GENERATION OF TRANSGENIC FLIES TO UNCOVER THE ROLE OF THE INTRINSICALLY DISORDERED REGIONS IN TRANSCRIPTIONAL REGULATION USING *DROSOPHILA MELANOGASTER* TRANSCRIPTION FACTOR BICOID

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By
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ABSTRACT

Generation Of Transgenic Flies to Uncover the Role of the Intrinsically Disordered Regions in Transcriptional Regulation Using *Drosophila melanogaster* Transcription Factor Bicoid

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Multicellular organisms develop from a single cell into a complex organism. Their development is strictly controlled by transcription factors that control the gene expression in a context-dependent manner, so that each gene is expressed at the right time and place. The specificity of transcription factors is determined mainly by their DNA-binding domains; however, their activity and interaction with DNA, proteins, and small molecules are modified by the effector domains (EDs). The EDs are generally low-complexity regions with high flexibility, often intrinsically disordered. Intrinsically disordered regions (IDRs), with their flexible and adaptable nature, help navigate protein activity in a context-dependent manner. The maternal morphogen Bicoid is a transcription factor responsible for the anterior development of *Drosophila melanogaster* embryos that regulates the expression of hundreds of genes responsible for *Drosophila* segmentation. It is absolutely required for embryo development and its absence results in the replacement of anterior structures by posterior ones. It consists of a 60 amino acid long structured homeodomain (HD) flanked by IDRs on both amino- and carboxyl termini. Through full Bicoid and HD swap experiments between *D.melanogaster*, *Lucilia sericata*, and *Calliphora vicina*, we found disordered EDs are required for Bicoid's full developmental functions. We used the *Drosophila* genetic manipulation tool recombinase-mediated cassette exchange to generate Bicoid ED mutant flies. We aim to elucidate the role of IDRs in regulating the activity of Bicoid via phenotypical and molecular analyses.

Keywords: Drosophila melanogaster, Embryonic development, Bicoid, RMCE, IDR, Transcription factor, Morphogen

ÖZET

Drosophila melanogaster Transkripsiyon Faktörü Bicoid Kullanılarak Kendiliğinden Düzensiz Bölgelerin Transkripsiyon Regülasyonundaki Rolünü Açığa Çıkarmak Üzere Transgenik Sineklerin Oluşturulması

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Ocak 2025

Çok hücreli canlılar tek hücreden kompleks bir organizmaya gelişirler. Gerekli genlerin ihtiyaç duyulan zamanda ve yerde ifade edilmesi için gelişimleri transkripsiyon faktörleri tarafından sıkı bir şekilde koşula bağlı şekilde kontrol edilir. Transkripsiyon faktörlerinin spesifitesi DNA bağlanma bölgeleri aracılığıyla belirlenirken aktiviteleri ile protein, DNA ve küçük moleküllerle olan etkileşimlerini efektör bölgeler düzenler. Efektör bölgeler çoğunlukla düşük kompleksite ve yüksek esnekliğe sahip olup genellikle kendiliğinden düzensizdir. Kendiliğinden düzensiz bölgeler (KDBler), esneklik ve uyumlulukları sayesinde protein aktivitesini koşula bağlı şekilde kontrol eder. Anneden aktarılan morfojen Bicoid, *Drosophila melanogaster* embriyolarında segmentasyondan sorumlu yüzlerce genin ifadesini düzenleyerek anterior gelişimden sorumlu olan bir transkripsiyon faktörüdür. Embriyo gelişimi için kesinlikle gereklidir ve yokluğu anterior yapılar yerine posterior yapıların oluşumunu sağlar. 60 amino asit uzunluğunda yapılı bir homeodomain (HD)den ve onu amino ve karboksil uçlarında çevreleyen KDBlerden oluşur. *D.melanogaster*, *Lucilia sericata* ve *Calliphora vicina* arasında yapılan tüm Bicoid ve HD değişim deneyleri aracılığıyla efektör bölgelerin Bicoid'in gelişimsel fonksiyonları için gerekli olduğunu bulduk. *Drosophila* genetik manipülasyon araçlarından rekombinaz-aracılığıyla kaset değişimi tekniğini kullanarak Bicoid efektör bölge mutantı sinekler geliştirdik. Bu sineklerle fenotipik ve moleküler analizler yaparak KDBlerin Bicoid aktivitesini nasıl kontrol ettiğini ortaya çıkarmayı amaçlıyoruz.

Anahtar Kelimeler: Drosophila melanogaster, Embriyonik gelişim, Bicoid, RMCE, KDB, Transkripsiyon faktörü, Morfojen

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DEDICATION

To my beloved Mother,

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List of Abbreviations

aa	Amino acid
AP	Anterior-Posterior
bcd	*bicoid* gene
btd	*buttonhead* gene
cad	*caudal* gene
CF	Cephalic furrow
Cv	*Calliphora vicina*
D	Dichaete
Dm	*Drosophila melanogaster*
DV	Dorsal-Ventral
Eb	*Episyrphus balteatus*
ems	*empty spiracles* gene
en	*engrailed* gene
eve	*even-skipped* gene
ftz	*fushi-tarazu* gene
GB	Germ band
gt	*giant* gene
hb	*hunchback* gene
HD	Homeodomain
HDR	Homology directed repair
IDR	Intrinsically Disordered Regions

kni	*knirps* gene
kr	*krüppel* gene
Ls	*Lucilia sericata*
MAFFT	Multiple Alignment using Fast Fourier Transform
Md	*Musca domestica*
NC	Nuclear Cycle
NHEJ	Non-homologous end joining
NMR	Nuclear magnetic resonance
otd	*orthodenticle* gene
prd	*paired* gene
PTM	Post-translational modifications
RH	Recognition helix
RMCE	Recombinase-mediated cassette exchange
Sb	Stubble
Ser	Serrate
Tb	Tubby
tll	*tailless* gene
VF	Ventral furrow
wg	*wingless* gene
Zen	Zerknüllt

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1. INTRODUCTION

Multicellular organisms develop from a single cell to form a complex organism (Barresi, 2020). It is critical for this development to progress precisely; thus, it is strictly regulated. Developmental regulation operates at multiple levels, starting from oogenesis (Barresi, 2020). In *Drosophila melanogaster* embryos, the regulation is achieved through maternal factors deposited to the egg (Avilés-Pagán & Orr-Weaver, 2018). In mammalians, however, this is achieved by intrinsic asymmetries and external clues (Shahbazi, 2020). Despite their differences, transcriptional regulation is what ensures the precision of development.

1.1 *Drosophila melanogaster*

D.melanogaster is an insect belonging to the order Diptera. It has a very small body of about 2-3 mm and is found near decaying fruits, where it gets the name fruit fly. With its short lifespan, it is also one of the most studied organisms in biology, especially in genetics and developmental biology.

1.1.1 *Drosophila melanogaster* Development

D.melanogaster undergoes 3 developmental stages, embryonic, larval, and pupal, before reaching adulthood. The embryo develops, hatches as a larva, and forms the pupa after going through 3 instar stages, where it goes through metamorphosis and ecloses as an adult after 10-12 days post-fertilization (at 25°C) (Barresi, 2020).

1.1.1.1 Embryonic Development

In *Drosophila* oogenesis, the oocyte develops in a series of stages where a single cell from 16 sister cells is selected to be the oocyte and the others to be the nurse cells (Avilés-Pagán & Orr-Weaver, 2018). During oocyte maturation, the nurse cells dump their cellular content into the oocyte and undergo cell death. The egg is activated by mechanical forces

and rehydration during ovulation and is fertilized in the uterus, which triggers parental nuclei fusion (Avilés-Pagán & Orr-Weaver, 2018). After egg laying, the embryo then undergoes 13 rapid mitotic divisions mainly under the control of maternal factors without cytokinesis, creating a multinucleated cytoplasm called the syncytium (Farrell & O'Farrell, 2014). Near the nuclear cycle (NC) 10, the nuclei in the syncytium migrate to the periphery of the embryo, cellularization begins, and the blastoderm forms. At around NC10 a group of nuclei cellularizes at the posterior pole and forms the pole cells, which will give rise to the germ line (J. E. Wilson & Macdonald, 1993). The zygotic transcription first starts at around NC8 and the zygotic genome is fully activated by NC14 (Blythe & Wieschaus, 2015; Harrison et al., 2023; Lécuyer et al., 2007).

