Development of Threshold sensitive NF-ĸB reporters for Live Imaging of NF-ĸB transcription in *Drosophila melanogaster*

Thesis

submitted in partial fulfillment of the requirements of

Five year BS-MS Dual Degree Programme

By

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&

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Certificate

This is to certify that the Thesis entitled, **Development of Threshold sensitive NF-ℽB reporters for Live Imaging of NF-ℽB transcription in *Drosophila melanogaster*,** and submitted by Srija Bhagavatula, Reg No. 20071028 in partial fulfillment of the requirement of Five year BS-MS Dual Degree Programme Thesis, embodies the work done by her under my supervision.

Signature of the Supervisor(s)

Date:

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Abstract

In *Drosophila*, NF-KB family of transcriptional regulators mediate early dorsoventral axis patterning and host defences through the Toll and Immunodeficient (IMD) pathways. The three major NF-KB molecules in *Drosophila* include Dorsal, Dorsal-like Immune factor (Dif) and Relish (REL). During dorsoventral (D/V) patterning in the early embryo, asymmetric Toll signalling sets up a nucleocytoplasmic Dorsal gradient in the ventral and lateral regions. This along with the Dpp/Sog gradient sets up the D/V axis through threshold dependent gene expression leading to the specification of Ectoderm, Neuroectoderm amd Mesoderm. In the larval and adult stages, the Toll/Dorsal-Dif and IMD/REL signal transduction pathways upregulate transcription of defense genes in response to infection and are also involved in blood cell proliferation.

Investigators have routinely used antibodies against Dorsal/Dif/REL and also antibodies or in-situ probes to monitor activation of the NF-K B targets in Development and host defense. In the last decade, a large number of fluorescence based methods have been developed to monitor the Dorsal gradient and also to measure NF-KB activation in the fat body and lymph gland. A very interesting problem in Development is the formation of the Dorsal nucleoctoplasmic gradient in the blastoderm and the effect of this gradient on cell-fate specification. The most recent efforts include use of Dorsal-fusions with fluorescent reporters such as eGFP to measure the spatiotemporal expression of the Dorsal gradient.

In this study, in order to avoid the problems inherent in a Dorsal-fluoroscent protein fusion, we attempt to develop an alternative method to visualize the Dorsal gradient without disturbing Dorsal. Our method relies on using promoter elements, both native and artificial, that will respond to different concentration of nuclear Dorsal (or Dif). We hypothesize, that by using promoters of different sensitivity, and by fusing these promoters to a variety of bright colored fluoresent reporters (eGFP, mCherry, Venus, Cerulean), we will be able to visualize increasing amounts of Dorsal in the nucleus by different colors. This concept, when applied to a fly embryo will help us visualize, live, the *Drosophila* gradient in multiple overlapping colors in the early embryo and will also allow us to visualize different strengths of Toll/Dorsal-Dif activation in the fat body and lymph gland.

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Introduction

1. Morphogen gradients

Development of a metazoan organism begins with a single cell, multiplying to give a distribution of cells, which eventually differentiate to give rise to the several tissues and organs that constitute an adult organism. Information initiating this fundamental process in development is acquired by the cells based on their spatial distribution, through molecules that act through a concentration gradient. Molecules that confer such information determining the morphology of the system, came to be known as morphogens. Cells respond to these morphogen gradients and acquire distinctive identities depending upon their spatial positions thus leading to axial patterning.

The early *Drosophila melanogaster* embryo has been studied extensively in this regard in order to understand the formation and dynamics of morphogen gradients. *Drosophila* as a model system is well established and therefore provides us with an extensive database to work with. It serves as an excellent genetic tool box owing to its short life span, and genetic features that include balancer chromosomes and absence of recombination in males. Most of the conserved pathways have been well characterized in the system and therefore act as a good reference to further our understanding on new phenomenon (www.flybase.com).

Fundamental developmental pathways mediated by morphogen gradients in the fly include Bicoid (Tautz., 1988), Dorsal (Anderson., 1985) and Dpp signalling (Spencer., 1982). These have been well characterized and best understood in the early embryo and wing disc and therefore are ideal for the understanding of how morphogen gradients determine cell fates. From these classic examples we know that cells detect small changes in the thresholds of morphogen gradient concentrations and convert it into distinct bands of downstream gene expression, thus increasing specificity. This is often referred to as the French Flag model (Ashe and Briscoe, 2006).

In this project, our subject of focus is the dorsal-ventral patterning of the

Drosophila embryo, an act of the "Dorsal" morphogen in collaboration with the Dpp/Sog system. Dorsal primarily patterns the ventral and lateral sides of the embryo while Dpp and Sog pattern the Dorsal and lateral embryo (refer to figure 2). Dorsal is a *Drosophila* homologue of the NF-kB transcription factor family in mammals (Reichhart et al., 1993). Memebers of this family are especially known for their importance in mediating rapid responses in a context-dependent, cell type and stimulus specific manner (Smale 2011). They have indispensible roles in inducing immune responses and also in regulating the growth, survival and proliferation of cells (Hayden et al., 2006). These proteins act as homo/hetero dimers. In mammals, they are retained in the cytoplasm by their interaction with the IkB proteins. Upon signalling from external stimulus transduced through the Toll Like Receptors (TLRs), the inhibitors are degraded, and the NF-kB dimers are recruited into the nucleus where they bind to a consensus sequence 5'-GGGRNNYYCC-3' in the promoters of its target genes and initiate transcription (Chen and Ghosh, 1999). All the above functions are mediated by their N-terminal Rel Homology domain which is a characteristic feature of all the members of this family. *Drosophila* is an unusal case where NFKB is downstream of a morphogen gradient and forms a nucleo-cytoplasmic transcription factor gradient. The protein binds to a similar consensus sequence GGGWWWWCYS, GGGW4–5CCM (W = A or T, M = C or A) (Markstein et al., 2001). This developmental feature has not been seen in vertebrates.

2. Dorsal-Ventral Patterning in *Drosophila*

2.1 **Toll signalling pathway:**

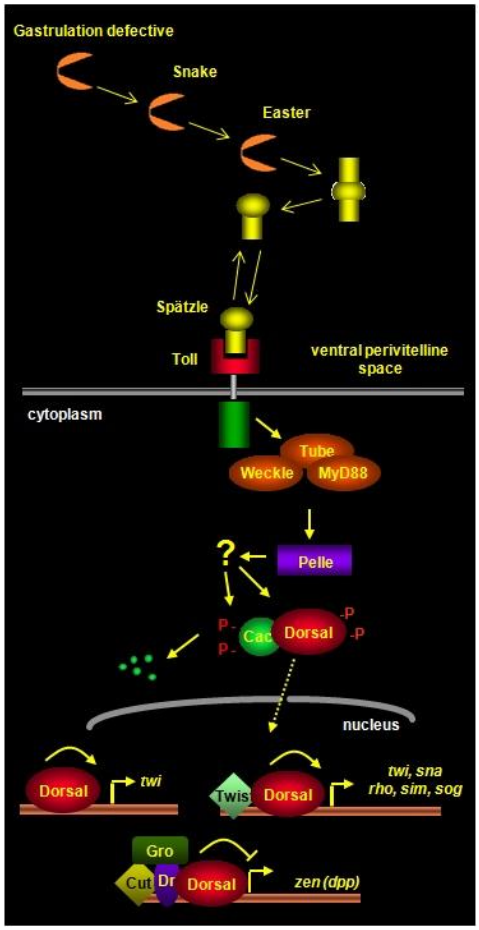


Figure1. *Illustration of the Toll-signalling pathway depicted the transduction of signal from extracellular protease cascade to Dorsal nuclear import. Dorsal acts both as an activator and repressor in early development. Genes activated include twist, snail and sog while genes repressed are dpp and zen.*

Asymmetric Toll signalling is the fundamental mechanism of dorsal-ventral axis determination in the early *Drosophila* embryo. A very important consequence of this signal transduction, is the nucleo-cytoplasmic gradient of Dorsal. Dorsal is a maternal transcription factor, that is transcribed uniformly throughout the embryo. It is sequestered in the cytoplasm by the IkB homolog Cactus (Lehming et al., 1995). The ventral follicle cells initiate a localized processing of the Spazle ligand leading to

differential Toll signalling. This signal is transduced via a series of other maternal factors which include Tube, Pelle, MyD88 and Weckle (Sun et al., 2004, Chen et al., 2006), leading to the degradation of Cactus. Cactus gets degraded in a gradient fashion across the dorsal-ventral axis (Belvin et al., 1995). This releases Dorsal, which is then recruited into the nucleus, forming a nuclear-cytoplasmic gradient inversely correlated to the Cactus gradient (Reeves and Stathopoulos., 2009).

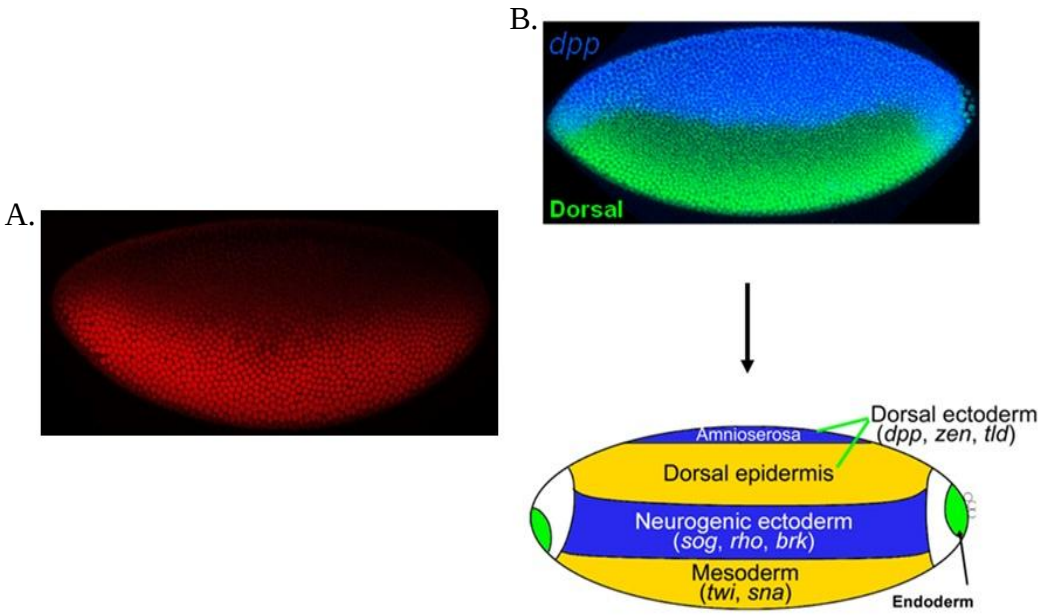


Figure 2: *A. Lateral view of a wildtype embryo of Drosophila melanogaster in stage 5, displaying the Dorsal gradient. Anterior end of the embryo is to the left. Embryos were fixed and stained with anti-Dorsal antibody. B. Lateral view of the embryo. Immunostaining image indicating opposing gradients of Dorsal and dpp. Both the gradients act in concert to pattern dorsal-ventral axis and determine the various germ layers. (Source: Girish Ratnaparkhi).*

Ectopic expression of Toll10b, a constitutively active mutant of the Toll receptor, fused with the 3'-UTR region of bicoid, in the background of a gd (*gastrulation defective*) mutant, replicated the expression bands of Dorsal target genes along the antero-posterior axis (Huang et al., 1997). Similar studies were done with an active form of Pelle Kinase (Stathopoulos and Levine, 2002). These studies reveal the linearity of signal transduction in the pathway, where Spazle activity gradient is converted to Toll activity gradient, interpreted further downstream

as a Pelle kinase activity gradient.

