clear up regulation of the gene, compared to the samples 1 hour and 71 hours APF. The expression level of *L14* is equally high throughout the pupal stage and is therefore used as a positive control to evaluate the quality and amount of cDNA used in the PCR reaction. The samples 0 hours, 1 hour and 48 hours APF show a slightly higher level of expression. Interestingly, the level of *Nelf-E* transcript does not follow the same pattern. The level of expression is strongest at 0 hours, whereas the samples 1 hour and 48 hours show a considerably reduced expression. A noteworthy coincidence is that pulses of ecdysone occur at 0 hours and 12 hours APF, signalling puparium formation and head eversion respectively. The considerable difference in expression level between 0 hours and 1 hour APF is also interesting, as it indicates a very tight regulation of *Nelf-E* expression.

4.4 Possible connection between the expression levels of *Nelf-E* and genes involved in cell adhesion

We showed that pupae depleted of *Nelf-E* transcript also show an altered expression level of genes mediating cell adhesion (results, figure 3.5.1). Semi-quantitative RT-PCR was used to measure the transcription level of *Nelf-E* and the potential target genes. Two reactions were run on the same total RNA isolations, and three series of samples were collected from each

developmental time, in order to exclude random errors in the RNA isolation step, RT reaction or in the PCR. We demonstrated that transcription of the genes if, mys and *by* is up regulated in the mutant pupae compared to transcription levels in wild type pupae. Transcripts of *if* are present during the entire pupal period but, the highest transcript level was detected in the samples 12 hours, 48 hours and 96 hours APF. The transcripts detected at 0 hours, 1 hours and 71 hours are hardly measurable. In mutant pupae lacking *Nelf-E* function, we observed a decrease in *if* transcripts. In wild type pupae we observed transcription of *by* at 0 hours APF, but no expression was detected later in the development. In the *Nelf-E* RNAi mutant we detected *by* transcripts at 24 hours, 48 hours, 71 hours and 80 hours APF. No transcripts were detected at 0 hours and 96 hours APF. In wild type pupae, transcription of *mys* is manifested as weak bands in pupae from 1 hour to 71 hours APF, and is undetected at 0 hours and 96 hours APF. In the mutant we detected a faint band from 48 hours APF, and stronger bands from 71 hours, to 96 hours APF, indicating an increase in *mys* mRNA in the absence of *Nelf-E* function. *by* transcripts are also measured to increase in the absence of *Nelf-E* function.

Since *Nelf-E* encodes a negative transcription elongation factor, the depletion of this protein is expected to result in higher transcription rates of its target genes, as was observed with *mys* and *mew*. This coincides with our expectation of NELF repressing transcription elongation of the *mys* and *by* genes. The hypothesized regulation of *if* by the NELF complex needs to be further investigated. A closer inspection of *if* mRNA level using Real Time PCR can help elucidate a difference in expression level between wild type expression and expression in *Nelf-E* knock-down individuals.

4.5 Over expression of *Nelf-E*

To further establish the function of *Nelf-E,* an over expression assay was performed. The coding sequence of *Nelf-E* was cloned in pPWG containing the GFP gene in the same reading frame. The vector also contains the UAS promoter, making it possible to induce expression of the construct in a spatial and temporal manner. Flies containing the construct were crossed to the GAL4 drivers, #1553, #4414, #3041, #5460, expressing GAL4 in *dpp*, *Actin 5C*, *ap* and *da* pattern

respectively. The progeny did not show any GFP signal during development, and further examination revealed no phenotypes.

Several misleading sources could be affecting our experiment. The pPGW vector, with inserted fragment, was sequenced and inspected for errors that could have lead to truncated transcript. No such errors were discovered. The vector was injected into embryos along with helper DNA to facilitate the transposition of the element into the *Drosophila* genome. The emerging flies were collected and crossed to flies containing the appropriate genetic background in order to select for flies with the element incorporated into their genome. The presence of the transgene DNA in the *Drosophila* genome can be monitored by using the *mini-white* gene contained in the element. In an appropriate genetic background, the *mini-white* gene will result in wild-type red eye pigmentation in flies carrying the element, while the flies lacking the element have white eye pigmentation. We selected for individuals with red eyes. Seven different lines were obtained, and they were further crossed to GAL4 drivers, to get expression of the element. The progeny failed to express GFP. Consequently we are questioning the element's capability to express the *Nelf-E* fragment, or if the lines obtained through selection of eye pigmentation in fact are harboring the insertion. To confirm that the element is present in the genome, a PCR using genomic DNA and specific primers for the element has to be run. For future over expression studies, a negative control with wild type flies transformed with the empty pPGW vector should be performed to confirm that it is *Nelf-E*, and not the vector itself, that induces the phenotype if any.