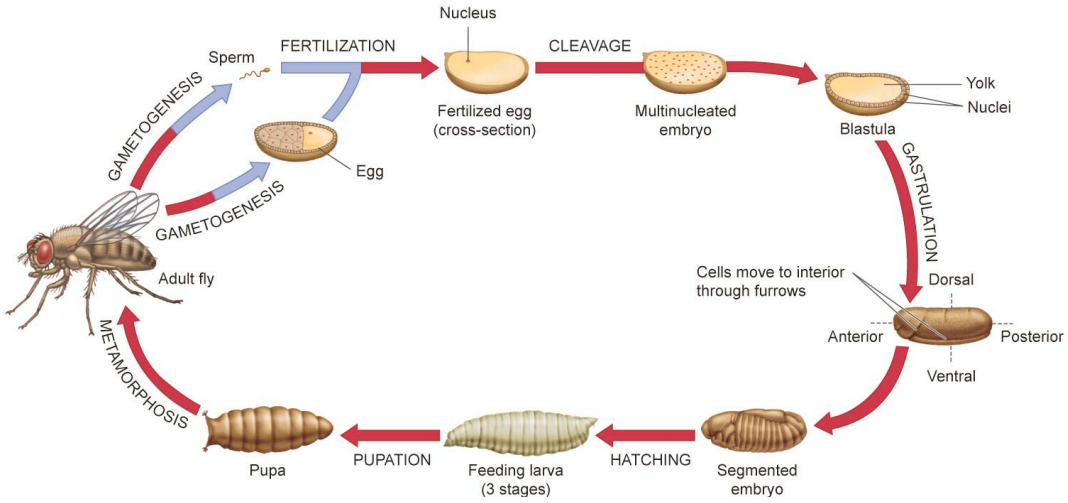

Figure 1: The developmental stages of *D.melanogaster*. The maternal factors are deposited to the egg during oogenesis. After fertilization inside the mother, the egg is laid and it starts developing. The egg hatches as a larva and goes through 3 larval stages. After the completion of the larval stages, it forms a pupa and undergoes metamorphosis to eclose as an adult. The development from egg to adult takes around 10-12 days at 25°C. Adapted from https://biology.kenyon.edu

The embryonic axes of the embryo are set during oogenesis via the deposited maternal factors. The anterior-posterior (AP) axis is defined by Gurken (Grk) and gradients of Bicoid govern this axis in the anterior and Nanos in the posterior of the embryo (J. Ma et al., 2016). These factors defining the AP axis control the expression of gap genes like

hunchback (*hb*), *giant* (*gt*), *knirps* (*kni*), *tailless* (*tll*) and *krüppel* (*kr*) (Gheisari et al., 2020). The gap genes control the expression and pattern of pair-rule genes like *even-skipped* (*eve*), *paired* (*prd*), and *fushi-tarazu* (*ftz*) that, when combined, define the expression of segment polarity genes such as *wingless* (*wg*) and *engrailed* (*en*) (Gheisari et al., 2020).

The dorsal-ventral (DV) axis of the embryo is defined by the ventral to dorsal gradient of the transcription factor Dorsal (Stein & Stevens, 2014). The gradients of these factors control the zygotic gene expression. In the DV axis, Dorsal concentration activates the expression of distinct zygotic genes that define the patterning of the DV, which is refined by the dorsal to ventral gradient of the signaling molecule Dpp, which opposes Dorsal activity (Gelbart, 1989).

Tissue architecture rearrangements occur during gastrulation. At the onset of gastrulation, cephalic furrow (CF) formation occurs. CF is an epithelial fold, forming in the anterior of the embryo, between the future head and thorax, and spanning the DV axis (Gheisari et al., 2020). CF formation is followed by the formation of the ventral furrow (VF) in the mid-ventral of the embryo, and the cells internalized by VF give rise to the mesoderm germ layer (Sweeton et al., 1991). Shortly after the VF forms, the germband (GB) starts expanding on the AP axis. Germband is made up of epithelial cells spanning the cephalic furrow to the posterior pole on the AP axis and invaginating cells of VF and ectoderm except for the amnioserosa (Gheisari et al., 2020). It expands 2.5 fold in length and reaches half of its width (Hartenstein & Campos-Ortega, 1985). The GB extension allows cells to align with the segmented body plan of the embryo and positions them to be involved in organogenesis (Gheisari et al., 2020). This positioning and alignment finalizes when the GB starts retracting (Lynch et al., 2013). During the GB retraction, the head structures start to form due to the force it applies and are retracted under the first thoracic segment in a process called head involution (Finkelstein & Perrimon, 1991). After completing organogenesis, the embryo secretes a protective cuticle layer and hatches from the egg as a first instar larva and larval development begins (Jürgens et al., 2024). Starting from the egg laying, it takes around 24 hours at 25°C for the larva to hatch.

1.1.1.2 Larval Development

The first instar larva (L1) hatches from the chorion and vitelline layers of the egg using its mouth structures and starts feeding off of foods. After about 24 hours, the larva grows and sheds its old cuticle (molting), turning into second instar larva (L2) (Jürgens et al., 2024). This larva also continues further growth and development, undergoes another molting, turning into third instar larva (L3). L3 persists in this stage for about 72 hours while feeding and wandering before immobilizing to form a pupa (Jürgens et al., 2024).

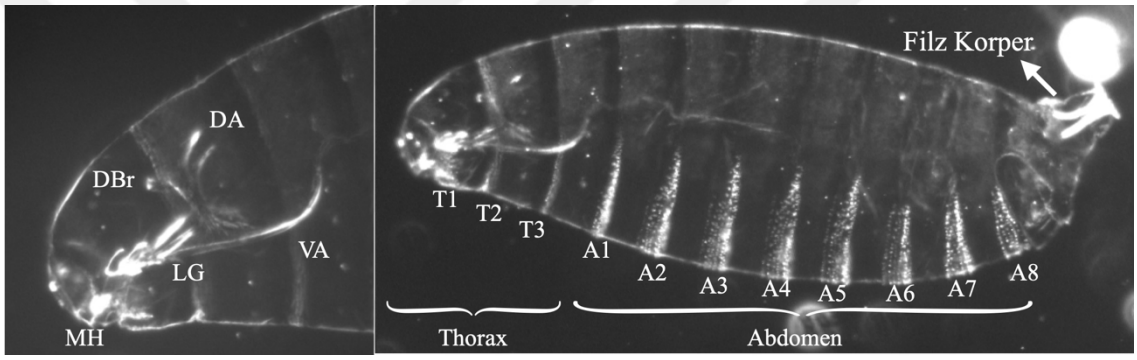

Figure 2: *D.melanogaster* larval morphology. Cuticle preparation of L1 larva under Darkfield microscope. The whole body is on the right, while the zoomed head of the same larva is on the left. Segments labeled T1-3 and A1-8 are thoracic and abdominal segments, respectively. MH: mouth hook, DBr: dorsal bridge, DA: dorsal arm, VA: ventral arm, LG: lateral gräten.

The *Drosophila* larva has a very pronounced body segmentation along the AP axis. The larval body has three thoracic and eight abdominal segments of cuticle with rows of chitinous denticle belts marking the segment borders, as seen in Figure 2 (Jürgens et al., 2024; Wipfler et al., 2013). The cuticle of the larvae can be prepared and analyzed using a mounting medium that digests the larvae except for the cuticle layer (Alexandre, 2008). In Figure 2, the abdominal and thoracic segments of the larva, as well as the head structures, can be seen in the cuticle preparation of a wild-type larva.

1.1.1.3 Pupal Development

Using its cuticle layer, the L3 larva forms a puparium, the rigid structure around a pupa. During metamorphosis, several organs reserved only for the adult, like wings, complex legs, and eyes, emerge (Jürgens et al., 2024). Once the pupa is formed, the adult structures are stimulated for growth, while larval structures that are no longer necessary go through histolysis (Bender, 1995). After the end of metamorphosis, an adult fly ecloses from the pupa.

1.2 Bicoid

The maternal transcription factor Bicoid is the first morphogen to be characterized (Frohnhöfer & Nüsslein-Volhard, 1986). During oogenesis, *bicoid* (*bcd*) mRNA is deposited to the anterior pole of the oocyte, and it is translated into Bicoid protein after the egg is laid to form a concentration gradient (Driever et al., 1989; Johnston et al., 1989; Struhl et al., 1989). This concentration gradient allows for the anterior patterning of the embryo by controlling the development of the head and thorax structures (Frohnhöfer & Nüsslein-Volhard, 1986).

Although Bicoid has an essential role in anterior development, it is not ubiquitously found in all fly species, let alone insects. Around 140 million years ago, a duplication event on the *hox3-like* gene resulted in the formation of *zerknüllt* (*zen*) and *bcd* genes (Sommer & Tautz, 1991; Stauber et al., 1999). While *zen* is ubiquitously found in all Drosophilids, *bcd* is only confined to the Cyclorrhaphan flies (Brown et al., 2001; Sommer & Tautz, 1991; Stauber et al., 2002). In other flies, the anterior development is controlled by different transcription factors, while Zen, like its ancestor Hox3, is only involved in extraembryonic tissue formation (Brent et al., 2007; Klomp et al., 2015; Rafiqi et al., 2008, 2010) .

1.2.1 Gene Regulation via Bicoid

Bicoid controls the expression of hundreds of genes responsible for the patterning of the embryo, including the gap genes like *hb*, *orthodenticle* (*otd*), *gt*, *kr*, *empty spiracles* (*ems*) and *buttonhead* (*btd*) and the pair-rule genes like *eve* and *runt* (Dalton et al., 1989; Driever & Nüsslein-Volhard, 1989; Q. Gao & Finkelstein, 1998; Struhl et al., 1989; Wimmer et al., 1993). Bicoid, in addition to regulating the expression of several zygotic genes, can interact with ubiquitously found mRNA to inhibit translation (Rivera-Pomar et al., 1996a).