2.1. **a) NF-KB and host defense:**

The Toll pathway is akin to the NF-kB pathway in mammals, and also has an important role in mediating the defense responses of the fly (Sandhya et al., 2011). Unlike early development, when a single NF-KB (Dorsal) is expressed and is used to pattern the ventral and lateral sides of the embryo, two additional NF-KB's are expressed and utilized in signaling during the innate immune response. There are three homologues of the NF-kB family in *Drosophila melanogaster*, Dorsal (Dorsal), Dorsal related immune factor (Dif) and Relish (Rel). All the three proteins retain the characteristic Rel homology domain, and are in fact the primary transcriptional regulators in transducing alarm signals in response to a pathogenic attack (Reichhart et al., 1993, Ip et al., 1993, Dushay et al., 1996).

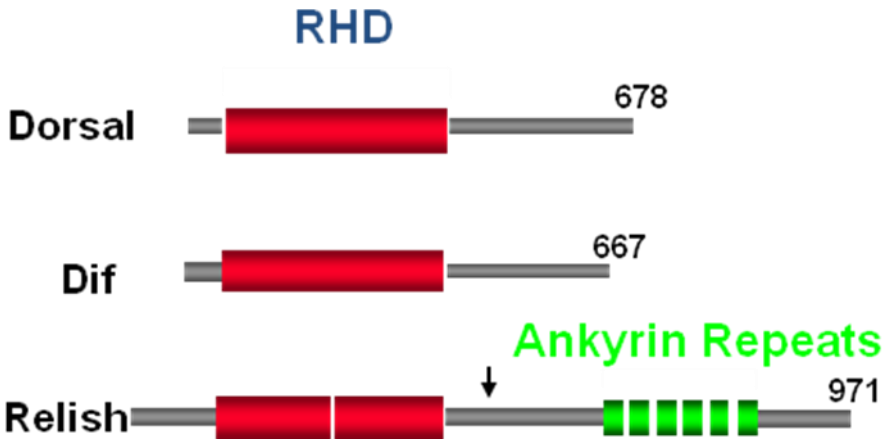


Figure 3: *Similarities of structure within the three NF-K B homologs of Drosophila, Dorsal (Dorsal), Dorsal like Immune Factor (Dif) and Relish (Rel). Dorsal and Dif are downstream of Toll signaling, while Relish is doenstream of the Immune deficient pathway. Dorsal and Dif are sequestered in the cytoplasm by Cactus. The Ankyrin C-terminal tail of Relish sequesters relish in the cytoplasm.*

Dif probably evolved from Dorsal because of a gene duplication event and is present in close proximity in the genome. Both Dif & Dorsal are expressed in larval and adult fat bodies and have redundant roles (Reichhart et al., 1993, Ip et al., 1993). Dorsal has been shown to be dispensable for host defense. Dorsal & Dif are

effectors of the Toll pathway (See Figure). Dorsal&Dif are usually found sequestered in the cytoplasm, bound to the mammalian IKB analog Cactus in the absence of infection. REL is an effector of the Immune deficient (IMD) signaling pathway. REL has a C-terminal Ankyrin chain which is homologous to the Cactus Ankyrin chain (Dushay et al., 1996). This chain sequesters REL in the cytoplasm.

In response to injury or infection, Toll and IMD receptors are activated, which thru a sequence of distinct intermediates lead to nuclear localization of Dorsal&Dif (Toll pathway) or REL (IMD pathway). The nuclear localization of these transcription factors is driven by the degradation of Cactus (for Toll) or the cleavage of the REL tail (for IMD) in response to injury/infection (Wu and Anderson., 1998). Once in the Nucleus, Dorsal, Dif and Rel bind as homo or heterodimers upstream of ~ 400 defense genes and activate transcription (Lemaitre and Hoffmann., 2007). The protein/RNA product of these target genes work to stave off the infection/ pathogen. Once the infection is under control, the Toll/IMD pathways get deactivated.

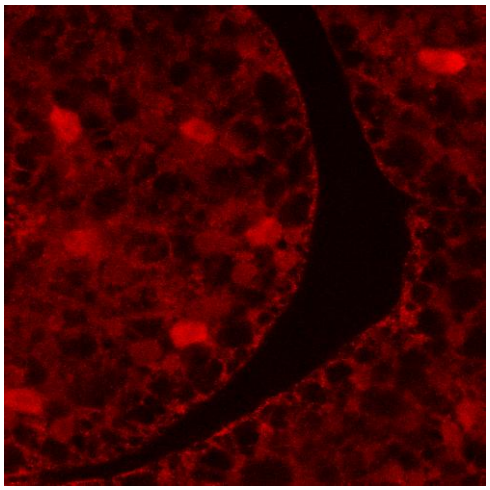


Figure 4: *Dorsal nuclear localization in the larval fat body in response to infection. (Source: Girish Ratnaparkhi).*

Dorsal, Dif and REL also have critical roles in rousing the cellular arm of the innate immune response. In response to injury/infection, these transcription factors are activated in the Lymph Gland, where they enhance proliferation of blood cells, which include plasmatocytes (Macrophages), Lamellocytes (to encapsulate parasites) and crystal cells (wound healing) (Qiu et al., 1998, Jung et al., 2005). Dif and Dorsal double mutants have more than 95% lethality, due to defects in blood-cell

morphology and number leading to death by microbial and fungal infection (Matova and Anderson, 2006). The activation of these pathways, in possibly a dose dependant manner in the lymph gland is not well understood.

2.2 **Dorsal**

a) **Functional domains:**

The highly conserved N-terminal Rel Homology domain (RHD) of Dorsal has functional domains that mediate homodimerization, interactions with Cactus, DNA binding and regulated nuclear localization (Steward R., 1987). The nuclear localization signal of Dorsal is a 6 aa stretch at the C-terminus of the RHD. However, this signal is insufficient to maintain the native nuclear-cytoplasmic gradient of the protein. Regulated nuclear import is brought about by the highly conserved N-terminal 40aa of the RHD. Nuclear import is adversely affected "even in the absence of Cactus" in the truncated constructs of Dorsal harbouring deletions of these 40 aa. Therefore this region has crucial information for regulated nuclear import which is independent of Cactus (Govind et al., 1996).

Dorsal interacts with several other transcription factors and functions both as an activator and a repressor depending upon the context of its binding sites (Jiang et al., 1992). The C-terminal domain (CTD) contains repeats of Glutamine (Q), Alanine (A) and Asparagine (N) which are implicated in transcriptional regulation. The first 150 aa of the C-terminal domain was shown to promote repression. Moreover, the last 18 aa show similarity to the eh1 motif, found in dedicated repressors like Engrailed (Flores-Saaib et al., 2001, Jimenez G et al., 1997). This is a signal sequence for the recruitment of the co-repressor Groucho. Also, deletion of the last 200 aa of the C-terminal domain causes a five fold reduction in transcriptional activation. This indicates two domains of activation within the protein, one in RHD and the other in CTD (refer to appendix for protein sequence).

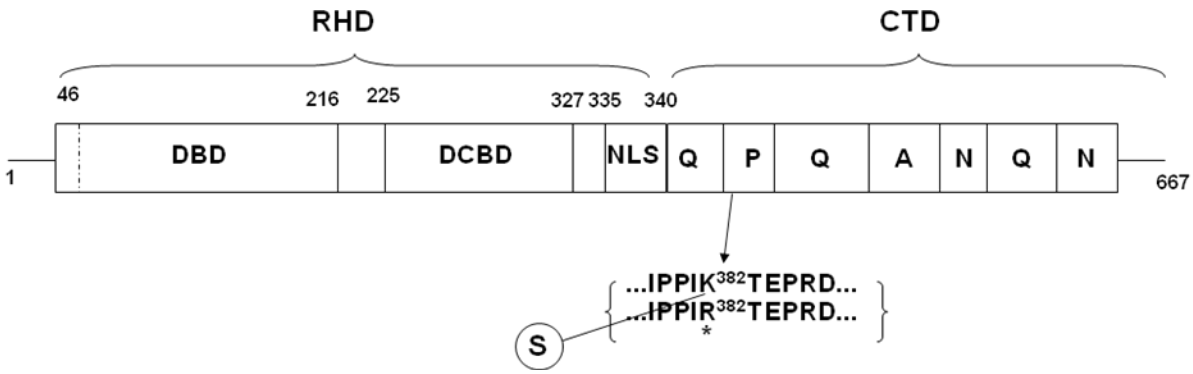


Figure 5: *Dorsal protein domain arrangement. DNA Binding Domain (DBD), Dimerization and Cactus binding domain (DCBD) and Nuclear Localization Signal (NLS) in the Rel Homology Domain (RHD) and the regions rich in Proline (P), Glutamine (Q), Arginine (N), in the C-terminal Domain (CTD). SUMOylation site which is at 382 position (lysine) is also shown in the figure.*

b) **SUMOylation:**

SUMOylation is a reversible post-translational modification that affects several aspects of protein function. SUMO is a small-ubiquitin like modifier, which upon attaching to a substrate protein, can affect its localization, stabilization, interactions with other proteins or confer other special functions to that protein. The first step in the SUMOylation pathway involves an activation enzyme (E1) which catalyzes the formaiton of a SUMO-adenylate conjugate.This is an intermediate in the formation of a thioester bond between the C-terminal carboxy group of SUMO and the Cys residue of E1. SUMO is then transferred to the E2 conjugating enzyme. This enzyme along with the E3 ligase, conjugates SUMO with the substrate protein with an isopeptide bond between the C-terminal Glycine of SUMO and an ε-amino group of a Lysine residue in the substrate protein (Friedlander and Melchior., 2007).

Drosophila orthologs of the SUMOylation conjugation machinery proteins are expressed in high levels throughout embryogenesis and in adult females (Proschel., 2006, Jasper., 2002). dmsmt3, the fly ortholog of verterbrate smt3, is maternally inherited and is known to play important roles in embryogenesis, morphogenesis and immune response (Talamillo et al., 2008). Recently, Dorsal also has been shown to

be a SUMOylation substrate. Smt3 conjugation has been shown to favour Dorsal nuclear localization and enhance Dorsal mediated reporter activity in S2 cells (Bhaskar et al., 2000).

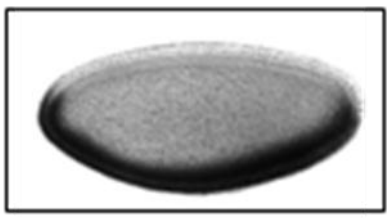
c) **Transcriptional regulation by Dorsal:**

Dorsal activates its downstream targets in a concentration dependent manner. There are three important factors that together govern the expression of Dorsal target genes in the early embryo. a) The nucleo-cytoplasmic Dorsal concentration gradient b) the affinity of Dorsal binding sites in the enhancer regions of the downstream targets c) the interaction of Dorsal with other transcription factors. These features are elaborated below, using the transcriptional regulation of *twist*, *snail* and *rhomboid* as examples.