4.6 Conclusions and further work

To investigate the functions of *Nelf-E*, genetic approaches have been carried out. Our gene silencing experiments identified that *Nelf-E* is essential throughout the developmental stages of *Drosophila*'s life cycle. Previous experiments have revealed a phenotype affecting wing development in the absence of *Nelf-E* function. The blisters observed on the wings of affected individuals, were caused by improper adhesion of the two wing epithelia sheets. Correct adhesion involves integrins and proteins associating with integrins. In addition similarities between the affected wings in *Nelf-E* mutants and integrin mutants, caught our attention, and a RT-PCR experiment was performed to investigate if knock-down of *Nelf-E* would affect the transcript

level of the integrin and laminin genes. In addition, a new phenotype not described for *Nelf-E*, pupal lethal phenotype, was identified and further investigated. First, we established a time frame where the *Nelf-E* function is essential for correct development. The time frame was identified as -20 hours relative to puparium formation and 30 hours APF. Secondly, a RT-PCR experiment was designed to detect differences in transcription levels of specific genes in wild type pupae and pupae expressing an RNAi construct for the *Nelf-E* gene. The specific genes encoding the integrin subunits and proteins associated with the subunits, implicated in cell adhesion and demonstrated to be important throughout the developmental stages of *Drosophila*. These genes were chosen based on a previous experiment, where abolishing *Nelf-E* function from wing tissues resulted in separation of the two epithelial sheets of the wing, indicating the lack of integrin function.

To confirm that the NELF complex is present in the promoter region of these genes, a chromatin immunoprecipitation (ChIP) analysis should be performed (Nelson et al., 2006). This method is widely used to explore *in vivo* interactions between proteins and DNA.

REFERENCES

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., George, R.A., Lewis, S.E., Richards, S., Ashburner, M., Henderson, S.N., Sutton, G.G., Wortman, J.R., Yandell, M.D., Zhang, Q., Chen, L.X., Brandon, R.C., Rogers, Y.-H.C., Blazej, R.G., Champe, M., Pfeiffer, B.D., Wan, K.H., Doyle, C., Baxter, E.G., Helt, G., Nelson, C.R., Gabor Miklos, G.L., Abril, J.F., Agbayani, A., An, H.-J., Andrews-Pfannkoch, C., Baldwin, D., Ballew, R.M., Basu, A., Baxendale, J., Bayraktaroglu, L., Beasley, E.M., Beeson, K.Y., Benos, P.V., Berman, B.P., Bhandari, D., Bolshakov, S., Borkova, D., Botchan, M.R., Bouck, J., Brokstein, P., Brottier, P., Burtis, K.C., Busam, D.A., Butler, H., Cadieu, E., Center, A., Chandra, I., Cherry, J.M., Cawley, S., Dahlke, C., Davenport, L.B., Davies, P., Pablos, B.d., Delcher, A., Deng, Z., Mays, A.D., Dew, I., Dietz, S.M., Dodson, K., Doup, L.E., Downes, M., Dugan-Rocha, S., Dunkov, B.C., Dunn, P., Durbin, K.J., Evangelista, C.C., Ferraz, C., Ferriera, S., Fleischmann, W., Fosler, C., Gabrielian, A.E., Garg, N.S., Gelbart, W.M., Glasser, K., Glodek, A., Gong, F., Gorrell, J.H., Gu, Z., Guan, P., Harris, M., Harris, N.L., Harvey, D., Heiman, T.J., Hernandez, J.R., Houck, J., Hostin, D., Houston, K.A., Howland, T.J., Wei, M.-H., Ibegwam, C., Jalali, M., Kalush, F., Karpen, G.H., Ke, Z., Kennison, J.A., Ketchum, K.A., Kimmel, B.E., Kodira, C.D., Kraft, C., Kravitz, S., Kulp, D., Lai, Z., Lasko, P., Lei, Y., Levitsky, A.A., Li, J., Li, Z., Liang, Y., Lin, X., Liu, X., Mattei, B., McIntosh, T.C., McLeod, M.P., McPherson, D., Merkulov, G., Milshina, N.V., Mobarry, C., Morris, J., Moshrefi, A., Mount, S.M., Moy, M., Murphy, B., Murphy, L., Muzny, D.M., Nelson, D.L., Nelson, D.R., Nelson, K.A., Nixon, K., Nusskern, D.R., Pacleb, J.M., Palazzolo, M., Pittman, G.S., Pan, S., Pollard, J., Puri, V., Reese, M.G., Reinert, K., Remington, K., Saunders, R.D., Scheeler, F., Shen, H., Shue, B.C., Simpson, M., Skupski, M.P., Smith, T., Spier, E., Spradling, A.C., Stapleton, M., Strong, R., Sun, E., Svirskas, R., Tector, C., Turner, R., Venter, E., Wang, A.H., Wang, X., Wang, Z.-Y., Wassarman, D.A., Weinstock, G.M., Weissenbach, J., Williams, S.M., Woodage, T., Worley, K.C., Wu, D., Yang, S., Yao, Q.A., Ye, J., Yeh, R.-F., Zaveri, J.S., Zhan, M., Zhang, G., Zhao, Q., Zheng, L., Zheng, X.H., Zhong, F.N., Zhong, W., Zhou, X., Zhu, S., Zhu, X., Smith, H.O., Gibbs, R.A., Myers, E.W., Rubin, G.M., and Venter, J.C. (2000). The Genome Sequence of *Drosophila melanogaster*. Science **287,** 2185-2195.