Bicoid-dependent gene expression is crucial for setting the anterior identity. In strong mutant alleles of *bcd*, such as bcd^{E1}, Bicoid-target gene expression is disturbed, and the anterior structures of the larva are lost, resulting in duplication of posterior structures (see Figure 3B) (Frohnhöfer & Nüsslein-Volhard, 1986). In addition, cytoplasmic transplantation of the anterior cytoplasm of bcd^{+} embryos to the bcd^{-} embryos can trigger cephalic and thoracic structure development at the injection site (Frohnhöfer & Nüsslein-Volhard, 1986).

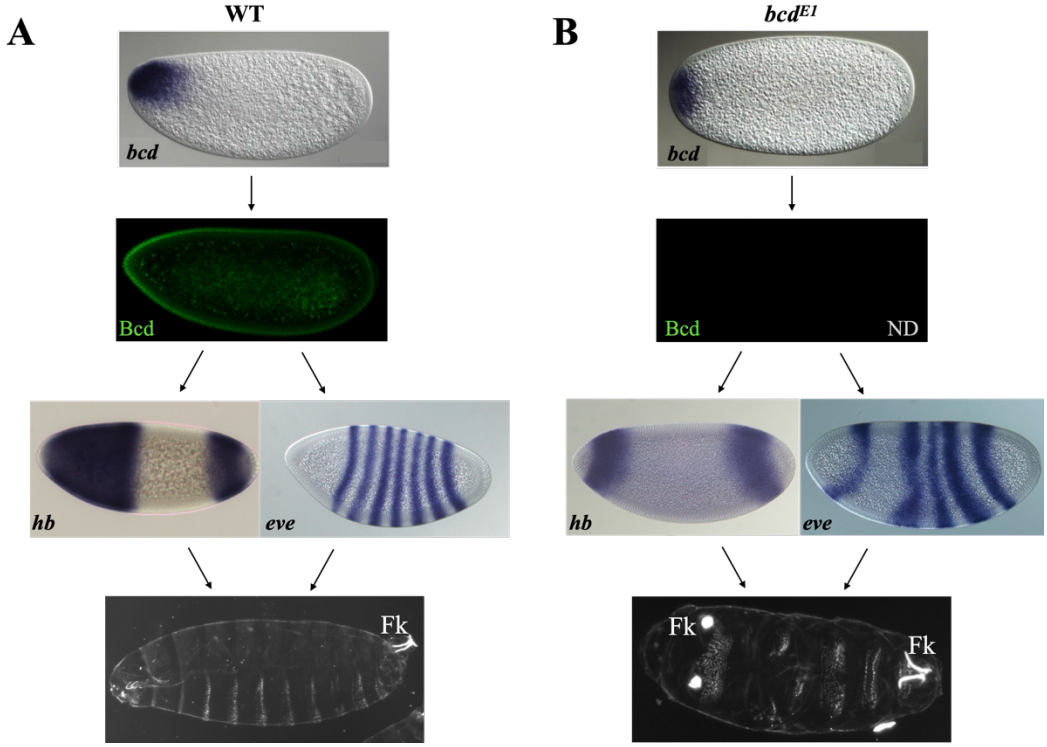

Figure 3: Developmental activity of *bcd* gene. A) mRNA expression pattern of wild-type *bcd* gene, its protein concentration gradient, expression pattern of Bicoid-target gap (*hb*) and pair-rule

(*eve*) genes and the phenotype of L1 larva. **B)** mRNA expression pattern of bcd^{E1} allele, expression pattern of Bicoid-target gap (*hb*) and pair-rule (*eve*) genes and the phenotype of L1 larva. Bicoid protein is undetectable in bcd^{E1} mutants. Anterior is on the left and dorsal is up. ND: Not detectable, Fk: Filz korper. *bcd* mRNA in situ hybridization images are modified from Datta et al., 2018.

1.2.2 Structure of Bicoid

Bicoid's longest isoform is made up of 489 amino acids (aa). It contains a well-conserved 60 aa-long DNA-binding homeodomain (HD) flanked by regulatory amino (N)- and carboxyl (C)-termini. Bicoid is a disordered protein, except for its HD region, which was structurally resolved by nuclear magnetic resonance (NMR) spectroscopy (Baird-Titus et al., 2006). AlphaFold protein structure prediction tool also cannot predict any structured regions with high confidence except for the HD (see Figure 4)

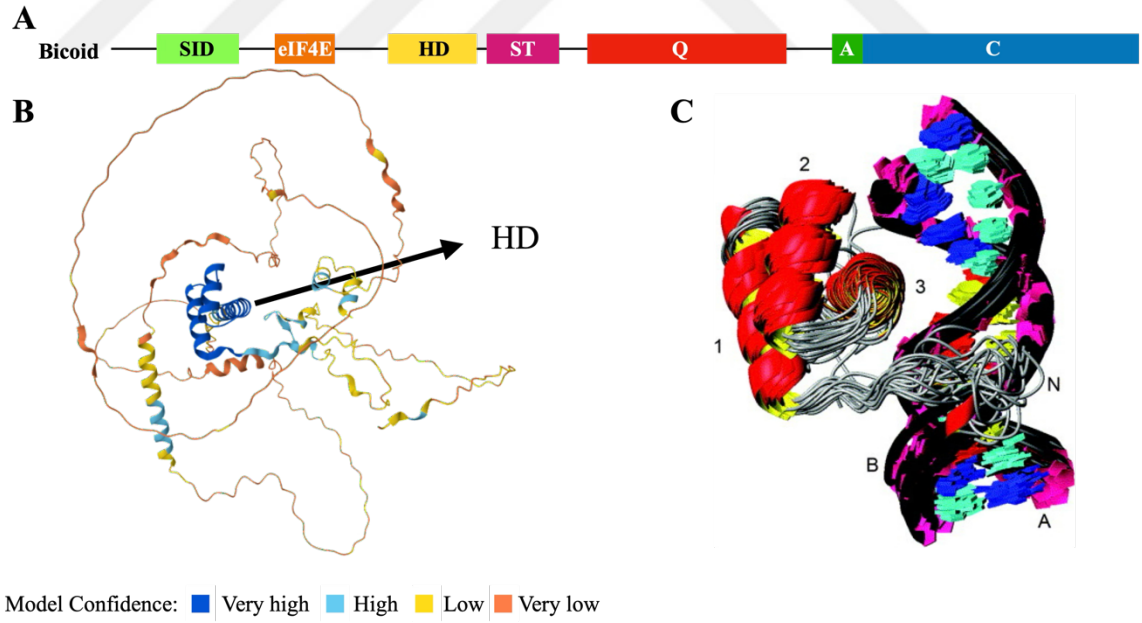

Figure 4: The structure of Bicoid. A) The domains of Bicoid. **B)** AlphaFold prediction of the Bicoid structure. The structured HD region is indicated with the black arrow. Modified from AlphaFold Protein Structure Database (Jumper et al., 2021; Varadi et al., 2024). **C)** The structure of Bicoid HD superimposed on DNA obtained by Baird-Titus et al., 2006 via NMR spectroscopy. Copyright © 2006, Elsevier Ltd.

1.2.2.1 The N-Terminal Region

The N-terminal region of Bicoid is a disordered region containing two important domains: the self-inhibitory domain (SID) and the eIF4E binding domain. Mutations in SID increase Bicoid transcriptional activity 20-fold, providing a regulatory role to the N-terminal of the protein (Zhao et al., 2003). The eIF4E binding domain is involved in the inhibition of mRNA translation by binding to the translation initiator eIF4E when Bicoid is bound to mRNA (Niessing et al., 2002).

1.2.2.2 The Homeodomain

Bicoid contains a very conserved 60 aa HD region having a helix-turn-helix motif around a recognition helix with DNA and RNA binding affinities (Dubnau & Struhl, 1996; Gehring et al., 1994). The recognition helix (RH) recognizes the specific 5'-TAATCC-3' sequence and binds DNA through the major groove while the N-terminal region interacts with the minor groove to stabilize the interaction (Baird-Titus et al., 2006; D. S. Wilson et al., 1996).

Although Zen and Bicoid share an ancestor, they have very different HD sequences (Q. Liu et al., 2018). Especially the K50 and R54 on the Bicoid HD found on the RH are critical for DNA and RNA binding specificity (Q. Liu et al., 2018; Trelsman et al., 1989). On the other hand, Bicoid HD sequence is highly conserved across species, indicating its importance in the transcriptional activity of Bicoid.