Regulation of Twist expression:

Twist is a bHLH domain containing protein that recognizes E box binding sequences. It acts in synergy with Dorsal to determine gene expression in the mesoderm and ventral neuroectoderm. The Twist promoter has four Dorsal binding sites that have a very low-binding affinity for Dorsal, thus restricting its expression to the ventral most regions of the embryo, where nuclear concentrations of Dorsal are the highest. Twist expression extends in a graded fashion upto ~10 cells on either side of the ventral midline by nuclear cycle 12. This includes the ventral most cells of the neuroectoderm (Jiang et al., 1992).

A.



B.

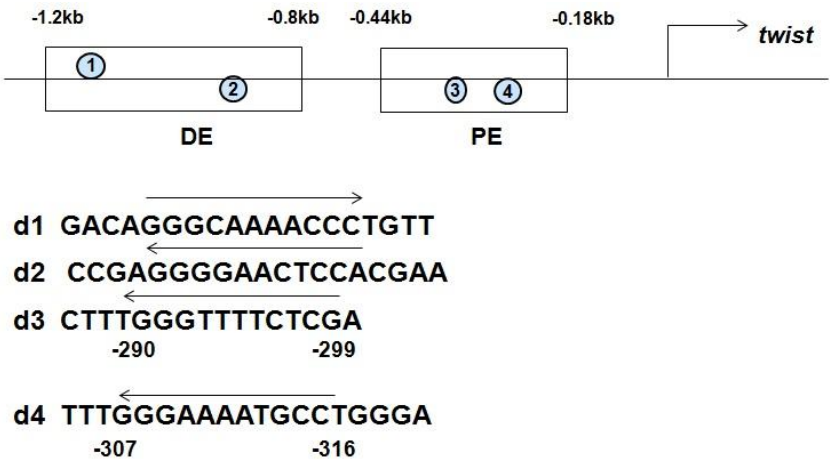


Figure 6: *A. Early embryo in pre-cellularization showing twist expression in the ventral mesoderm. B. Illustration showing the distribution of Dorsal binding sites in the twist promoter. Modified from Jiang et al., 1992*

The promoter has two stretches, Distal and Proximal, which contain the Dorsal binding sites. As shown in the figure, TD2 and TD3 Dorsal binding sites are in the distal enhancer (-1.2kb to -0.8kb), while TD4&5 are in the proximal enhancer (-0.44kb to -0.18kb). Another study by Pan et al., recognized DNA elements in the twi promoter region bound by several other nuclear factors including zeste, a transcription factor that mediates transvection. This also shows that Dorsal acts in synergy with several other nuclear factors to drive target gene expression.

Interestingly, Twist expression is compromised in *twi*$^{-}$ mutants though the promoter region lacks E-box binding sequences. This could possibly be mediated through the recruitment of Twist to the promoter, by protein-protein interactions with bound Dorsal (Shirokawa and Courey, 1997).

Regulation of Snail Expression:

Snail is a zinc finger encoding target of Dorsal transcription factor that acts as a repressor and restricts all the neuroectodermal determining gene expression to the lateral limits. This keeps the presumptive mesoderm from taking an alternative developmental fate. and demarcates the boundary between mesoderm and neuroectoderm. Unlike Twist expression which is graded, Snail shows uniform

expression pattern throughout the ventral mesoderm. The expression territory includes 14-18 cells on both the sides of the ventral midline and extends into the poles (Ip et al., 1991, Kosman et al., 1991).

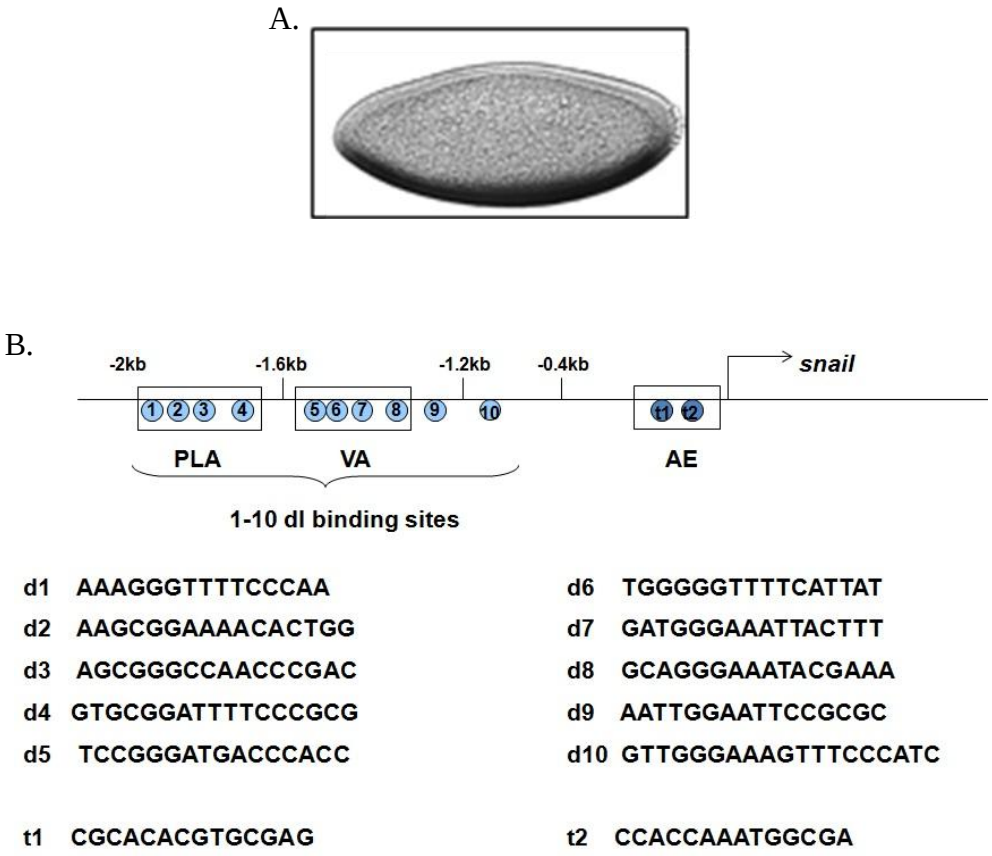


Figure 7. *A. Early Drosophila embryo in pre-cellularization stage showing snail expression in the ventral mesoderm. The pattern is much sharper than twist. B. Organization of the Dorsal and Twist binding sites in the snail promoter The regulatory region harbours 10 Dorsal binding sites and 2 Twist binding sites. Truncation studies reveal that the Polar Lateral Activator (PLA: -2kb to -1.6kb) is crucial for expression at the poles, while the ventral activator (VA: (-1.6kb to -1.2kb) mediates snail expresion in the ventral regions. (Modified from Ip et al., 1992a).*

Truncation studies done with *snail* promoter – lacZ fusions revealed 10 Dorsal binding sites and 2 Twist binding sites within 2 kb upstream of the transcriptional start site. Dorsal-Twist interaction is required for the uniform expression shown by *snail*. Mutations in the Twist binding sites do not render the sharp boundaries that are characteristic of Snail. Though mutations in Dorsal or Twist binding sites have a very

small effect on snail expression, mutations in both the sites together, show a drastic decrease in gene expression.

Regulation of rhomboid gene expression:

Rhomboid codes for a transmembrane receptor and is crucial for the differentiation of ventral epidermis. Transcripts are detected by the onset of nuclear cycle 13. Its ventral limits are defined by Snail repression and therefore expression is restricted to neural ectoderm (Ip et al., 1992b).

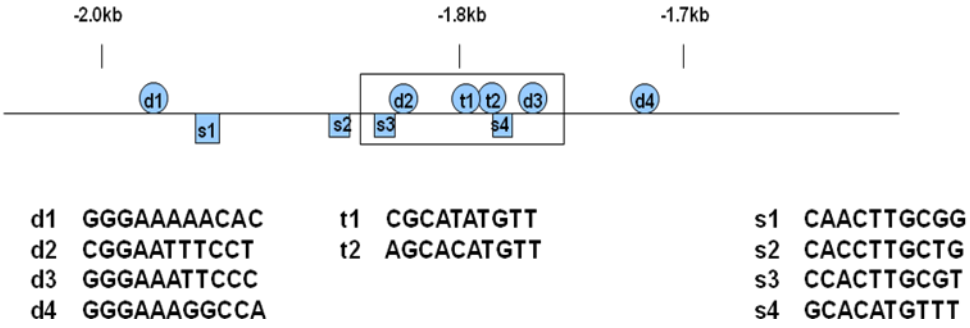


Figure 8: *Organization of various transcription factor binding sites (Dorsal, Twist and Snail) in Neuroectodermal Element (NEE) of the rhomboid promoter. Inset shows the overlapping binding sites of twist and snail. (Modified from Ip et al., 1992b).*

The regulatory region contains two clusters of binding sites. The cluster between 2.2kb and 1.6kb (Neuroectoderm element (NEE)) contains four high-affinity Dorsal binding sites alongside two Twist binding sites. The two transcription factors act in synergy to activate early rhomboid expression in the ventral neuroectoderm. The Snail binding sites that show a partial overlap with those of Twist indicating a competitive interaction between the repressor and enhancer in regulating gene expression.

Synthetic promoters:

Synthetic promoter constructs were also used with different arrangements of Dorsal and Twist binding sites to understand Dorsal mediated transcriptional regulation. Szymanski and Levine (1995) showed that a 57bp minimal enhancer

showed transcriptional activation of a downstream reporter throughout the presumptive mesoderm and ventral neuroectoderm. They then tested reporter gene expression fused with multimerizations of a synthetic oligonucleotide containing high affinity Dorsal and twist binding sites and obtained activation throughout the presumptive mesoderm. Moreover, they also showed that the organization of the dl and twi binding sequences, does not affect transcriptional activation.

Synthetic constructs were later fused with Chloramphenicol Acetyl Transferase (CAT) reporter vectors and used by Shirokawa and Courey (1997) to dissect the synergistic interactions between Dorsal and Twist. The constructs were multimerizations of 5' – CCGAGAG***CATATG***TTTTGG***GGGATTTTCCC***AAC – 3' sequence element. The spacing and orientation of the Dorsal and Twist binding sites in these constructs mimic the arrangement in the native rhomboid promoter. The constructs we use in our study are modifications of these oligonucleotides.

Main features of Dorsal transcriptional regulation:

Examining the regulatory regions of these three targets of Dorsal reveal a few important principles that determine gene expression patterns regulated by this morphogen.

- All the three enhancers contain defined regions with combinations of transcription factor binding sites indicating the importance of interactions between the different transcription factors defining expression limits. The Ventral Activator Region of *twist*, the Polar and lateral activator region in s*nail* enhancer and the Neuroectoderm Element region of *rhomboid* gene exemplify this.
- The binding site arrangement could either mediate synergy as in the Dorsal-Twist interactions, or promote competition, as seen in the overlap of the Snail and Twist binding sites, or simply show redundancy as shown by the function of multiple Dorsal binding sites.
- Activation and repression of Dorsal is dependent on the context of the binding sites. Just as the competitive binding of Snail to *rho* promoter is crucial for its repression in ventral regions, the Ventral Repressor Element (VRE) in *zen* promotes repression by Dorsal throughout the ventral mesoderm and neuroectoderm where

other targets like *twi*, *sna* and *rho* are activated (Jiang et al., 1991).