Aiyar, S.E., Sun, J.-l., Blair, A.L., Moskaluk, C.A., Lu, Y.-z., Ye, Q.-n., Yamaguchi, Y., Mukherjee, A., Ren, D.-m., Handa, H., and Li, R. (2004). Attenuation of estrogen receptor {alpha}-mediated transcription through estrogen-stimulated recruitment of a negative elongation factor. Genes Dev. **18,** 2134-2146.

Archambault, J., and Friesen, J.D. (1993). Genetics of Eukaryotic RNA Polymerases I, II, and III. Microbiological Reviews **57,** 703-724.

Bellen, H.J., Levis, R.W., Liao, G., He, Y., Carlson, J.W., Tsang, G., Evans-Holm, M., Hiesinger, P.R., Schulze, K.L., Rubin, G.M., Hoskins, R.A., and Spradling, A.C. (2004). The BDGP Gene Disruption Project: Single Transposon Insertions Associated With 40% of *Drosophila* Genes. Genetics **167,** 761-781.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409,** 363.

Brabant, M.C., and Brower, D.L. (1993). PS2 Integrin Requirements in *Drosophila* Embryo and Wing Morphogenesis. Developmental Biology **157,** 49-59.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118,** 401-415.

Brower, D.L., and Jaffe, S.M. (1989). Requirement for integrins during *Drosophila* wing development. Nature **342,** 285-287.

Brower, D.L., Piovant, M., and Reger, L.A. (1985). Developmental analysis of *Drosophila* position-specific antigens. Developmental Biology **108,** 120-130.

Brower, D.L., Bunch, T.A., Mukai, L., Adamson, T.E., Wehrli, M., Lam, S., Friedlander, E., Roote, C.E., and Zusman, S. (1995). Nonequivalent requirements for PS1 and PS2 integrin at cell attachments in *Drosophila*: genetic analysis of the alpha PS1 integrin subunit. Development **121,** 1311-1320.

Cohen, B., Simcox, A.A., and Cohen, S.M. (1993). Allocation of the thoracic imaginal primordia in the *Drosophila* embryo. Development **117,** 597-608.

D'Avino, P.P., and Thummel, C.S. (2000). The Ecdysone Regulatory Pathway Controls Wing Morphogenesis and Integrin Expression during *Drosophila* Metamorphosis. Developmental Biology **220,** 211-224.

Devenport, D., and Brown, N.H. (2004). Morphogenesis in the absence of integrins: mutation of both *Drosophila* {beta} subunits prevents midgut migration. Development **131,** 5405-5415.