1.2.2.3 The C-Terminal Region

The C-terminal of Bicoid is a disordered region made up of several domains of aa repeats. These domains are Serine-Threonine (ST)-rich (also known as PEST domain), Glutamine (Q)-rich, Alanine (A)-rich, and acidic C-terminal (C) domains. This region is important for Bicoid activity as the inhibition by Torso and the stabilizing phosphorylations by MAPK target the C-terminal region (Bellaïche et al., 1996; Janody et al., 2000, 2001;

Ronch et al., 1993). The C-terminal region is also considered to be the transactivation domain, regulating Bicoid transcriptional activity (Bellaïche et al., 1996). Unlike Zen, Bicoid HD can also bind RNA to regulate mRNA translation and this is achieved with the help of the PEST sequence on the C-terminal (Chan & Struhl, 1997; Niessing et al., 1999).

Schaeffer and colleagues made deletion mutants of this region to understand its contribution to the transcriptional activity of Bicoid via interactions with RNA polymerase II-mediated transcription machinery (Schaeffer et al., 1999). It was previously suggested that Bicoid effector domains and Hunchback (Hb) interact with TATA-associated factors 60 and 110 *in vitro* and synergize in activating their targets *in vivo* (Sauer et al., 1995b, 1995a). However, this was disproven by the effector domain mutants, and several mutants were able to function like full-length Bicoid (Schaeffer et al., 1999).

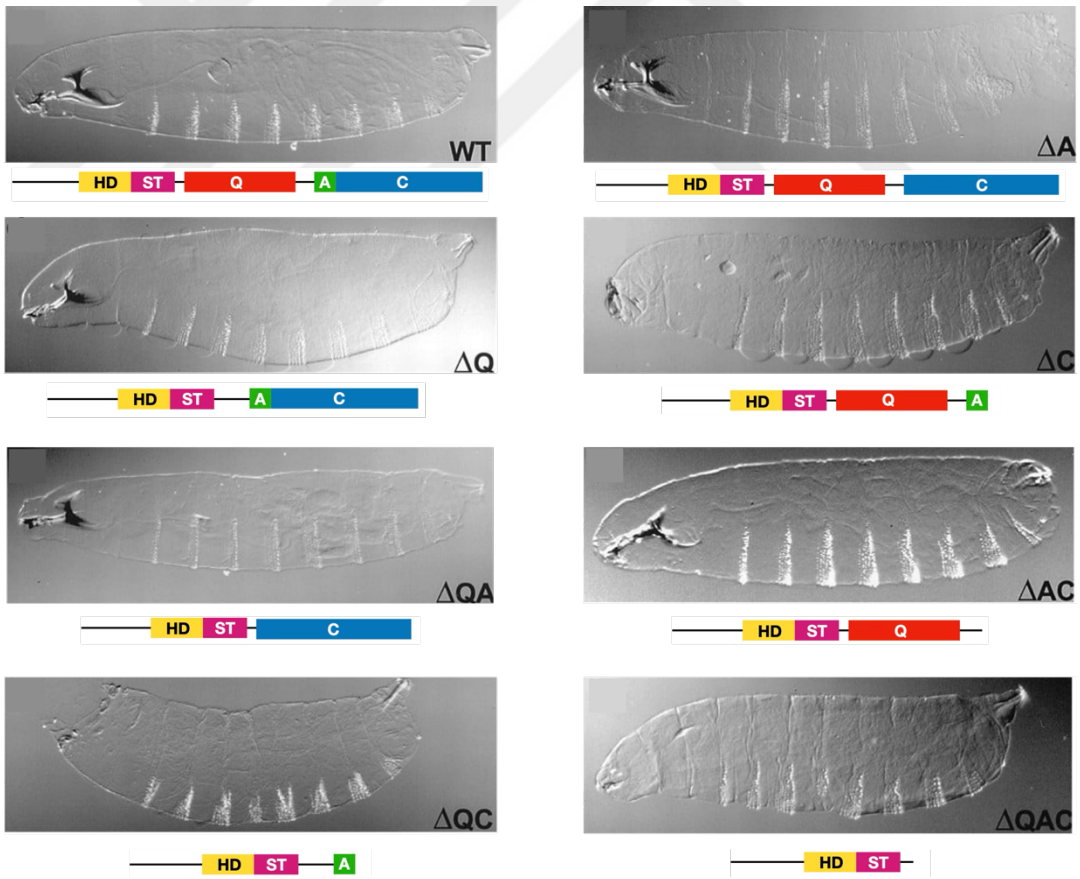

Figure 5: The larval phenotypes of the effector domain mutants. The L1 larval phenotypes of the Bicoid effector domain mutants are shown. The deletion constructs are given below. Anterior is on the left and dorsal is up. WT: Wild-type. Modified from Schaeffer et al., 1999. Copyright © 1999, The National Academy of Sciences.

Although these deletion mutants mostly rescued Bicoid activity, as can be seen in Figure 5, the well-defined *bcd* mutant alleles obtained via ethyl-methane sulfonate-induced mutagenesis, some of which correspond to effector domain deletions are not able to rescue the anterior development (Berleth et al., 1988). One example of these alleles is bcd^{E1}, which has a deletion downstream of HD, causing a 55 aa frameshift before causing translation to stop prematurely (Rivera-Pomar et al., 1996b). This mutation causes the loss of the entire effector domain and is the strongest *bcd* mutant allele, causing the loss of anterior structures (see Figure 3B). Other alleles that cause deletions downstream of the ST-rich region are also identified as lethal, indicating the importance of this region (Frohnhöfer & Nüsslein-Volhard, 1987). Thus, without a structure like that of HD has, the effector domain still has important roles in regulating transcriptional activity.

1.3 Intrinsically Disordered Regions

Intrinsically disordered regions (IDRs) are structurally unidentifiable and unpredictable regions found in the proteome (J. Liu et al., 2006). In general, the prevalence of IDRs in the proteome increases with the complexity of the organism, distinguishing prokaryotes from eukaryotes, which could have contributed the evolution of more complex biological processes (C. Gao et al., 2021). Their lack of structure relieves them from structural constraints and allows for a more rapid evolution than structured proteins, which in turn, makes it complicated to characterize the relay of functional information between homologues and predict their function in general (van der Lee et al., 2014). IDRs are present in around 4% of bacterial and more than 30% of the eukaryotic proteome (C. Gao et al., 2021; Pancsa & Tompa, 2012). In humans, 37%-50% of the proteome contains disordered regions longer than 30 amino acids, mainly the transcription factors (Minezaki et al., 2006; Oates et al., 2013).

IDRs were first found during X-ray crystallography experiments when there were invisible regions in electron density maps (Romero et al., 1997). They are usually composed of polar and charged aa repeats, and are very flexible that allows for several

types of target-specific, low-affinity interactions regulating protein function (J. Liu et al., 2006). For example, the Q-rich regions are often associated with biomolecular condensate formation by interacting with other factors, the ST-rich regions are phosphorylation hotspots for many kinases regulating protein activity, localization, and interactions, and the C-terminal acidic domains usually modulate protein-protein interactions and protein activity via its charged nature and post-translational modifications (Harlen & Churchman, 2017; Hsin & Manley, 2012; Hu et al., 2023; Mo et al., 2015; Sen et al., 2017).

IDRs are also hubs for post-translational modifications (PTMs) that control protein function (Pejaver et al., 2014). Many proteins involved in critical cellular processes, like SOX2, FOXO4, and RUNX1, contain PTMs introduced to the IDRs to regulate their activity, interaction partners, structure, and even subcellular localization (Bjarnason et al., 2024; Bourgeois et al., 2021; Guo & Friedman, 2011).

Mutations of IDRs can disturb protein activity, and in some cases lead to diseases. Expansion of Q-rich regions leads to formation of aggregates, resulting in diseases like brachyphalangy, polydactyly and tibial aplasia syndrome, Huntington's Disease, and spinobulbar muscular atrophy (Mensah et al., 2023; Ripaud et al., 2014). In p53 the C-terminal domain responsible for interactions with coactivators and DNA, and its mutations renders p53 inactive, leading to cancer (Kim et al., 2012).

With such involvement in protein function and evolution, uncovering the roles of IDRs in protein regulation is very important.

1.4 *Drosophila melanogaster* as a Model Organism

With its genome highly homologous (around 60%) to human, *Drosophila* has been a powerful model organism for the past 100 years (Chien et al., 2002; Ugur et al., 2016). They are inexpensive to care for, have a very short lifespan, and have a high reproductive rate, providing larger sample sizes studied in shorter amounts of time (Roberts, 2006).

It was first used as a model for making ground-breaking research in genetics, but the introduction of new mutations by X-ray led to the discovery of genes responsible for segmentation, opening the doors to developmental biology (Frohnhöfer & Nüsslein-Volhard, 1986; Roberts, 2006). Most of the human disease-associated genes have homologs in *Drosophila*, which made it important for modeling human diseases too (Pfleger & Reiter, 2008). As it kept on leading to more discoveries, newer tools for research had been developed, leading to an exponential increase in findings.