- Moreover, previous studies done using fusions of modified forms of the *twi* PE element with reporter constructs revealed that the level of target gene expression is governed by number of Dorsal binding sites while the limit is affected by affinity of the binding sites (Jiang and Levine, 1993). Also, the binding sites of Dorsal within the *zen* regulatory region show a five fold higher activation potential than those within the *twi* PE (Jiang et al., 1991). This leads to Dorsal induced repression of *zen* in the lateral regions where twist cannot be activated.

The enhancer regions of its downstream targets thus respond differently to different thresholds of the dorsal nuclear concentration and express in distinctive bands across the dorsal-ventral axis of the embryo. This differential expression eventually leads to the specification of the presumptive mesoderm, neuroectoderm and the dorsal ectoderm. Twist and Snail as given above are expressed in the presumptive mesoderm, rhomboid is expressed in the ventral limits of the lateral neuroectoderm and sog is expressed in the junction between the neuroectoderm and dorsal ectoderm with lowest levels of nuclear Dorsal (Reeves and Stathopoulos., 2009). This is a classic example wherein cells respond to morphogen gradients during early development and hence acquire different distinctive identities depending on their spatial position leading to axial patterning.

Concept of the project

Dorsal Gradient:

Dorsal nuclear concentration gradient is thus the determining factor of dorsal-ventral patterning. This gradient is formed for barely thirty minutes and in this time it sets the D/V axis for the rest of the animals life. Therefore, it becomes important to understand its spatial and temporal dynaimcs.

Tools developed to monitor Dorsal mediated activation in the past

Several direct and indirect approaches have been taken in order to obtain a read-out of this gradient during the early developmental stages. Liberman et al (2009) used antibody staining and confocal imaging on fixed embryos to detect and quantify the nuclear dorsal concentration spectrum.The primary tool used to measure Dorsal activity, live, in the animal is the use of Dorsal protein fusions with fluorescent proteins (FP). The Dorsal-FP fusion can be viewed by fluorescence microscopes in the developing embryo, and its concentration measured over time. Kanodia et al (2009) performed live-cell imaging on embryos expressing dorsal tagged with GFP to get a temporal read-out.

Problems associated with Dorsal-FP measurements

Fixed embryos do not give an accurate temporal dynamics of the Dorsal gradient. Although the Dorsal-FP experiments have generated excellent data for Dorsal concentration gradients in the early embryo and have given us insight into D/V patterning, the tagging of the FP to Dorsal may change the partitioning of Dorsal between the Nucleus and Cytoplasm and may thus affect the slope of the Dorsal D/V nucleocytoplasmic gradient. Dorsal-FP constructs do not, when expressed in a dl null animal rescue the animal beyond embryonic development. These animals usually do not hatch into larvae, indicating that the Dorsal-FP does not mimic Dorsal completely.

Our Idea

Generate a reporter construct that reads the Dorsal concentration gradient, WITHOUT tagging Dorsal with a FP. This could be done by creating fluorescent reporters that read out the concentration of the Dorsal gradient without perturbing or tagging Dorsal protein.

The plan

- Generate artificial Dorsal binding sites that respond to different concentrations of Dorsal& Dif. Generate Fusions of these aritificial promoters with a variety of FP's (e.g eGFP, mCherry, Venus).
- Use natural promoters that respond to different threshold concentrations of Dorsal & DIf. Generate Fusions of these aritificial promoters with variety of FP's(e.g eGFP, mCherry, Venus).
- Combine 3 different promoter-FP fusions in a single transgenic animal. Different FP's would respond to different Dorsal/Dif concentrations, creating an overlap of colors, which would be a quantitative representation of the Dorsal concentration threshold/ gradient.
- Demonstrate the utility of the Tri-colored transgenic fly by testing the system in a genetic background that perturbs the Dorsal nuclear concentration gradient.

Materials and Methods

Cloning:

The twi-tk-luc, rho-tk-luc and sna-tk-luc constructs were obtained from Y Tony Ip. The inserts were subcloned into the clontech vectors pVenus, pmCherry and pCerulean respectively using XmaI (for twi) and KpnI (for rho).

Restriction Digestion Mixture:

NEB Buffer :	5 ul
BSA:	0.5ul
Enzyme:	10units
Vector DNA:	2 ug
Distilled H_2O:	to make up reaction volume to 50ul.

The reaction mixture was incubated at 37^{o}C for 3-5 hrs followed by heat inactivation for 15 min. at 65oC. The digested product was run on a 0.8% agarose gel alongside GeneI Supermix DNA ladder, DNA bands of relavant size were sliced out and gel purified using Qiagen Gel Purification Kit.

The insert was then ligated into the vector using the following ligation mix:

Promega T4 Ligation Buffer (10X):	1ul
Promega T4 DNA ligase:	1-3units
Vector:	50-100ng
Insert:	calculated according to the furmula given below
Distilled H_2O:	to make up the volume to 10ul

Amt of Insert (ng) = [amt of vector (ng) / length of the vector (bp)] x length of insert x [ratio of insert:vector]

An insert:vector ratio of 3:1 was used for all the ligations.

The ligation mixture was incubated at 16^{o}C o/n and transformed into Stbl2 strain of E.coli. The colonies were innoculated in Lysogeny Broth and plasmid preparations were done using the standard alkaline lysis method or Qiagen plasmid mini-prep kit. Positive clones were confirmed by digestions with relevant restriction enzymes.

Transgenics:

Plasmid midi-preps of the native promoter-FP constructs of twi-Venus and sna-Cerulean were done using the Qiagen midi-prep kit. A Dorsal sumo mutant encoding construct (K382R) in pUASP vector was similarly midi-preped and sequenced. The constructs were quantified for DNA concentration and sent to Trangenic Fly Facility, National Centre for Biological Sciences, Bangalore.

Maintainence of flies:

Fly stocks were maintained at 25^{o}C on Corn Meal Agar media. The second generation crosses were set up in cages and maintained at 25^{o}C for embryo collection.

Immunostaining protocol:

Embryos were collected 3hrs after egg laying, washed and dechorionated in 100% bleach. They were fixed in a 1:1 ratio of heptane and 4% formaldehyde. The embryos were then de-vitellinized in 1:1 ratio of heptane and chilled methanol and transferred to an eppendorf tube. They were washed thrice with PBST and then blocked for an hour in 2% BSA solution for an hour. The embryos were then incubated overnight in anti-dorsal Rabbit antibody at 4^{o}C.

The embryos were given PBST washes and incubated for an hour in Alexa Fluor 633 / 488 Goat Anti-Rabbit IgG. They were then washed again and stained with DAPI followed by another wash in PBST. They were then mounted onto a slide in Slow fade Gold (Invitrogen).

Microscopy:

Details of Confocal Microscopy:

System: Zeiss LSM 710

Objective lens: W Plan-Apochromat 40X/1.3M DIC

Laser: HeNe 633nm, Argon laser 488nm.

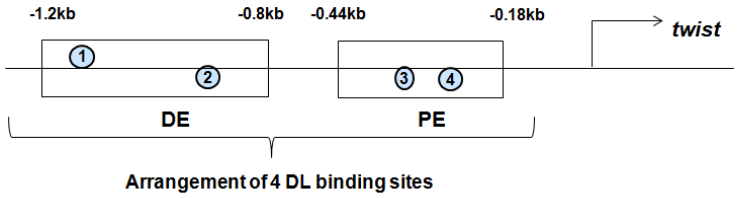
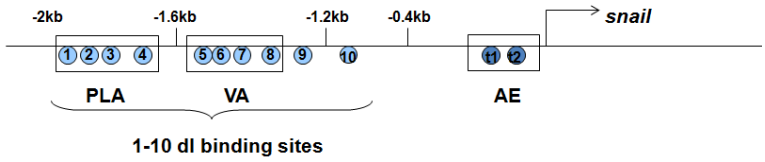
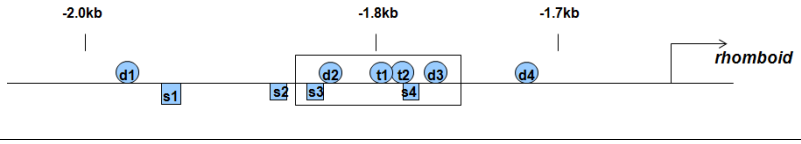
Image processing software used: Image J

Results

Choice and Design of promoter constructs:

The native promoters of *twist, snail* and *rhomboid* were chosen for the native promoter-reporter fusion constructs. A *twist* promoter spaning ~2kb, *snail* promoter of ~2.8kb and *rhomboid* gene promoter of ~2.2kb of the 5' flanking region upstream of the transcription start site were found to reproduce the wildtype expression profile of the three proteins in the early *Drosophila* embryo (Jiang et al., 1991, Ip et al., 1992a, and Ip et al., 1992b). Clones of these promoters were procured from Y Tony Ip. Additional information of the organization of these promoters is available in the introduction and Materials and Methods of this thesis.

Table 3: *List of native promoters used*

Dorsal Target	Length of the insert	Illustration
twist	~2kb	
snail	~2.8kb	
rhomboid	~2.2kb	

The synthetic promoters designed were a modification of the oligonucleotides used by Shirokawa and Courey (1997) in their study of the synergistic interactions between Dorsal and Twist mediated transcriptional activation. The oligonucleotides were designed taking into consideration the previous studies that use various minimal promoters and other synthetic constructs that could reproduce expression patterns generated by the wildtype enhancer regions of Dorsal targets (Jiang and

Levine, 1993., Szymanski and Levine, 1995).

Table 4: *List of oligonucleotides designed to generate Artificial promoters*

2X *dorsal* (D2)	C***GGGATATCCG***CAGTATT***GGGATTCTCCC***G CATGG***CCCTATAGGC***GTCATAA***CCCTAAGAGGG***CCTAG
5X dorsal (D5)	AAAGGTACC***GGGATATCCGC***AGTATT ***GGGATTTTCCC***AGTATT***GGGATATCCG***CAG CCATGG***CCCTATAGGCG***TCATAA ***CCCTAAAAGGG***TCATAA***CCCTATAGGC***GTC TCTT***GGGATTTTCCC***AGTGCAGTATT ***GGGATATCCG***CGGATCC AGAA***CCCTAAAAGGG***TCACGTCATAA ***CCCTATAGGC***GCCTAGGAAA
2X *dorsal* + 2X *twist* (DE2)	C***CACATGT***TTTGG***GGGATATCCG***CAACGAG***CACATGT***TTT GG***GGGA TTTT CCC***G CATGG***GTGTACA***AAACC***CCCTATAGGC***GTTGCTC***GTGTACA***AAA CC***CCCT AAAAGGG***CCTAG
5X *dorsal* + 5X *twist* (DE5)	AAAGGTACCGAG***CACATGT***TTTGG***GGGATATCCG***AACGAG***CACATGT***TTTGG***GGGATTTTCCC***AACGAG***CACATGT***TTTGG CCATGGCTC***GTGTACA***AAACC***CCCTATAGGC***TTGCTC***GTGTACA***AAACC***CCCTAAAAGGG***TTGCTC***GTGTCA*** AAACC ***GGGATATCCG***CAACGAG***CACATGT***TTTGG***GGGATTTTCCC***AACGAG***CACATGT***TTTGG***GGGATATCCG***CGGATCC ***CCCTATAGGC***GTTGCTC***GTGTACA***AAACC***CCCTAAAAGGG***TTGCTC***GTGTACA***AAACC***CCCTATAGGC***GCCTAGGAAA

D2 and DE2 were designed to incorporate KpnI and BamHI sticky ends so as to facilitate direct cloning into the appropriate vector systems. The D5 oligonucleotide contains A overhangs for feasible cloning into the pGEMT easy vector. DE5 sequence cloned into pGL3 – basic vector was obtained from Promega.