Diaz-Benjumea, F.J., and Cohen, S.M. (1993). Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila*. Cell **75,** 741-752.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001a). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15,** 188-200.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001b). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. **20,** 6877-6888.

Enerly, E., Larsson, J., and Lambertsson, A. (2002). Reverse genetics in *Drosophila*: From sequence to phenotype using UAS-RNAi transgenic flies. genesis **34,** 152-155.

Espen Enerly, J.L.A.L. (2002). Reverse genetics in Drosophila: From sequence to phenotype using UAS-RNAi transgenic flies. genesis **34,** 152-155.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391,** 806-811.

Fischer, J.A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). GAL4 activates transcription in *Drosophila*. Nature **332,** 853.

Fortini, M.E., Skupski, M.P., Boguski, M.S., and Hariharan, I.K. (2000). A Survey of Human Disease Gene Counterparts in the *Drosophila* Genome. J. Cell Biol. **150,** 23F-30.

Friedman, R., and Hughes, A.L. (2001). Pattern and Timing of Gene Duplication in Animal Genomes. Genome Res. **11,** 1842-1847.

Fujinaga, K., Irwin, D., Huang, Y., Taube, R., Kurosu, T., and Peterlin, B.M. (2004). Dynamics of Human Immunodeficiency Virus Transcription: P-TEFb Phosphorylates RD and Dissociates Negative Effectors from the Transactivation Response Element. Mol. Cell. Biol. **24,** 787-795.

Garcia-Bellido, A., Ripoll, P., and Morata, G. (1976). Developmental compartmentalization in the dorsal mesothoracic disc of *Drosophila*. Developmental Biology **48,** 132-147.

Gelbart. (1998). FlyBase: a *Drosophila* database. Flybase Consortium. Nucl. Acids Res. **26,** 85-88.

Giniger, E., Varnum, S.M., and Ptashne, M. (1985). Specific DNA binding of GAL4, a positive regulatory protein of yeast. Cell **40,** 767.

Glass, H.B. (1934). New mutants report. *Drosophila* Information Service **2,** 7-8.

Hamilton, A.J., and Baulcombe, D.C. (1999). A Species of Small Antisense RNA in Posttranscriptional Gene Silencing in Plants. Science **286,** 950-952.

Hammond, S.M., Caudy, A.A., and Hannon, G.J. (2001). POST-TRANSCRIPTIONAL GENE SILENCING BY DOUBLE-STRANDED RNA. Nature Reviews Genetics **2,** 110.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404,** 293.

Hannon, G.J. (2002). RNA interference. Nature **418,** 244.

Hartley, J.L., Temple, G.F., and Brasch, M.A. (2000). DNA Cloning Using In Vitro Site-Specific Recombination. Genome Res. **10,** 1788-1795.

Hutvagner, G., and Zamore, P.D. (2002). RNAi: nature abhors a double-strand. Current Opinion in Genetics & Development **12,** 225.

Hynes, R.O. (1987). Integrins: A family of cell surface receptors. Cell **48,** 549-554.

Hynes, R.O. (2002). Integrins: Bidirectional, Allosteric Signaling Machines. Cell **110,** 673-687.

Jiang, C., Baehrecke, E.H., and Thummel, C.S. (1997). Steroid regulated programmed cell death during *Drosophila metamorphosis*. Development **124,** 4673-4683.

Kennerdell, J.R., and Carthew, R.W. (2000). Heritable gene silencing in *Drosophila* using double-stranded RNA. Nat Biotech **18,** 896-898.

Kornberg, T., Siden, I., O'Farrell, P., and Simon, M. (1985). The *engrailed* locus of *Drosophila*: In situ localization of transcripts reveals compartment-specific expression. Cell **40,** 45-53.

Landy, A. (1989). Dynamic, Structural, and Regulatory aspects of lambda Site - Specific Recombination. Annual Review of Biochemistry **58,** 913- 949.

Lawrence, P.A., and Morata, G. (1976). Compartments in the wing of *Drosophila*: A study of the *engrailed* gene. Developmental Biology **50,** 321-337.

Lawrence, P.A., and Morata, G. (1977). The early development of mesothoracic compartments in *Drosophila* : An analysis of cell lineage and fate mapping and an assessment of methods. Developmental Biology **56,** 40-51.