The characterization of inverted chromosomes upon X-ray irradiation that did not crossover like a normal chromosome does and carries recessive lethal alleles resulted in the establishment of balancer chromosomes, which are used for keeping recessive lethal allele stocks (Miller et al., 2019). Currently, there are many balancer chromosomes used in *Drosophila* research. Some of the commonly used ones are the II. chromosome balancers Sm6 and CyO, carrying the dominant *curly* gene, and the III. chromosome balancers Tm6B carrying Tubby (Tb), and Tm3 carrying Serrate (Ser), sometimes combined with Stubble (Sb); all of which are easily screened by the wing and bristle phenotypes (Greenspan, 1996).

In addition to genetics, *Drosophila* is also an important model in developmental biology. Many eggs can be collected from a single female each day and they develop outside the female's body. The embryos are almost transparent, making it easy to observe their development under microscope. Transparent nature of the embryos and the high sample size allows for detailed examination of development. Another advantage of *Drosophila* over models for embryonic development is the fact that the developmental defects can be observed, categorized, and quantified later, in the larval stages (Nüsslein-Volhard et al., 1984). In other models, any embryonic lethality would stop the progression of development, preventing from observing any downstream effects. However, in *D.melanogaster*, cuticle preparations of L1 larvae can clearly portray segmentation defects that occurred during embryonic development, allowing for examination of embryonic lethality defects.

With the development of new tools for genetic manipulation, such as the P-element transposon system, recombination-mediated cassette exchange, and CRISPR-Cas9, the focus shifted more toward specific genes, and studying specific mutations *in vivo* became possible. As the need to introduce specific mutations in the genome arose, the *Drosophila* P-element mediated transposon system that randomly inserted the constructs into the genome was developed (Rubin & Spradling, 1982). Although it was a great approach, site-specific modifications became more important due to the position effects influencing the gene expression (Groth et al., 2004; Levis et al., 1985).

1.4.1 Recombinase-Mediated Cassette Exchange (RMCE)

The need to avoid the position effect resulted in the development of the recombinase-mediated cassette exchange method in which a recombinase catalyzes a strand-exchange event between short target sites (Baer & Bode, 2001). ΦC31 phage integrase-mediated recombination system was established in *D.melanogaster* to achieve site-specific integration of DNA (Groth et al., 2004). ΦC31 integrase mediates recombination between attB and attP sites, leaving attR and attL sites that the integrase can no longer recognize (Thorpe & Smith, 1998). Using P-element mediated transposition, a construct of two attP sites surrounding the *mini-white* gene was integrated into the *Drosophila* genome (Bateman et al., 2006). After screening for the position effect, several lines that can be used for RMCE via ΦC31-mediated recombination were obtained (Bateman et al., 2006). By introducing a vector containing attB sites to the germ line via microinjection, the insert between the attB sites can be inserted into the genome, replacing the *mini-white* gene that, in turn, can be used as a selection marker for transformants (Bateman et al., 2006; Groth et al., 2004). The working principle of this system is illustrated in Figure 6.

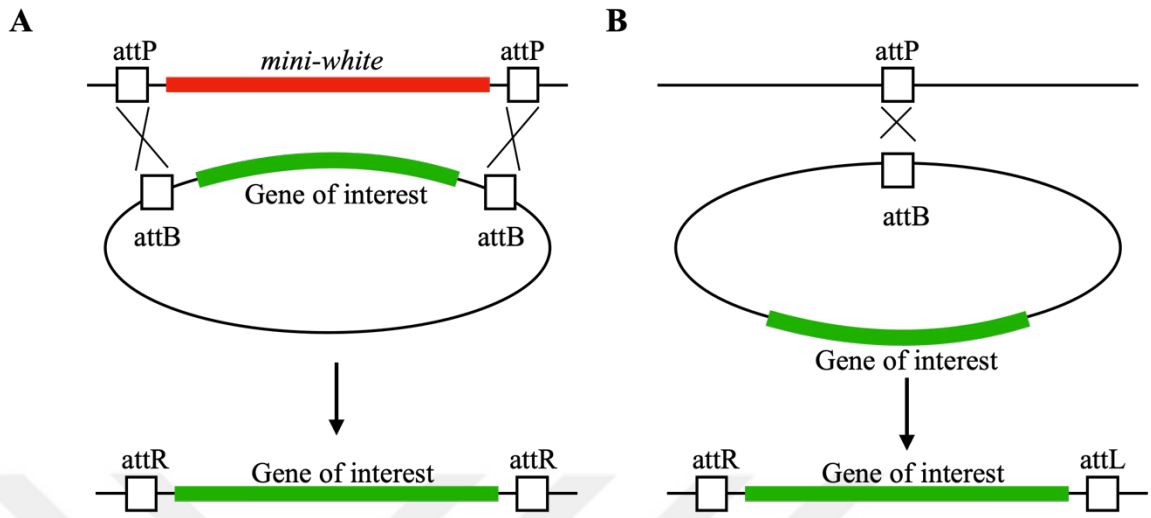

Figure 6: Working principle of ΦC31-mediated RMCE. A) In the system with marker, when the vector containing attB sites is introduced, ΦC31 integrase mediates recombination between the attP and attB sites, eventually eliminating *mini-white* and inserting the gene of interest. **B)** In the system without the marker and a single recombination site, the donor plasmid is inserted as a whole.

1.4.2 Genome Editing via CRISPR-Cas9

Although the RMCE approach allowed for more site-specific modifications, it was still not possible to modify a gene in its locus. The CRISPR-Cas9 system, which was first identified as an immune system in bacteria, was engineered to allow for site-specific genome editing (Jinek et al., 2012). In this system, short guide RNAs (gRNAs) are used for targeting a site to introduce a double-strand break via Cas9 enzyme and triggering non-homologous end joining (NHEJ) or homology-directed (HDR) DNA repair (Gratz et al., 2015). Unless a donor plasmid with homology to the site is introduced, the break is repaired via NHEJ, causing a deletion, while the donor plasmid triggers HDR and introduces a new sequence to the locus (Gratz et al., 2015; Y. Ma et al., 2014). Thanks to the site-directed activity of the CRISPR-Cas9 system, it is now possible to introduce modifications to specific regions of the genome (Gratz et al., 2015).

2. OBJECTIVES AND AIMS

Transcription is an important step in gene regulation that is mediated by transcription factors at its core. Transcription factors, through their DNA binding domains (DBDs), recognize and bind specific DNA sequences, and control the transcription machinery. To be controlling the transcription machinery, they require modularity which is provided by their intrinsically disordered effector domains that have a flexible nature. IDRs contribute to protein activity by regulating the interactions with proteins, DNA and small molecules, but the extent of their contribution is still unclear.

Utilizing *Drosophila* genetic manipulation tools and classic molecular cloning techniques, we aim to generate *Drosophila* maternal transcription factor Bicoid effector domain mutant flies. Using these mutant lines, we aim to understand the individual and synergistic roles of the IDRs in regulating Bicoid activity, if they play any role in Bicoid protein stability and gradient formation, the target-specific use of these IDRs, and if there are any redundant regions.

3. MATERIALS

3.1 Fly Stocks

Line Name	Properties
yw	Used as a wild-type control
$\mathrm{DmBcd}\ ;\ \mathrm{bcd^{E1}}$	*bcd*^{E1} rescue line containing *D.melanogaster bcd* on 38F1 locus
$\frac{\mathrm{CvBcd}}{\mathrm{CyOBcd}}\ ;\ \mathrm{bcd^{E1}}$	*bcd*^{E1} rescue line containing *Calliphora vicina bcd* on 38F1 locus, balanced with CyO balancer chromosome expressing maternal *bcd*
$\frac{\mathrm{LsBcd}}{\mathrm{CyOBcd}}\ ;\ \mathrm{bcd^{E1}}$	*bcd*^{E1} rescue line containing *Lucilia sericata bcd* on 38F1 locus, balanced with CyO balancer chromosome expressing maternal *bcd*
$\frac{\mathrm{DmBcdCvHD}}{\mathrm{CyOBcd}}\ ;\ \mathrm{bcd^{E1}}$	*bcd*^{E1} rescue line containing *D.melanogaster bcd* with *Calliphora vicina* Bicoid HD on 38F1 locus, balanced with CyO balancer chromosome expressing maternal *bcd*
$\frac{\mathrm{DmBcdLsHD}}{\mathrm{CyOBcd}}\ ;\mathrm{bcd^{E1}}$	*bcd*^{E1} rescue line containing *D.melanogaster bcd* with *Lucilia sericata* Bicoid HD on 38F1 locus, balanced with CyO balancer chromosome expressing maternal *bcd*
eGFPBcd	N-terminal eGFP-tagged Bicoid line made through 2-step CRISPR in endogenous *bcd* locus
$\Phi\mathrm{C31(y^{+})}\ ;\ \mathrm{38F1\ (w^{+})}$	Injection line for RMCE containing w gene between two attP sites at 38F1 locus
$\Phi\mathrm{C31(y^{+})}\ ;\ \frac{\Delta\mathrm{7kb\ bcd^{-}, RFP^{+}}}{\mathrm{Tm3, Ser}}$	Injection line for RMCE containing dsRED between two attP sites in *bcd* locus
$\frac{\mathrm{D}}{\mathrm{Tm3, Sb, Ser}}$	III. chromosome balancer line
$\frac{\mathrm{Sco}}{\mathrm{CyOBcd}}\ ;\ \mathrm{bcd^{E1}}$	II. chromosome modified balancer line expressing maternal *bcd* on CyO balancer chromosome in *bcd*^{E1} background

Table 1: List of fly lines used in this study.