Cloning

The native promoters of twi (~2kb) from the twi-tk luc vector, sna (~2.8kb) from the sna-tk-luc vector, and rho (~2.2kb) from the rho-tk-luc vector were subcloned into pVenus, pCerulean and pmCherry vectors, and the positive clones were confirmed with restriction digestion using XmaI (twi-Venus) and KpnI (sna-Cerulean and rho-mCherry) restriction enzymes.

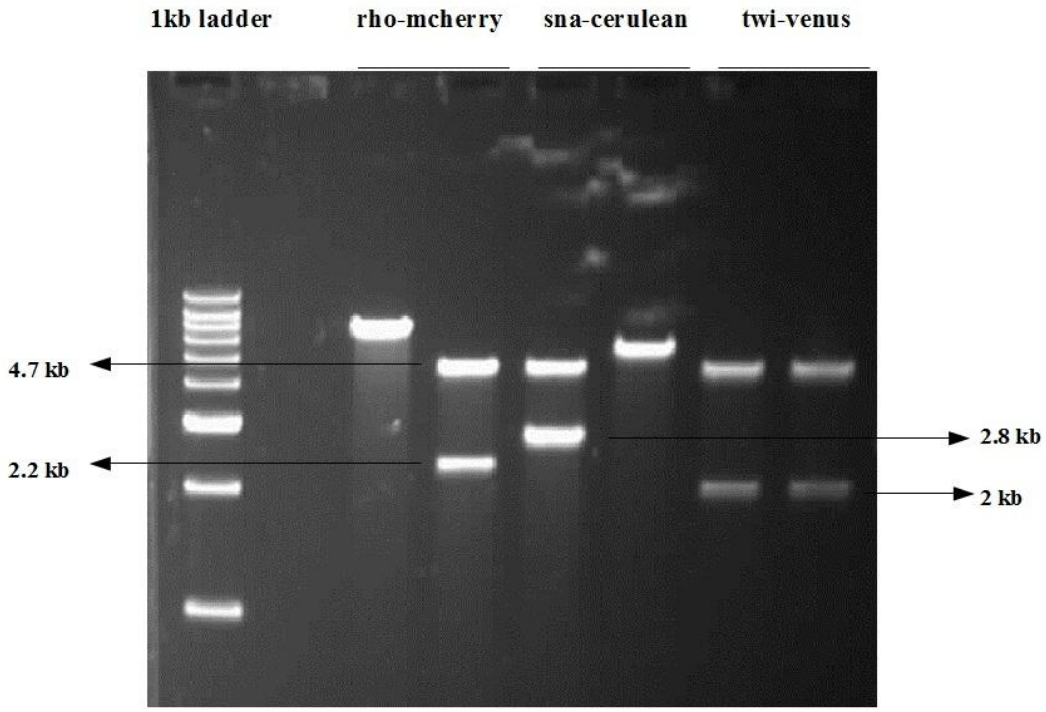


Figure 9: *Restriction digestion to confirm ligation of native promoter inserts into Clontech vectors.*

sna-Cerulean and rho-mCherry were then subcloned from the clontech vectors into the pCasper4 vector. The positive clones were confirmed by restriction digestions with XhoI-XbaI (twi-Venus) and KpnI-NotI (sna-Cerulean).

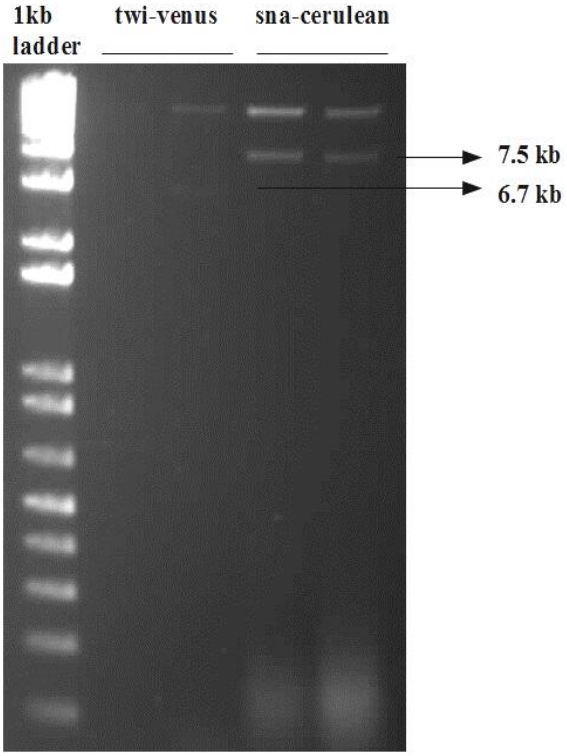


Figure 10: *Restriction digestions to check the ligation of promoter-reporter fusions into pCasper4 Pelement transformation vector.*

Transgenics

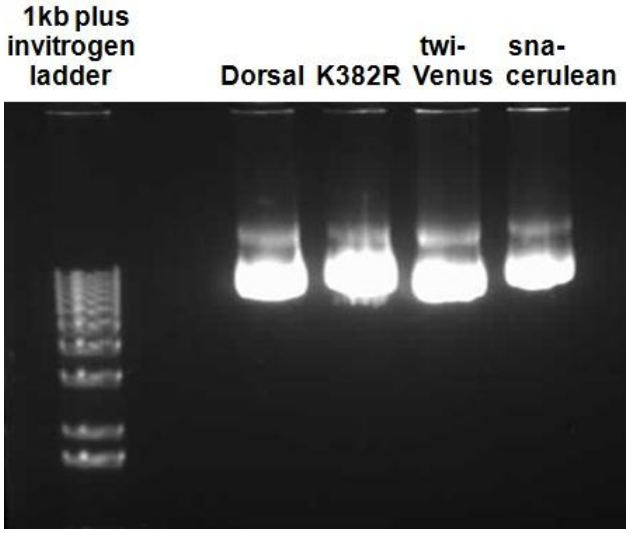
DNA of high Quality was generated for all the constructs by using the Qiagen Midi-preparation kit. Table 5 gives the concentration of DNA sent while Figure 11 shows the quality of DNA. The DNA sent to NCBS was re-sequenced before sending as a confirmatory mechanism. At the NCBS transgenic facility, the DNA was injected into w118 *Drosophila melanogaster* embryos for the generation of transgenics. Refer to appendix for sequence and vector maps of the constructs.

Once the transgenic lines were sent by NCBS, we identified the chromosome where each P-element was inserted for each line and stabilized each line using balancer chromosomes for that line.

TABLE 5: *Details of constructs sent to NCBS for generating transgenics*

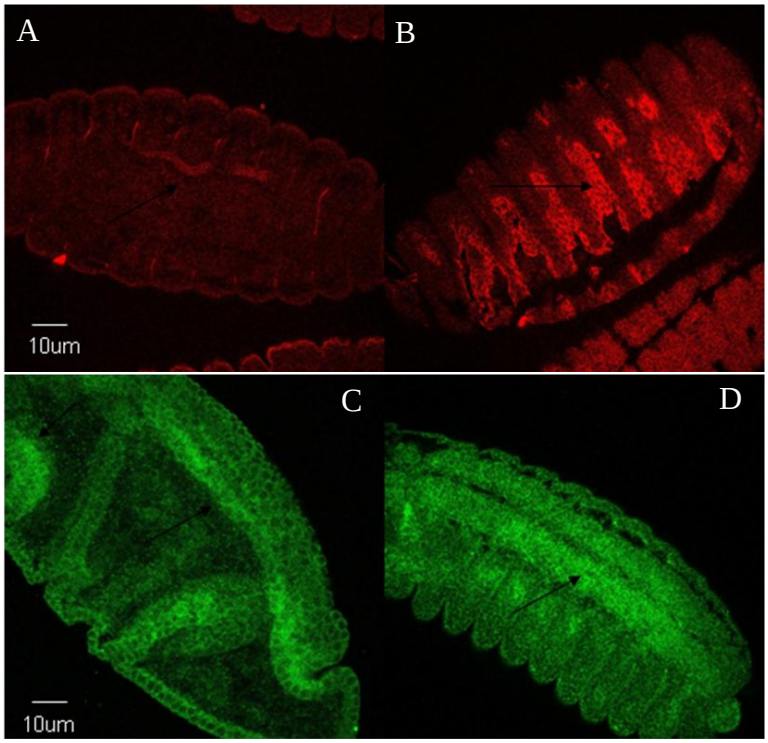
Construct	DNA Concentration	Stable lines generated
Sna-cerulean	0.97 ug/ul	3 Lines on Chromosome II and 2 Lines on Chromosome III
Twi-venus	1.34 ug/ul	2 Lines on Chromosome II and 3 Lines on Chromosome III
pUASp-*Dorsal*	1.00 ug/ul	1 Lines on Chromosome II and 3 Lines on Chromosome III
pUASp-*Dorsal (K382R)*	1.00 ug/ul	3 Lines on Chromosome II and 3 Lines on Chromosome III

Figure 11: *1ul of the plasmid midi-preps were run on a 0.8% agarose gel alongside a 1kb plus invitrogen ladder and resequenced before sending it for transgenics.*



The early embryos of transgenic lines were immunostained with anti-GFP anti-body to check for the expression of the reporter constructs during early development. However, expression is seen only during the later stages of development, around stage 7 - 12 (refer to figure).

Figure 12: *twi-venus expression seen in the tracheal system (A)and the muscle fibres (B) and sna-cerulean expression seen in (C) cephalic furrow and (D)neural tube in stage 7 – 12 embryos*

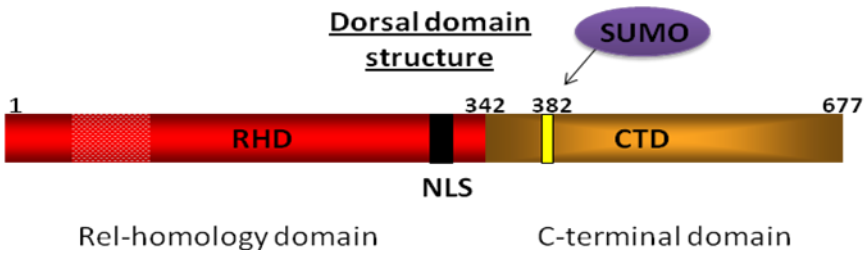


The twi-venus construct shows expression in the muscle fibres and the developing tracheal system (Thisse et al., 1998). sna-construct shows expression in the cephalic furrow and the central and the peripheral nervous system (Ip et al., 1994). However the delay could be a consequence of the folding kinetics of the fluorescent protein.

SUMOylation of Dorsal

In this part of our study, we would like to study the implications on localization of the Dorsal protein in the absence of SUMOylation. Dorsal gets sumoylated at the 382nd amino acid, Lysine. We use a mutant contruct of Dorsal, in which this lysine is substituted with arginine. This is a part of the C-terminal domain of Dorsal which is important for its repressor activities. It has been previously suggested that SUMOylation of Dorsal blocks its repressor activities, thereby enhancing its function as an activator.