Laybourn, P.J., and Dahmus, M.E. (1989). Transcription-dependent structural changes in the C-terminal domain of mammalian RNA polymerase subunit IIa/o. J. Biol. Chem. **264,** 6693-6698.

Lee, S.B., Cho, K.S., Kim, E., and Chung, J. (2003). *blistery* encodes *Drosophila* tensin protein and interacts with integrin and the JNK signaling pathway during wing development. Development **130,** 4001-4010.

Leptin, M. (1987). *Drosophila* position-specific antigens resemble the vertebrate fibronectin-receptor family. The EMBO Journal **6,** 1037-1043.

Leptin, M., Bogaert, T., Lehmann, R., and Wilcox, M. (1989). The function of PS Integrins during *Drosophila* embryogenesis. Cell **56,** 401-408.

Li, Y.-X., Farrell, M.J., Liu, R., Mohanty, N., and Kirby, M.L. (2000). Double-Stranded RNA Injection Produces Null Phenotypes in Zebrafish. Developmental Biology **217,** 394.

MacKrell, A.J., Blumberg, B., Haynes, S.R., and Fessler, J.H. (1988). The Lethal *myospheroid* Gene of *Drosophila* Encodes a Membrane Protein Homologous to Vertebrate Integrin {beta} Subunits. PNAS **85,** 2633-2637.

Martin, D., Zusman, S., Li, X., Williams, E.L., Khare, N., DaRocha, S., Chiquet-Ehrismann, R., and Baumagartner, S. (1999). *wing blister*, A New *Drosophila* Laminin Alpha Chain for Cell Adhesion and Migration during Embryonic and Imaginal Development. The Journal of Cell Biology **145,** 191-201.

Montgomery, M.K., Xu, S., and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. PNAS **95,** 15502-15507.

Mullins, K.B., and Faloona, F.A. (1987). Spesific synthesis of DNA in vitro via a polymerase catalysed chain reaction. Methods in Enzymology **155,** 350-355.

Narita, T., Yamaguchi, Y., Yano, K., Sugimoto, S., Chanarat, S., Wada, T., Kim, D.-k., Hasegawa, J., Omori, M., Inukai, N., Endoh, M., Yamada, T., and Handa, H. (2003). Human Transcription Elongation Factor NELF: Identification of Novel Subunits and Reconstitution of the Functionally Active Complex. Mol. Cell. Biol. **23,** 1863-1873.

Nelson, J.D., Denisenko, O., Sova, P., and Bomsztyk, K. (2006). Fast chromatin immunoprecipitation assay. Nucl. Acids Res. **34,** e2-.

Newman, S.M., and Wright, T.R.F. (1981). A histological and ultrastructural analysis of developmental defects produced by the mutation, *lethal(1)myospheroid*, in *Drosophila melanogaster*. Developmental Biology **86**.

Nikolov, D.B., and Burley, S.K. (1997). RNA polymerase II transcription initiation: A structural view. PNAS **94,** 15-22.

North, G., and French, V. (1994). Insect Wings: Patterns upon patterns. Current Biology **4,** 611-614.

Nykanen, A., Haley, B., and Zamore, P.D. (2001). ATP Requirements and Small Interfering RNA Structure in the RNA Interference Pathway. Cell **107,** 309.

Price, D.H. (2000). P-TEFb, a Cyclin-Dependent Kinase Controlling Elongation by RNA Polymerase II. Mol. Cell. Biol. **20,** 2629-2634.

Restifo, L.L., and Wilson, T.G. (1998). A juvenile hormone agonist reveals distinct developmental pathways mediated by Ecdysone-inducible broad complex transcription factors. Developmental Genetics **22,** 141-159.

Robertson, C.W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. Journal of Morphology **59,** 351-399.

Rorth, P. (1998). Gal4 in the *Drosophila* female germline. Mechanisms of Development **78,** 113-118.

Rubin, G.M., and Lewis, E.B. (2000). A Brief History of *Drosophila's* Contributions to Genome Research. Science **287,** 2216-2218.

Sambrook, J., and Russel, D.W. (2001). Molecular cloning: A laboratory manual, the third edition. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Schramm, L., and Hernandez, N. (2002). Recruitment of RNA polymerase III to its target promoters. Genes Dev. **16,** 2593-2620.

Sharp, P.A. (1999). RNAi and double-strand RNA. Genes Dev. **13,** 139-141.