3.2 Materials for Fly Maintenance, Buffers, Reagents, and Glassware

Product Name	**Manufacturer**	**Catalog Number**
Agar Agar	Benosen	E406
Corn Flour	Yazar	N/A
Granulated Sugar	Torku	N/A
Propionic Acid	Sigma Aldrich	800605-2500
Methyl 4-hydroxybenzoate	Carlo-Erba	354007
Hoyer's Medium	Home-Made	N/A
Narrow *Drosophila* Vials	Biologix	51-0500
60 mm x 15 mm Not TC-treated Bacteriological Petri Dish	Falcon	351007
Heptane	Sigma Aldrich	H2198-2.5L
20 mL Glass Scintillation Vials	LLG-Wheaton	DWK986541
Proteinase K	Genaxxon	M3036.0100
NBT/BCIP Stock Solution	Roche	11681451001
Anti-Digoxigenin-AP, Fab Fragments	Roche	11093274910
Anti-Fluorescein-AP, Fab Fragments	Roche	11426338910
Flypad, CO_2 Anesthetizing Apparatus	FlyStuff	59-114
Halocarbon Oil 700	Sigma Aldrich	H8898
Halocarbon Oil 27	Sigma Aldrich	H8773
Single Barrel Borosilicate Glass Capillaries	World Precision Instruments	1B100F-4
RNase A	Grisp	GE011.0100
Sample Collection Containers, Plastic	Fıratmed	8870000004
37% Formaldehyde Solution	Sigma Aldrich	8187081000

10x PBS	Home-made	N/A
20x SSC	Home-made	N/A
Lactic Acid	Merck	100366
Yeast	Pakmaya	N/A
Salmon Sperm DNA	Invitrogen	15632011
Heparin	Millipore	375095

Table 2: List of materials, reagents, buffers, and glassware used in fly maintenance, cuticle preparation, embryo fixation, *in situ* hybridization, and microinjection.

3.3 Foods for Fly and Embryo Maintenance

Food	**Ingredient**	**Amount**
Fly Food (Classic Cornmeal Recipe)	Corn Flour	28.60 g/L
	Granulated Sugar	42.85 g/L
	Yeast	15.00 g/L
	Agar Agar	9.60 g/L
	Propionic Acid	3.80 mL/L
	10% Nipagin (in Ethanol)	7.15 mL/L
Apple Juice Agar	Apple Juice	170.00 mL/L
	Agar	25 g/L

Table 3: The ingredients and recipe of the food used for maintaining the fly stocks and embryos.

3.4 Buffers for *in situ* Hybridization and Immunostaining

Buffer	Contents
10x PBS	100 mM Na2HPO4 18 mM KH2PO4 1.37 M NaCl 27 mM KCl
PBTw	10 mM Na2HPO4 1.8 mM KH2PO4 13.7 mM NaCl 2.7 mM KCl 0.5% Tween-20
in situ Buffer	13 mM Na2HPO4 23.4 mM KH2PO4 17.81 mM NaCl 3.51 mM KCl 67 mM EDTA pH 8.0
Hybridization Solution	150 mM NaCl 15 mM Sodium Citrate pH 8.0 50% Formamide 0.1 mg/mL Salmon Sperm DNA 0.05 mg/mL Heparin 0.5% Tween-20
20x SSC	3 M NaCl 300 mM Sodium Citrate pH 8.0
AP Staining Solution	0.5 M NaCl 50 mM MgCl2 100 mM Tris-HCl pH 9.5 0.1% Tween-20

Table 4: List and the contents of the buffers and solutions used in immunostaining and *in situ* hybridization.

3.5 Buffers for Plasmid and Genomic DNA Isolation

Buffer	Contents
Resuspension Buffer	25 mM Tris-HCl pH 8.0 50 mM glucose 10 mM EDTA pH 8.0
Lysis Buffer	0.2 M NaOH 1% SDS
Neutralization Buffer	3 M Potassium Acetate 11.5% Glacial Acetic Acid
Squishing Buffer	10 mM Tris-HCl pH 8.0 0.5 mM EDTA pH 8.0 125 mM NaCl 0.2 mg/mL Proteinase K

Table 5: List of buffers used in plasmid DNA isolation from bacteria and genomic DNA isolation from flies and their contents.

3.6 Materials for Molecular Cloning and Kits

Product Name	Manufacturer	Catalog Number
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific	SM0311
GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific	SM0241
6x DNA Gel Loading Dye	Thermo Fisher Scientific	R0611
QIAquick PCR Purification Kit	QIAGEN	28104
QIAquick Gel Extraction Kit	QIAGEN	28704
Plasmid *Plus* Midi Kit	QIAGEN	12943

VITA™ Taq DNA Polymerase	GDD Biolabs	G0078T-500U
VITA™ Pfu DNA Polymerase	GDD Biolabs	G00508P
BspEI	New England Biolabs	R0540S
AscI	New England Biolabs	R0558S
MluI-HF	New England Biolabs	R3198S
RsrII	New England Biolabs	R0501S
NheI-HF	New England Biolabs	R3131S
T4 DNA Ligase	Thermo Fisher Scientific	EL0014
Ampicillin	Glentham Life Sciences	GA0283
DH5α Competent *E. coli*	New England Biolabs	C2988J

Table 6: List of materials and kits used for molecular cloning.

3.7 Primers and Gene Blocks Used in Molecular Cloning and Sequencing

Primer Name	**Primer Sequence (5' to 3')**	**Purpose**
Q_R2	ACATGCACATGCAGTATCCTTCCGG	Common primer used for obtaining BspEI to RsrII or MluI PCR fragments. Also used for SDM.
HD_Control	CCACGTCGCACCCGCACCACTTTTA	Colony PCR primer to confirm the insert size
5_UTR_R	GCGTCTCGAAAGTAACCGGTTACTG	Primer used in SDM for the 5'-fragments of the ΔST and ΔSTQAC truncations. Binds to the 5' UTR of *bcd*
UTR_MluI_F	CTACGCGTAGATATCTACAATCACTTGG	Primer used in SDM for obtaining all of the 3'-fragments. Binds to the 3' UTR of *bcd*, downstream of MluI recognition site

sjs939_R	GAGCAGCCACCCAGATACAT	Sequencing primer binding to the 3'UTR of *bcd*
sjs584	AACAACACTATTATGCCCACCA	Primer used for obtaining PCR fragments from fly genomic DNA to send for sequencing. Binds to a site near the 5'-attP site on 38F1 locus of the II. chromosome
sjs586	CTGGAATTCGGCTTCGACT	Primer used for obtaining PCR fragments from fly genomic DNA to send for sequencing. Binds to a site near the 3'-attP site on 38F1 locus of the II. chromosome
HD_F_out	TCGCCGCATCACCAGAGAGT	Sequencing primer used for 5' of the HD of *bcd*
ΔA_F_in	CTCGGAGCCCGGCGAGCCAGACATTCCCGATCGCA	Primer used for obtaining the 5'-fragment of all ΔA truncations
ΔA_R_in	TCGGGAATGTCTGGCTCGCCGGGCTCCGAGGTCTA	Primer used for obtaining the 3'-fragment of all ΔA truncations
ΔAC_F_in	CTCTCGTCCAGGCTAGCCAGACATTCCCGATCGCA	Primer used for obtaining the 5'-fragment of ΔAC truncation of the eGFP-tagged Bicoid.
ΔAC_R_in	TCGGGAATGTCTGGCTAGCCTGGACGAGAGGCGTG	Primer used for obtaining the 3'-fragment of ΔAC truncation of the eGFP-tagged Bicoid.
ΔST_F_in	GTAGTAGGCGTTGAAGGGGGGATCGCCATCGCTCT	Primer used for obtaining the 3'-fragment of all ΔST and ΔSTQAC truncations
ΔST_R_in	GATGGCGATCCCCCCTTCAACGCCTACTACAACTA	Primer used for obtaining the 5'-fragment of all ΔST and ΔSTQAC truncations
ΔQA_F_in	CTCGGAGCCCGGCGAGCCGCCATTGACATTGGTCG	Primer used for obtaining the 5'-fragment of all ΔQA truncations

ΔQA_R_in	AATGTCAATGGCGGCTCGCCGGGCTCCGAGGTCTA	Primer used for obtaining the 3'-fragment of all ΔQA truncations
ΔQC_F_in	CTCTCGTCCAGGCTAGGCAGCGCCTCGGGCCACAG	Primer used for obtaining the 5'-fragment of ΔQC truncation of the eGFP-tagged Bicoid.
ΔQC_R_in	GCCCGAGGCGCTGCCTAGCCTGGACGAGAGGCGTG	Primer used for obtaining the 3'-fragment of ΔQC truncation of the eGFP-tagged Bicoid.
Q_Stop_RsrII_F1	ATACGGACCGCTAGCCAGACATTCCCGATCGCAT	Primer used for obtaining the ΔAC fragment for the Bicoid rescue plasmid. Contains a stop codon, RsrII recognition site and a 3 bp (ATA) flanking site as an overhang
Q_Stop_RsrII_F3	CGGACCGCTAGCCAGACATTCCCGATCGCATGTAGTACG	Primer used for obtaining the ΔQAC fragment for the Bicoid rescue plasmid. Contains a stop codon, RsrII recognition site and a 3 bp (ATA) flanking site as an overhang
A_Stop_RsrII_F2	ATACGGACCGCTAGGCAGCGCCTCGGGCCACAGCGGAT	Primer used for obtaining the ΔC and ΔQC fragment for the Bicoid rescue plasmid. Contains a stop codon, RsrII recognition site and a 3 bp (ATA) flanking site as an overhang

Table 7: List of the primers used for cloning and sequencing the constructs, their 5' to 3' sequences, and their purpose.