A.



B.

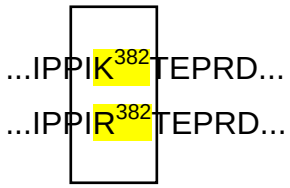


Figure 13: A. *An illustration of the Dorsal protein structure showing Rel Homology Domain and C-terminal Domain. B. Also shown is the SUMOylation site lysine (K)382 which is mutated to code for an asparagine (R) in the project.*

We use the UAS-Gal4 system to overexpress Dorsal wild type and the *SUMO* mutant (Dorsal K382R). The Gal4 is under the regulation of a Nanos promoter, which is a maternally expressed promoter. This would lead to maternal over-expression and deposition of the *dorsal* mRNA in the egg. Staining the embryo with an anti-Dorsal antibody would aid in the visualization of Dorsal gradient.

Fly Crosses:

Set #1: In this set of crosses Dorsal or Dorsal(K382R) was expressed maternally in a Dorsal-null background

$$+;\frac{dl'}{Cyo};\frac{13.4\,Mat\,Gal4}{TM3Ser}$$ Line 1

$$\frac{ywK382R}{ywK382R};\frac{J4(dif,dl)}{Cyo};+$$ Line 2

$$+;\frac{dl',UASpdl}{Cyo};+$$ Line 3

Cross A: Line 1 females were crossed with Line 3 males.

$$+;\frac{dl'}{Cyo};\frac{13.4\,Mat\,Gal4}{TM3Ser}\ \text{x}\ +;\frac{dl',UASpdl}{Cyo};+$$

Cross B: Line 1 females were crossed with Line 2 males. An egg lay

$$+;\frac{dl'}{Cyo};\frac{13.4\,Mat\,Gal4}{TM3Ser}\ \text{x}\ \frac{ywK382R}{ywK382R};\frac{J4(dif,dl)}{Cyo};+$$

An egg lay of F1 generation females expressing Dorsal (dl^{l}/dl^{l} females from Cross A) and Dorsal sumo mutant (K382R) (dl^{l}/J4 females from Cross B) was done.

Set #2:

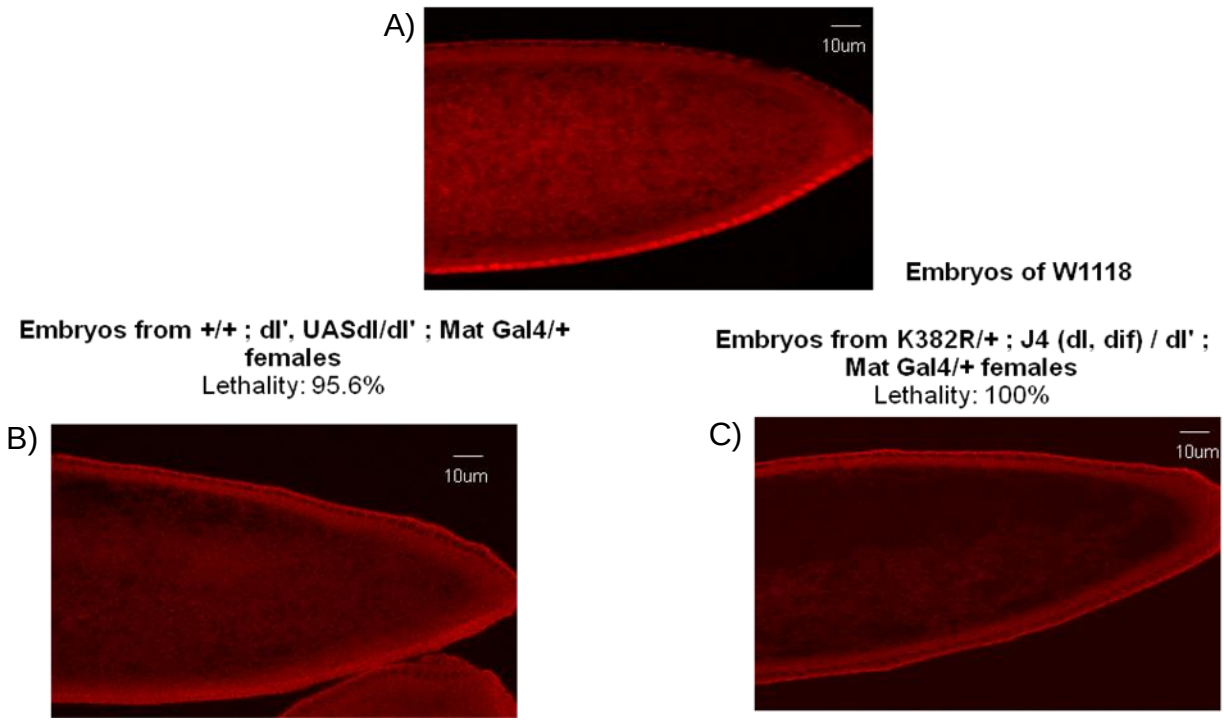
The following crosses were set up to follow the fate of over-expressed Dorsal sumo mutant during early development (Cross C and Cross D). In these crosses, wild type Dorsal was already present in the embryos.

Cross C $+;+;\frac{Nanos\ Gal4}{Nanos\ Gal4}$ x $+;\frac{dl',UASpdl}{Cyo};+$

Cross D $+;+;\frac{Nanos\ Gal4}{Nanos\ Gal4}$ x $\frac{yw,K382R}{yw,K382R};\frac{J4(dif,dl)}{Cyo};+$

As above for Crosses A and B, egg lay was done for F1 generation females over-expressing Dorsal (**+; dl', UASdl / + ; Nanos gal4 / +** females from Cross C**)** and the Dorsal sumo mutant (**K382R / + ; J4 (Dif, Dorsal) / + ; Nanos Gal4 / +** females from cross D) These eggs were fixed and stained with anti-Dorsal, to image for the localization of the Dorsal protein.

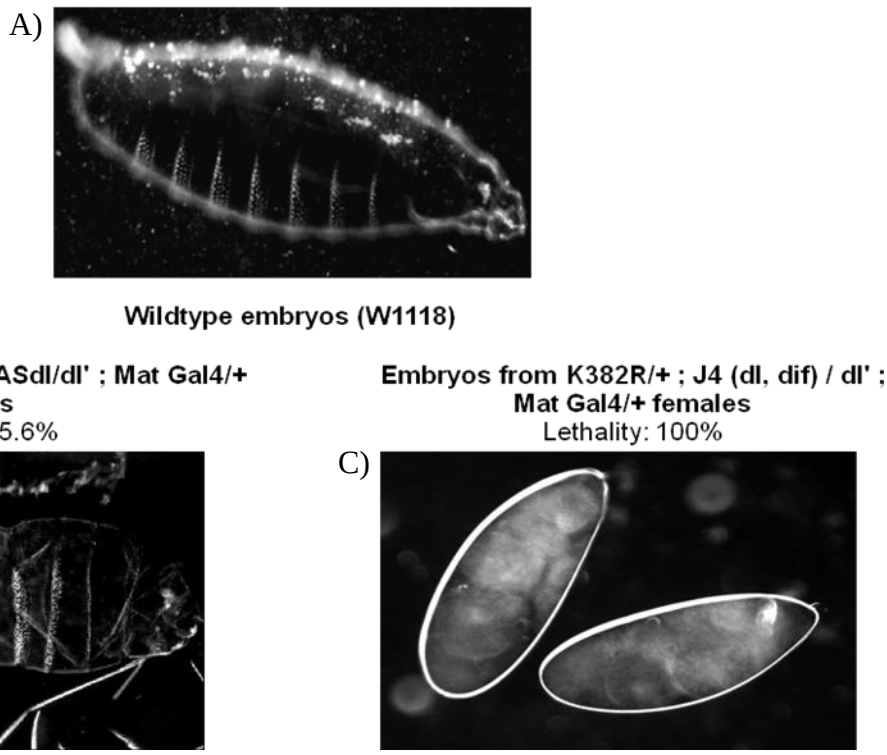
Figure 14: *Immunostaining of 3hr embryo collection with anti-Dorsal anti-body. A) wildtype embryos, B) embryos of females overexpressing dorsal in a Dorsal null background. C) embryos of females overexpressing Dorsal sumo mutant in a Dorsal null background.*



Preliminary data shows that the Dorsal sumo mutant shows reduced or no

localization in the nucleus, as compared to the wild-type or the Dorsal rescue. This was followed by cuticle preparations of 20hr old embryos.

Figure 15: A*) Cuticle preparations of wildtype embryos B) embryos of females overexpressing Dorsal and C) embryos of females overexpressing Dorsal sumo mutant in a Dorsal null background.*



Females overexpressing Dorsal give embryos with denticle patterns similar to wildtype patterns while the embryos of those overexpressing Dorsal sumo mutant do not hatch into larvae. Cuticle preparations along with lethality tests show that overexpression of the sumo mutant does not rescue the Dorsal null phenotype completely and have developmental defects. However, the experiments need to be reproduced and these preliminary results need to validated.

Summary

In the above project, our goal was to develop fluoresent reporters for monitoring concentration dependent NF-KB activity, live, in *Drosophila* development. A major focus was to visualize the Dorsal nucleoplasmic gradient in multiple overlapping colours, based on the concentration of nuclear Dorsal, across the D/V axis. A parallel experiment was to test our reagents by visualizing changes in the activity in Toll10b which shows dramatic changes in the Dorsal gradient and also test possible changes in the Dorsal gradient in flies expressing Dorsal (K382R), a Dorsal variant that cannot be SUMOylated.

We were able to plan, design and generate most of the reporter vectors with the promoter-FP fusions. For the native promoters *snail* and *twist,* we have achieved our primary goals of generating stable fly lines that should be sensitive to Dorsal activity in the cell. Transgenic flies that could express Dorsal and Dorsal(K382R) maternally have also been generated.

Due to the time component involved in cloning, generating transgenics and setting up maternal expression in a Dorsal null background, many of the crosses that help us validate the reporter - constructs are still underway at the time of writing this thesis. The experiments will be continued and results will be presented during the thesis defense, which is 40 days hence.

Work in Progress

Due to the long time component in generating transgenics and setting up crosses in Dorsal null background, the following experiments are in progress and are expected to be completed in May/June 2012.

- Characterize expression of sna-Cerulean, twi-Venus and rho-mCherry
- Measure the DorsalL gradient in Dorsal(K382R) mutants and compare to wild type.
- Clone all artificial promoters in pStinger vectors. Send constructs to NCBS to generate transgenics.

Future Work

The work presented in this thesis is a positive step forward in trying to develop reagents to monitor Dorsal activation, live, to indirectly measure Dorsal concentration in the nucleus and visualize concentration gradients. Once the artificial promoters fusion/ transgenic flies are generated, we will characterize the expression patterns of the native and aritificial promoters during embryonic and larval development. Based on their expression pattern and sensitivity to Dorsal/Dif levels, different inserts will be balanced in a single fly which expresses at least three colors and responds to 3 different thresholds of Dorsal activity.

If the above goal is successful, we would have generated a very useful reagent to monitor NF-K B concentration in the nucleus, and this could be monitored live at all stages of *Drosophila* development.