Sharp, P.A. (2001). RNA interference{---}2001. Genes Dev. **15,** 485-490.

Spradling, A.C., Stern, D.M., Kiss, I., Roote, J., Laverty, T., and Rubin, G.M. (1995). Gene Disruptions Using P Transposable Elements: An Integral Component of the *Drosophila* Genome Project. PNAS **92,** 10824-10830.

Spradling, A.C., Stern, D., Beaton, A., Rehm, E.J., Laverty, T., Mozden, N., Misra, S., and Rubin, G.M. (1999). The Berkeley *Drosophila* Genome Project Gene Disruption Project: Single P-Element Insertions Mutating 25% of Vital *Drosophila* Genes. Genetics **153,** 135-177.

St Johnston, D. (2002). The art and design of genetic screens: *Drosophila melanogaster*. Nature Reviews Genetics **3,** 176-188.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, A.H., and Hoffmann, F.M. (1994). Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in *Drosophila* embryos induced by decapentaplegic but not 60A. Cell Growth Differ **5,** 585-593.

Timpl, R., and Brown, J.C. (1996). Supramolecular assembly of basement membranes. BioEssays **18,** 123-132.

Torgler, C.N., Narasimha, M., Knox, A.L., Zervas, C.G., Vernon, M.C., and Brown, N.H. (2004). Tensin Stabilizes Integrin Adhesive Contacts in *Drosophila*. Developmental Cell **6,** 357-369.

Walsh, E.P., and Brown, N.H. (1998). A Screen to Identify *Drosophila* Genes Required for Integrin-Mediated Adhesion. Genetics **150,** 791-805.

Wehrli, M., DiAntonio, A., Fearnley, I.M., Smith, R.J., and Wilcox, M. (1993). Cloning and characterization of [alpha]PS1, a novel *Drosophila melanogaster* integrin. Mechanisms of Development **43,** 21-36.

Weigmann, K., Klapper, R., Strasser, T., Rickert, C., Technau, G., Jackle, H., Janning, W., and Klambt, C. (2003). FlyMove - a new way to look at development of *Drosophila*. Trends in Genetics **19,** 310.

Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. Nat Cell Biol **2,** 70.

Wieschaus, E., and Gehring, W. (1976). Clonal analysis of primordial disc cells in the early embryo of *Drosophila melanogaster*. Developmental Biology **50,** 249-263.

Wright, T.R.F. (1960). The phenogenetics of the embryonic mutant, *lethal (1) myospheroid*, in *Drosophila melanogaster*. Journal of Experimental Zoology **143,** 77-99.

Wu, C.-H., Lee, C., Fan, R., Smith, M.J., Yamaguchi, Y., Handa, H., and Gilmour, D.S. (2005). Molecular characterization of *Drosophila* NELF. Nucl. Acids Res. **33,** 1269-1279.

Wu, C.-H., Yamaguchi, Y., Benjamin, L.R., Horvat-Gordon, M., Washinsky, J., Enerly, E., Larsson, J., Lambertsson, A., Handa, H., and Gilmour, D. (2003). NELF and DSIF cause promoter proximal pausing on the hsp70 promoter in *Drosophila*. Genes Dev. **17,** 1402-1414.

Yamaguchi, Y., Inukai, N., Narita, T., Wada, T., and Handa, H. (2002). Evidence that Negative Elongation Factor Represses Transcription Elongation through Binding to a DRB Sensitivity-Inducing Factor/RNA Polymerase II Complex and RNA. Mol. Cell. Biol. **22,** 2918-2927.

Yamaguchi, Y., Wada, T., Watanabe, D., Takagi, T., Hasegawa, J., and Handa, H. (1999a). Structure and Function of the Human Transcription Elongation Factor DSIF. J. Biol. Chem. **274,** 8085-8092.

Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999b). NELF, a Multisubunit Complex Containing RD, Cooperates with DSIF to Repress RNA Polymerase II Elongation. Cell **97,** 41-51.

Yang, D., Lu, H., and Erickson, J.W. (2000). Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. Current Biology **10,** 1191.

Yin, V.P., and Thummel, C.S. (2005). Mechanisms of steroid-triggered programmed cell death in *Drosophila*. Seminars in Cell & Developmental Biology **16,** 237-243.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals. Cell **101,** 25.