Name	Sequence (5' to 3')	Purpose
ΔQ_BP	TCCGGAGGGGGGCCAGGACCTGGGTCGACCAA TGTCAATGGCGGCGCCAGCGCCTGTCGCGTCCT GGTCAAGGACGAACCGGAGGCCGACTACAACT TCAACAGCTCGTACTACATGCGATCGGGAATGT CTGGCGCCACTGCATCGGCATCCGCTGTGGCCC GAGGCGCTGCCTCGCCGGGCTCCGAGGTCTACG AGCCATTAACACCCAAGAATGACGAAAGTCCG AGTCTGTGTGGCATCGGCATCGGCGGACCTTGC GCCATCGCCGTTGGCGAGACGGAGGCGGCCGA CGACATGGACGACGGAACGAGCAAGAAGACGA CGCTACAGATCTTGGAGCCTTTGAAGGGTCTGG ACAAGAGCTGCGACGATGGCAGTAGCGACGAC ATGAGCACCGGAATAAGAGCCTTAGCAGGAAC CGGAAATCGTGGAGCGGCATTTGCCAAATTTGG CAAGCCTTCGCCCCCACAAGGCCCTCAGCCGCC CCTCGGAATGGGGGGCGTGGCCATGGGCGAATC GAACCAATATCAATGCACGATGGATACGATAAT GCAAGCGTATAATCCCCATCGGAACGCCGCGGG CAACTCGCAGTTTGCCTACTGCTTCAATTAGCG GTCCG	Geneblock of BspEI to RsrII fragment required for the ΔQ truncation of Bicoid rescue plasmid
ΔQ_GFP	TCCGGAGGGGGGCCAGGACCTGGGTCGACCAA TGTCAATGGCGGCGCCAGCGCCTGTCGCGTCCT GGTCAAGGACGAACCGGAGGCCGACTACAACT TCAACAGCTCGTACTACATGCGATCGGGAATGT CTGGCGCCACTGCATCGGCATCCGCTGTGGCCC GAGGCGCTGCCTCGCCGGGCTCCGAGGTCTACG AGCCATTAACACCCAAGAATGACGAAAGTCCG AGTCTGTGTGGCATCGGCATCGGCGGACCTTGC GCCATCGCTGTTGGCGAGACGGAGGCGGCCGAC GACATGGACGACGGAACGAGCAAGAAGACGAC GCTACAGGTCAGGCATGAGTCCACAACCTTTTT TGATCTCTTGATTCTGAGTGTGGCGTTTATAAAT TGAAGCTTTAAGCTTTGTAACTTTCAAACTGTCT GGTTTGAGATGTTATTCTGAAAGTACTTCTATTT CCGATCGATGAGATTTGGGAGTTCTTCAATATTT AACATTTAACTTATTAAGTTTTTGTTTTCTAAAT TAGACATGGCATTTCTGAAAGGGAAGTACAAGT GTTAAAGATGTATTTTAATATAGAATTTGTATCA AAGGTTAAGATTTCAACCGTTTGAAAGCCCTTA GTTTTCAGGGTTTTTTACTTTTTTATTCATGTAAT CACTCTTAATACACTGCAAGTTAAAATAGCATT TCTTTGACCAGAAAAATAAGATCTATGCATTTT	Gene block of BspEI to MluI fragment required for the ΔQ truncation of eGFP-Bcd plasmid

	AAAAGTGAAAACAGACTCATATGCTGATGAAC ATTTTTAGCTATAAATTGTAACAATAATTTAGCA ATTTCAATTGAATTTATTTATGTTCTAAATGCGT TCGCTCTCTCCCTAGATCTTGGAGCCTTTGAAGG GTCTGGACAAGAGCTGCGACGATGGCAGTAGC GACGACATGAGCACCGGAATAAGAGCCTTAGC AGGAACCGGAAATCGTGGAGCGGCATTTGCCA AATTTGGCAAGCCTTCGCCCCCACAAGGCCCTC AGCCGCCCCTCGGGATGGGGGGCGTGGCCCTGG GCGAATCGAACCAATATCAATGCACGATGGATA CGATAATGCAAGCGTATAATCCCCATCGGAACG CCGCGGGCAACTCGCAGTTTGCCTACTGCTTCA ATTAGCCTGGACGAGAGGCGTGTTAGAGAGTTT CATTAGCTTTAGGTTAACCACTGTTGTTCCTGAT TGTACAAATACCAAGTGATTGTAGATATCTACG CGT	
ΔQAC_GFP	TCCGGAGGGGGGCCAGGACCTGGGTCGACCAA TGTCAATGGCGGCTAGCCTGGACGAGAGGCGTG TTAGAGAGTTTCATTAGCTTTAGGTTAACCACTG TTGTTCCTGATTGTACAAATACCAAGTGATTGTA GATATCTACGCGT	Gene block of BspEI to MluI fragment required for the ΔQAC truncation of eGFP-Bcd plasmid
ΔC_GFP	TCCGGAGGGGGGCCAGGACCTGGGTCGACCAA TGTCAATGGCGGCCAGTTCTTCCAGCAGCAGCA GGTCCATAATCACCAGCAGCAACTGCACCACCA GGGCAACCACGTGCCGCACCAGATGCAGCAGC AGCAACAGCAGGCTCAGCAGCAGCAATACCAT CACTTTGACTTCCAGCAAAAGCAAGCCAGCGCC TGTCGCGTCCTGGTCAAGGACGAACCGGAGGCC GACTACAACTTCAACAGCTCGTACTACATGCGA TCGGGAATGTCTGGCGCCACTGCATCGGCATCC GCTGTGGCCCGAGGCGCTGCCTAGCCTGGACGA GAGGCGTGTTAGAGAGTTTCATTAGCTTTAGGT TAACCACTGTTGTTCCTGATTGTACAAATACCA AGTGATTGTAGATATCTACGCGT	Gene block of BspEI to MluI fragment required for the ΔQ truncation of eGFP-Bcd plasmid

Table 8: List of the gene blocks used for cloning, their 5' to 3' sequences, and their purpose.

3.8 Probes and Antibodies Used in Immunostaining and *in situ* Hybridization

Name of the Probe	**Sequence**
hb-dig	Whole *hb* coding region anti-sense sequence
eve-dig	1 kb *eve* coding region anti-sense sequence

Table 9: List of the probes used for in situ hybridization and their sequences.

Name of the Antibody	**Manufacturer**	**Catalog Number**
Rabbit anti-Bcd Antibody	Home-made	N/A
Goat anti-rabbit Antibody, Alexa Fluor 488-conjugated	Invitrogen	A11008

Table 10: List of the antibodies used for immunostaining.

4. METHODS

4.1 *Drosophila melanogaster* Fly Stock Maintenance

The fly lines used in this study are listed in Table 1. The fly stocks were kept in narrow *Drosophila* vials (20 mm) containing fly food in an 18°C room. The room's humidity was monitored and kept between 50-55%. The stocks were flipped every 1-2 weeks, depending on the health of the food.

To increase their numbers for experiments, the flies were transferred from the vials to 250 mL glass bottles containing fly food and grown at 25°C. The bottles were flipped every 2-3 days until the number required by the experimental setup was reached.

The fly food was prepared by mixing the dry ingredients with water and boiling it to activate the agar. After the mixture reached a temperature below 67°C, Propionic Acid and 10% Nipagin solutions were added, and the food was distributed to either vials or bottles. After the food was covered with a cheesecloth, it was let dry at room temperature overnight and plugged with cotton. Excess bottles and vials were covered with bags and kept at +4°C until needed.

4.2 Embryo Collection

Embryo collection cages were prepared from plastic sample collection containers. The bottom of the container was cut and melted onto a 106 μm stainless steel mesh to allow air to flow into the cage. Apple juice agars were used to provide an egg-laying surface for the flies. To feed the flies in the cage, yeast paste was spread to the center of the plate. The apple juice agar was prepared by mixing agar with dH_2O and boiling it 3 times. After the mixture was boiled, pre-warmed apple juice was added and boiled once more. The mixture was left to sit at a 60°C oven to remove the foam and then poured into 60 mm cell culture dishes. They were stored in a humid box at +4°C and warmed to room temperature before use.