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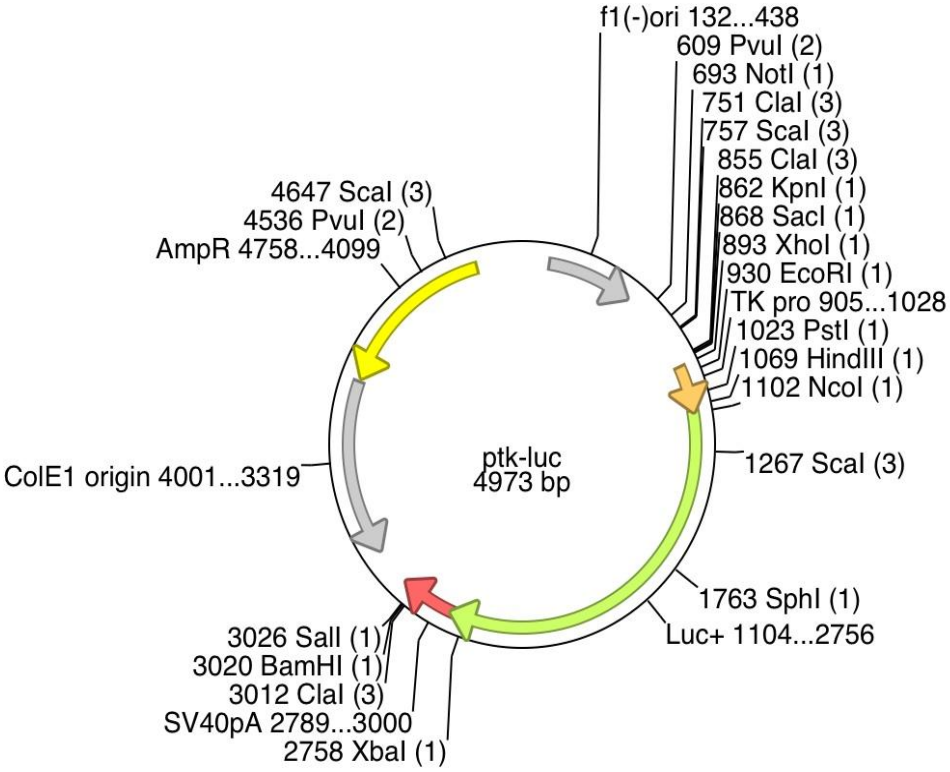
Appendix

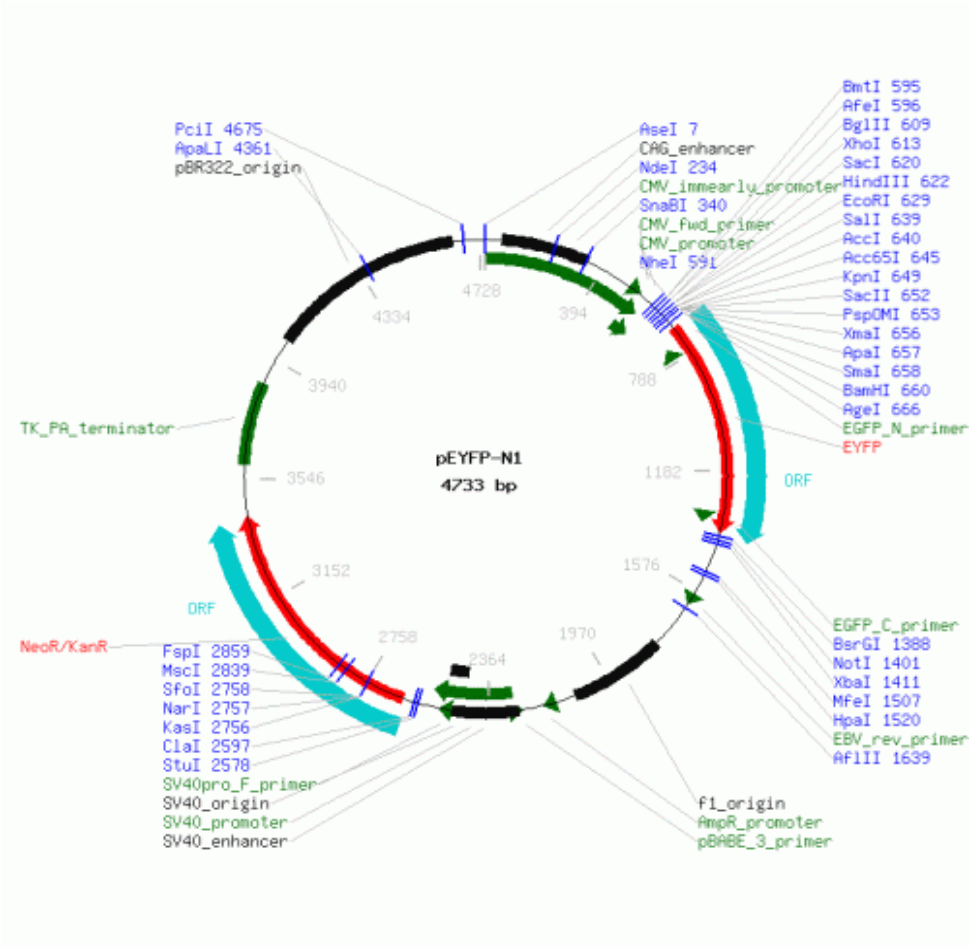
Vector Maps:

1. ptk-luc



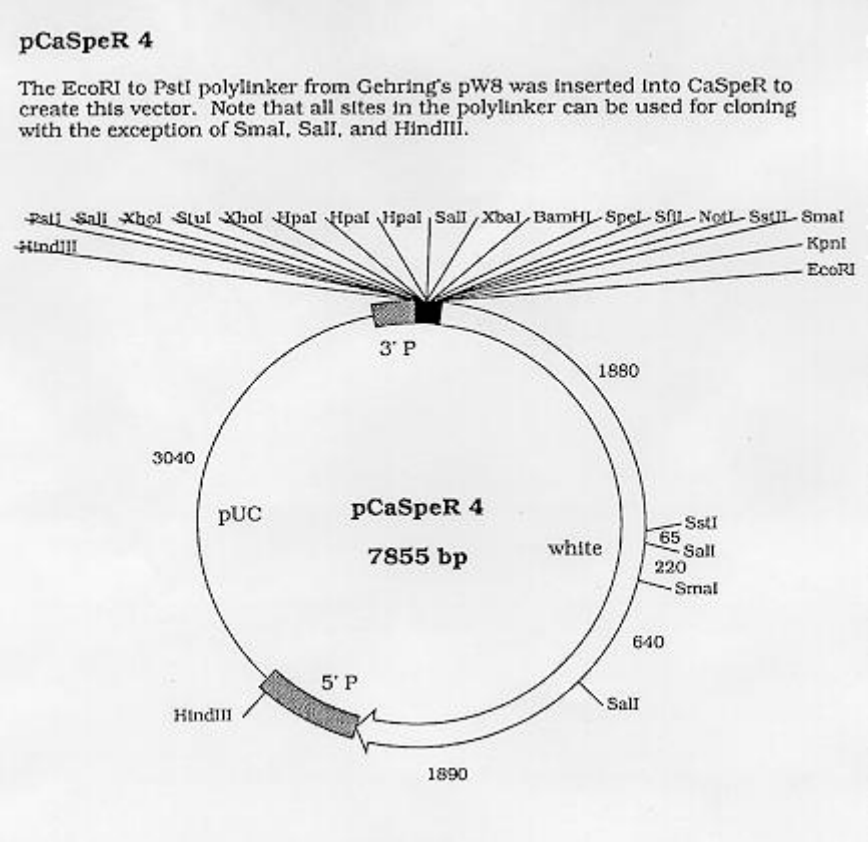
The native promoters for twist and snail were originally in this vector.

2. pYFP-N1



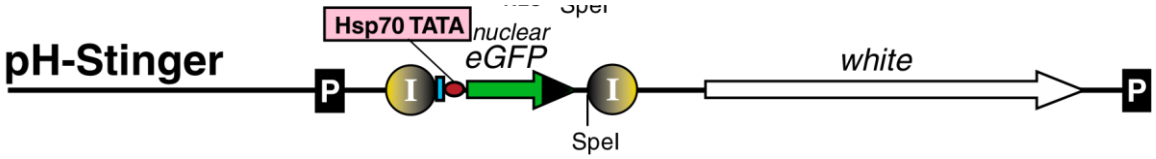
Variants of this vector, containing eYFP, eGFP, dsRed or Cerulean were used for cloning in the native and artificial promoters.

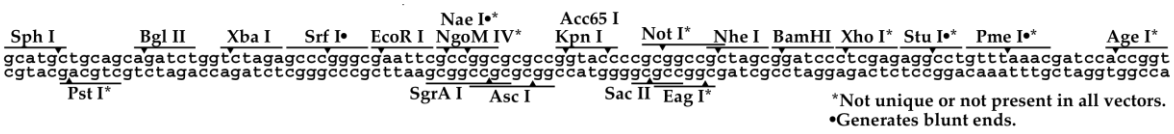
3. pCasPer4



Native promoter elements, fused to a fluorescent protein were cloned into the P-element vector. After succesful cloning, the vector-constructs were injected into w118 embryos to generate transgenic flies.

4. pH-Stinger





pH Stinger is the vector chosen to clone in the artificial promoters. The vector contains a minimal hsp70 TATA for efficeint transcription.

Dorsal protein sequence (from NCBI database)

N-terminal end:

M[1]FPNQNNGAAPGQGPAVDGQQSLNYNGLPAQQQQQLAQSTKNVRKK[46]P[47]YVKITEQPAGKALRF
RYECEGRSAGSIPGVNSTPENK[84]TYPTIEIVGYKGRAVVVVSCVTKDTPYRPHPHNLVGKEGCKKGV
CTLEINSETMRAVFSNLGIQCVKKKDIEAALKAREEIRVDPFKTGFSHRFQPSSIDorsalNSVRLCFQV
FMESEQKGRFTSPLPPVVSEPIF[216]DKKAM[225]SDorsalVICRLCSCSATVFGNTQIILLCEKVAKEDISV
RFFEEKNGQSVWEAFGDFQHTDVHKQTAITFKTPRYHTLDITEPAKVFIQLRRPSDGVTSEALPFEY
VPM[327]DSDPAHLR[335]RKRQK[340]TG[342]
GDPMHLLLQQQQKQQLQNDHQDGRQTNMNCWNTQNIPPIKTEPRDTSPQPFGLSYRAPPELTPSP
QPLSPSSNYNHNSTPSPYNMASAVTPTNGQQQLMSPNHPQQQQQQQQYGATDorsalGSNYNPFAQ
QVLAQQQQHQQQQQQHQHQHQQQHQQQQQQQQQQQQQQSLQFHANPFGNPGGNSWESKFSAA
AVAAAAATATGAAPANGNSNNLSNLNNPFTMHNLLTSGGGPGNANNLQWNLTTNHLHNQHTLHQQQ
QLQQQQQQQYDNTAPTNNNANLNNNNNNNNTAGNQADNNGPTLSNLLSFDSGQLVHINSEDQQILR
LNSEDorsalQISNLSIST - C-terminal end

Rel Homology Domain : 46-342 aa

DNA Binding Domain : 47-216 aa

Dimerization and Cactus Binding Domain : 225-327 aa

Nuclear Localization Signal : 46-84 (regulated nuclear import) and 335-340 aa

C-terminal Domain: 343-677 aa

Sequenced DNA (Transgenics)