Zhou, X., and Riddiford, L.M. (2002). Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. Development **129,** 2259-2269.

Zusman, S., Patel-King, R.S., Ffrench-Constant, C., and Hynes, R.O. (1990). Requirements for integrins during *Drosophila* development. Development **108,** 391-402.

ABBRIVIATIONS

A	Adenine
amp	Ampecillin
AP	Anteriorposterior
ap	*apterous*
APF	After puparium formation
ATP	adenosintriphosphate
BAC	Bacterial artificial chromosome
BDGP	The Berkely *Drosophila* Genome Project
BLAST	Basic local alignment search tool
bp	base pair
by	*blistery*
C	Cytosine
C. elegans	*Caenorhabditis elegans*
ccdB	*Controller of cell division or death B*
cDNA	Complementary DNA
CDS	Coding sequence
CTD	Carboxy-terminal domain
da	*daughterless*
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Dpp	Decapentaplegic
dpp	*decapentaplegic*
Drosophila	*Drosophila melanogaster*
DRB	5,6-di-chloro-1-β-D-ribofuranosylbenzimidazole
DSIF	DRB Sensitivity-inducing factor
dsRNA	double-stranded RNA
DV	Dorsoventral
E. coli	*Escherichia coli*
ECM	Extracellular matrix
eh	*eclosion hormone*
EMS	Ethylmethane sulphonate
en	*engrailed*
EtBr	Ethidium bromide
F_1	First generation
F_2	Second generation
GFP	Green Fluorescent Protein
hpRNA	hairpin-loop RNA
if	*inflated*
IHF	Integration host factor
IMBV	Department of Molecular Biosciences
Int	integrase
IR	Inverted repeat

JH	Juvenile hormone
kb	kilo base
LA	Luria Broth medium with agar
LB	Luria Broth medium
mew	*multiple edematous wings*
mRNA	messenger RNA
mys	*myospheroid*
NELF	Negative transcription elongation factor complex
Nelf-E	Negative elongation factor E
Nelf-E	*Negative elongation factor E*
nm	nano meters
ON	Over night
P	Parental line
P-elements	transposable elements
P-TEFb	Positive transcription elongation factor b
PCR	Polymerase Chain Reaction
Pfu	*Pyrococcus furiosus*
PIC	Preinitiation complex
PS	Position-specific
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II
rRNA	ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
S.O.B	Super Optimal Broth
S.O.C	S.O.C cell growth medium, derived from S.O.B
SAP	Shrimp Alkaline Phosphatase
SB	Squishing buffer
Sb	*Stubble*
SDS	Sodium dodecyl sulfate
siRNA	small interfering RNAs
snRNAs	small nuclear RNAs
T	Thymine
TAE	Tris-acetate
TEC	Transcription elongation complex.
tRNA	transfer RNA
UAS	Upstream Activating Sequences
UTR	Untranslated region
vg	*vestigal*
wb	*wing blister*
wg	*wingless*
Wg	Wingless
Xis	Excisionase

APPENDIX 1

Name	Oligo Sequence
Blisanti	5'-ACCAATGGCCAACAGCAGAT-3'
Blissense	5'-TTGACTGCATTAGTAGTGCTTAAGC-3'
Byantisense	5'-GGTACCAAGAACTTCTTGCGCGTATT-3'
Bysense	5'-GGATCCAAAAACTATAATGTGAATGGC-3'
Ifantisense	5'-TGTCCACAGTGCTGTTCCATAA-3'
Ifsense	5'-CGACCAGGTGTTCATCTTTAAGTCG-3'
Mewantisense	5'-GGTACCGCCTGAGGACTCAGTGATGT-3'
Mewasense	5'-CATTGAACCACAACCCACATGACC-3'
mys2anti	5'-ATGACCGGAGCAGATCTCGC-3'
mys2sense	5'-CTCCTGCATCCCGAATCATCC-3'
Mysantisense	5'-GGTACCATGTATCGTTGGACTCCTGG-3'
Myssense	5'-GAATTCCATCGAAGGAAAAGTGTCACACC-3'
nelfattB1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGTTTACATACACTTC-3'
nelfattB2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGAGCAAGAAGTCTTCATC-3'
sense447nelf	5'-GCCATCCAGAAGCAGACCAAGC-3'
anti898nelf	5'-GGCCATGAATCTCCGCAATG-3'