Rho native promoter construct (Partial sequence):

AAGCTAGCTTTTCCTCTGCTCAAAATGAATATGATTAAAATAACAGTTTGATACGAATTTTAATTCCC
CTTTTTGCTGCGGAGTCAGTTGAGTGAGCCGCTTTCAGGACTCAGGGCATCATCCAGATCGCAC
GATCCCATTTGCATCTGCCTTCTCAGAAGCTGCTTGAAAGACGCGCCCCTGTCGGATGATTAGTG
CTAAGATCCTTGGGCAGGATGGAAAAATGGGAAAACATGCGGTGGGAAAAACACACATCGCGAAA
CATTTGGCGCAACTTGCGGAAGACAAGTGCGGCTGCAACAAAAAGTCGCGAAACGAAACTCCGG
GAAGCGGAAAAAGGACACCTTGCTGTGCGGCGGGAAACGCAAGTGGCGGGCGGAATTTCCTGA
TTCGCGATGCCATGAGGCACTCGCATATGTTGAGCACATGTTTTGGGGGAAATTCCCGAGCGACG
GGCCAGGAATCAACGTCCTGTCCTGCGTGGGAAAGGCCACCGTCCCACCCACGCCCACTCGGT
TACCTGAATTCCAGCTCGAGTGTTTTGGGTGGCTGGGATTGCTTTGGTACGGTGGCTGACCTTGC
CAGTGCCAGTGGGTCCATGTCCGTGGAGTGCTGGCAATGCGTGTCTGGCACAATGAAAACGCAG
CAATGGAGGATGGAGAAACTCGACCTGAGGGAACGTGGGAAAAAAGGATTTACGATGAAGATGA
GCTGAGAAACTCCGAGCTGATGACGTGCCTGGTCATGGGCATGTAGTGCAGCAACTGTGTTTTC
GTATCTGCTTACCTGTTGCACGCACATGTTAAAAAGTCATCATAAATCTCAGTGGACGAGGACGAG
GACTCTGAATCTGTTTTGCCTCTCTTTTGCGCCCTGTGAAAATATTTTCACTTTTCATGCGGGAGTT
TTCATGCCGCCGAAGGTCGATGAGGATGACTGGCCAGGATGGGTATATCTATCTATTAGATCTGTG
TAAATTCCCAGCTTATCATGCAAAACACGTAACCACGTCGTCTCGATCCTTTCGGCCAGGAAGCC
GAAAGCTACTCCTTTTGTGCAACATGCCGTGGTGACCTTTCCCGCGAAATCGTGACTGCAACGGC
TCAGGCTGCTT

Snail native promoter construct (Partial sequence):

TACAGGTCAACAGTCGAGCACGCTCTTTTATACCCGGAACTCTGCGCCTCTGCCGACGTCGCTGT
CGCCATTTGGTGGTGGTTCTTCTTCAAAACAGAGAAACACTCGCACGTGTGCGTCGCTACCTAGC

AACAACAACAGCACTTTTCCCATACCCTTATTGCGTATTTCCCTCACTTCCCTCTACCTGCTCTCTC
ACTCTCTCTCTCTCTCTTCCTCCATTTCCCACCTCTCTCTCGGAAGAGCAGACGCAGGCGCACCC
TCCCAGGAATAAGGATCCCCCGTATAAGTTATGACAGATCTGGTGGCTCTCCAATTTCCCGATACG
CTCACAACCCACCCGTAATAACTCGTGCAGGTCCGGGTCCGAAATGCAAGTTACAATATCGCCTC
CCTACCTATGGATCACCCTAAGTAATTTTCTATTTTTAAGCACGAGTCAAATAGGACTCCTAGTACTC
TTCGTTCATGGGGTGGTTTCCTCACTTAGATTGAGTGCTTCAAGTACATCTGCCGATGCTCTAAAT
TCGGTTTTTGGTGGGGAGTTGAACCTGAGCGATACGCACTGACAAAAATGACACCTTCGCAGAGA
GGTGTTCCAAAAATAGCACTGATAATGAGCCGAATTCCGAGACACCAAGTGTCAACTCTGGGGTG
CACTCTACGCTGACTTATGGATCATCTCCAGTGGCTCATTGGCAGCGATCGATGAGAGCTAGGAA
AACATAGCTCAGGGCATAGGAGATGTCATTAAAAACTTGACAAGGAATAGACGAAGAGTGTCAAGT
CTCCTGTGAGCTTGGATCGAAGAGATCTCCTGTTCCCACGATTGGTCCGTGAAAGAATGACGATT
CGAATACGAGTTACACATTTTAACTAATCCCATGGTCATCTTATTAGTATGTGAAGTTCTGCTGTCTT
AAAATCAATTCGATTGATAACGTAAAAAAAATCATACTGCCCCTCAGCATCCAGCTGAATCCTGGAC
GATTCCCACAATTCTCGCTTTCCAACTCCCCTGATCTTTGACTCCTTGCCTCATCGCGCAAACACA
ACTTTCCCAGCTAGCACCTCCAGACTTTCAAGCCACCCCTCCAACTATCGATGGGAAACTTTCCC
AACTCCCACTCCCCCATGGAAGCT

Twist native promoter construct (Partial sequence):

GCGGGCCCGGGCTGCAGCGTGTCCAATTTGAACATTAAGATCATTTTGAATGGTTGTATCTACAGC
ATTGCTACACTCACTTTGGCGCCATCTGCCAGGGAAAATAAAGGCAAGATATGTTTATTAAAATTAT
TTGTATTTTTAGTGAAACAAGTTATCCTAAGTTATATTCACGTGAGTTAGCTCACAAATGTCTAAAATT
AAGGCATGATCTAAAATTGAATTTAAAACACACATTTCAATGCATACTTTCAGGCGCGCATTGAGAT
TGATTATCTTCAAATTTATTTATGCACCTTATCCAAAAAGTTGGCAACATTTATTTTGTCTTTTTGACC
AATAAGAACACAAATTCGCTCTTACTTTAAAAATAATCTCATCGTTAAGTATATTTCTGCATTAATTAC
CGACCAATAGTTTAAGCTTATATTACTTTGTGTTTTATAACAATAATAATAAATGTGCTGAGGGCAGTA
AATCATATTGCTCATCATGTCCAAGCTCCTAAGTCCAGGTAGTTTTGGGACAGGGCAAAACCCTGT
TGGTGGTTTTTCTAAGGGGACCATTTCGAGTCCTGGGTTTTGCTATTACCTAAGCCGGCGATCGG
CGATCTGCGATCGGAGATCTTCGATCGTGGTTTTTTCCAGCGGAAGTTCGCGCTCTGCATTAATC
GGGTATTTTTGGTGGCCCCGGCAGGCAAACAGATAATTATATCCGGAAATTTGACTTTTCGCTCGT
ATTTTTCTGGATTTTCGGAGCTCCGAGCCGCATTCGCCTGCGATTTTCTCGGTACGTGTGTGTGG
GAATTCACTAATTAGGCATAATGAAACCTTTTCGTGGAGTTCCCCTCGGTTAGGGTTGTGGATTTG
CACGCTTTACGATGGTTGGCAACTAACTGATGATTATTTAATAGCGGAATGATTTCGATGGGCGAG
CGTCTAAACATTTCGGCTTGGTTCCTGGGAAATTCCTGCGATCCCAAAGTATATACAAATGGAAAAT
CCTCGAA

Dorsal (wild type; Sequence #1) aligned with Dorsal (K382R; Sequence #2)

Underlined bases indicate the mutation

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Seq_1  1     ----AAAGCCAAAGGGTTGTGGCGAGGTATCTCTTGGTTCCGTTTTAATGGGCGGTATGT  56
                 |||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Seq_2  1     CGAAAAAGCCAAAGGGTTGTGGCGAGGTATCTCTTGGTTCCGTTCTAATGGGCGGTATGT  60


Seq_1  57    TTTGTGTATTCCAGCAATTCATGTTAGTTTGTCTGCCATCCTGGTGGTCATTCTGCAATT  116
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    TTTGTGTATTCCAGCAATTCATGTTAGTTTGTCTGCCATCCTGGTGGTCATTCTGCAATT  120


Seq_1  117   GCTGTTTCTGCTGCTGCTGGAGCAGCAGGTGCATGGGATCACCGCCAGTCTTCTGACGTT  176
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   GCTGTTTCTGCTGCTGCTGGAGCAGCAGGTGCATGGGATCACCGCCAGTCTTCTGACGTT  180


Seq_1  177   TCCGCCTCAAGTGCGCTGGATCTGAGTCCATTGGCACGTACTCGAAGGGCAGGGCCTCGC  236
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Seq_2  181   TCCGCCTCAAGTGCGCTGGATCTGAGTCCATTGGCACGTACTCGAAGGGCAGGGCCTCGC  240


Seq_1  237   TGGTAACTCCATCCGAGGGACGTCGCAGTTGAATAAAAACCTTGGCGGGCTCTGTGATGT  296
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Seq_2  241   TGGTAACTCCATCCGAGGGACGTCGCAGTTGAATAAAAACCTTGGCGGGCTCTGTGATGT  300

Seq_1  297   CCAGGGTATGATAGCGCGGCGTCTTAAAGGTAATGGCAGTCTGCTTGTGGACATCCGTGT  356
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Seq_2  301   CCAGGGTATGATAGCGCGGCGTCTTAAAGGTAATGGCAGTCTGCTTGTGGACATCCGTGT  360

Seq_1  357   GCTGGAAGTCACCAAAGGCCTCCCAAACACTCTGGCCATTCTTCTCCTCAAAGAATCGCA  416
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Seq_2  361   GCTGGAAGTCACCAAAGGCCTCCCAAACACTCTGGCCATTCTTCTCCTCAAAGAATCGCA  420

Seq_1  417   CGGAGATGTCCTCCTTGGCCACCTTCTCGCAGAGCAGGATGATCTGGGTGTTGCCGAAAA  476
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CGGAGATGTCCTCCTTGGCCACCTTCTCGCAGAGCAGGATGATCTGGGTGTTGCCGAAAA  480

Seq_1  477   CGGTGGCCGAGCAGCTACACAGCCGGCAGATGACCAGGTCGGACATGGCCTTCTTATCGA  536
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Seq_2  481   CGGTGGCCGAGCAGCTACACAGCCGGCAGATGACCAGGTCGGACATGGCCTTCTTATCGA  540

Seq_1  537   AGATGGGCTCCGAAACTACCGGCGGCAGTGGCGAGGTGAATCGACCCTTCTGCTCGCTCT  596
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Seq_2  541   AGATGGGCTCCGAAACTACCGGCGGCAGTGGCGAGGTGAATCGACCCTTCTGCTCGCTCT  600

Seq_1  597   CCATGAATACTTGAAAGCACAATCGCACCGAATTCAGATCTATGCTCGAGGGCTGGAAAC  656
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Seq_2  601   CCATGAATACTTGAAAGCACAATCGCACCGAATTCAGATCTATGCTCGAGGGCTGGAAAC  660

Seq_1  657   GATGCGAAAAGCCAGTCTTAAACGGATCCTCTAGAGTCGACCTCGAACGTTAACGTTAAC  716
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Seq_2  661   GATGCGAAAAGCCAGTCTTAAAC----------------------------------------  683
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