The flies were transferred into the cages at least 2 days before the collections started to allow them to get used to the container. The cage opening was closed with the apple juice agar plates, and the cage was kept in a 25°C incubator with 12 12-hour day-night cycle.

Depending on the experiment, the embryos were collected by changing the plates after a specific duration.

4.3 Cuticle Preparation

Embryos were collected for 4-5 hours and incubated between 22-24 hours to reach the first instar larval stage. 4% bleach (1:1 dilution of 8% hypochlorite with dH_2O) was applied for a maximum of 2 minutes by swirling to dechorionate the embryos. The embryos were collected by passing through a stainless-steel mesh and washed with dH_2O to remove any residual bleach. They were then transferred into a 1.5 mL tube containing 1:1 mixture of heptane and methanol using a dry brush.

The tube was then shaken vigorously for 30 seconds for devitellinization of the embryos, and the solution was left to settle for a while. The upper and mid-phase of the solution was discarded and the embryos were washed twice with methanol. After removing the second wash, 0.1% Triton-X was added to the embryos for rehydration. The embryos were then transferred to a glass slide with some Triton-X solution. The excess Triton-X was dried using filter papers, and the embryos were mounted using a 1:1 mixture of Hoyer's Mounting Medium: Lactic Acid enough to cover the slide. The coverslip was placed after the embryos were dispersed individually on the slide using a toothpick. The slide was then incubated at 60°C for at least 24 hours to allow for digestion of the insides of the larvae, and two 0.25 ₺ coins were placed on them after a few hours to flatten the larvae.

The larvae were visualized using Zeiss Axio Imager.A1m upright microscope. The light path of the Brightfield microscope was obstructed using a 1 TL coin to allow Darkfield

imaging. The whole larvae images were taken with a 10x lens, while the head zoom images were taken with a 20x lens.

4.4 Embryo Fixation

The embryos were collected for 2 hours and incubated for 1 hour to enrich embryos at nuclear cycle 13-14. They were then dechorionated by applying 4% bleach on the plate for a maximum of 2 minutes, and the embryos were collected by passing through a mesh sieve. After washing the remnants of bleach with dH_2O, the mesh sieve was placed inside a 20 mL scintillation vial containing 8 mL fixation solution to transfer the embryos into the solution, and the mesh was removed. The vial was shaken vigorously for 25 minutes. After the lower phase of the solution was discarded, 8 mL methanol was added, and the vial was shaken vigorously for 1 minute. The upper and mid-phase of the solution was discarded and 4 mL of methanol was added to the vial. After the embryos had settled, most of the methanol was discarded while keeping the vial tilted, and the embryos were transferred to a 1.5 mL tube. In the tube, they were washed 2 times with methanol and 2 times with ethanol. After the last ethanol was removed, 1 mL ethanol was added, and the embryos were stored in a -20 °C freezer until further use.

4.5 *In situ* Hybridization of *Drosophila* Embryos Using RNA Probes

The fixed embryos were rinsed twice with ethanol and twice with methanol. They were then incubated by rocking for 5 minutes in 1:1 methanol: PBTw + 5% formaldehyde solution. Then, they were incubated by rocking in PBTw +5 % formaldehyde solution for 25 minutes. The fixative was washed off with PBTw thrice by rocking for 5 minutes. This was followed by 8 minutes of incubation in PBTw containing 4 µg/mL Proteinase K. After washing 3 times by rocking in PBTw for 5 minutes, they were post-fixed in PBTw +5 % formaldehyde solution for 25 minutes by rocking. The embryos were washed 4 times for 5 minutes each in PBTw and incubated in 1:1 PBTw: Hybridization Solution for 5 minutes. They were pre-hybridized by incubating in 1 mL Hybridization Solution in a 55°C water bath for 2 hours. The probe was diluted in Hybridization Solution, heated to

80°C for 3 minutes, and put immediately on ice. After removing the Hybridization Solution from the embryos, they were incubated with probe overnight in the 55°C water bath.

The next day, the embryos were washed twice with Hybridization Solution for 30 minutes in a 55°C water bath. Then, they were washed for 10 minutes with 1:1 PBTw: Hybridization Solution by rocking. After washing 4 times with PBTw for 10 minutes by rocking, they were incubated in Alkaline Phosphatase conjugated anti-digoxigenin or anti-fluorescein antibodies diluted 1:2000 in PBTw for 2 hours. After the incubation, they were washed 4 times with PBTw for 10 minutes each, followed by 2 washes with AP Staining Buffer for 5 minutes by rocking. The staining solution was prepared by adding 40 µL NBT/BCIP solution into 1 mL AP Staining Buffer. After removing the embryos' last wash, the staining solution was added and incubated in the dark by rocking. The embryos were checked for staining every few minutes under the stereo microscope. Once the embryos have become dark brown, PBTw was added immediately to stop the reaction. The stained embryos were washed twice with PBTw for 5 minutes each, once with 1 mL 1:1 PBTw: Ethanol for 5 minutes, once with Ethanol for 25 minutes, and once with 1 mL 1:1 PBTw: Ethanol for 5 minutes, by rocking. The residual ethanol was removed by washing with PBTw 3 more times for 5 minutes each by rocking.

The embryos were transferred onto a coverslip that had a guide slide underneath with lines on it. The embryos of the wrong age and bad condition were removed under the stereo microscope. A drop of Aqua/Polymount was added to the remaining embryos and mixed with the small amount of PBTw remaining to prevent drying and crystallization. After placing each embryo laterally on the coverslip in straight lines, the Aqua/Polymount was let harden at 37°C incubator. Once the mounting medium hardened, two drops of Aqua/Polymount were added on a slide, and the coverslip was mounted. The slide was left overnight for the mounting medium to solidify, and several 0.25 ₺ coins were placed on the slide to let embryos flatten. They were stored at room temperature. The slides were visualized under the Zeiss Axio Imager.A1m Brightfield microscope.

4.6 Immunostaining of *Drosophila* Embryos

The fixed embryos were rinsed twice with ethanol and twice with methanol. They were washed for 5 minutes by rocking with methanol and 5 minutes with 1:1 PBTw: Methanol solution. After washing them in PBTw 4 times for 10 minutes each by rocking, they were incubated in primary antibodies diluted in PBTw containing 1% BSA by rocking overnight at 4°C.

The next day, they were washed with PBTw 4 times for 10 minutes each and incubated with a secondary antibody diluted in PBTw for 1 hour at room temperature by rocking in the dark. After the incubation, ensuring they were not exposed to light, they were washed with PBTw 4 times for 10 minutes each by rocking. They were mounted, as explained in the previous section. The slides were kept in the dark and stored at -20°C. The samples were visualized using Leica SP8 Confocal Microscope with 20x Dry objective using LASX software.

4.7 Cloning

The backbone and the template for the Bicoid rescue injection plasmids was the piattB40-Bcd plasmid used in Liu et al., 2018. This plasmid contains two ΦC31-specific attB40 recombination sites, a Gmr-GFP reporter that is expressed in the eyes, and the 1.9 kb *bcd* promoter and the 0.8 kb 3'-UTR flanking the *bcd* cDNA sequence on its 5' and 3', respectively.

Some of the truncated *bcd* sequences were obtained via standard PCR using a forward primer flanked by a BspEI recognition site and a reverse primer flanked by a stop codon followed by an RsrII recognition site on the template plasmid (ΔQAC-, ΔAC-, and ΔC-Bcd) or on the truncated *bcd* plasmid (for ΔQC-Bcd, piattB40-ΔQ-Bcd). ΔQ-Bcd truncation was obtained by using a gene block from Gene Universal, Inc. All the remaining truncations were done via a modified version of the overlap-based site-directed

mutagenesis (SDM) deletion method depicted in Figure 7 (Wu et al., 2013). The 3'-fragment was obtained using a reverse primer with an overhang complementing the first 15 bases after the deletion site. The 5'-fragment was obtained using a forward primer with an overhang complementing the last 15 bases before the deletion site. Both fragments were PCR purified and used overlap-extension PCR reaction using Pfu High-Fidelity DNA Polymerase for 1 to 10 cycles, depending on the yield, in which no primers were used. The forward primer used for the 3'-fragment and the reverse primer used for the 5'-fragment were then added to the reaction, and a standard PCR was applied for 35 cycles to amplify the deletion fragment to be cloned. ΔQ-, ΔA-, ΔC-, ΔQA-, ΔQC-, ΔAC-, and ΔQAC-Bcd truncations were ligated through the BspEI and RsrII sites to the piattB40-Bcd plasmid, while ΔST- and ΔSTQAC-Bcd truncations were ligated through AscI and BspEI sites to the piattB40-Bcd and piattB40-ΔQAC-Bcd plasmids, respectively.

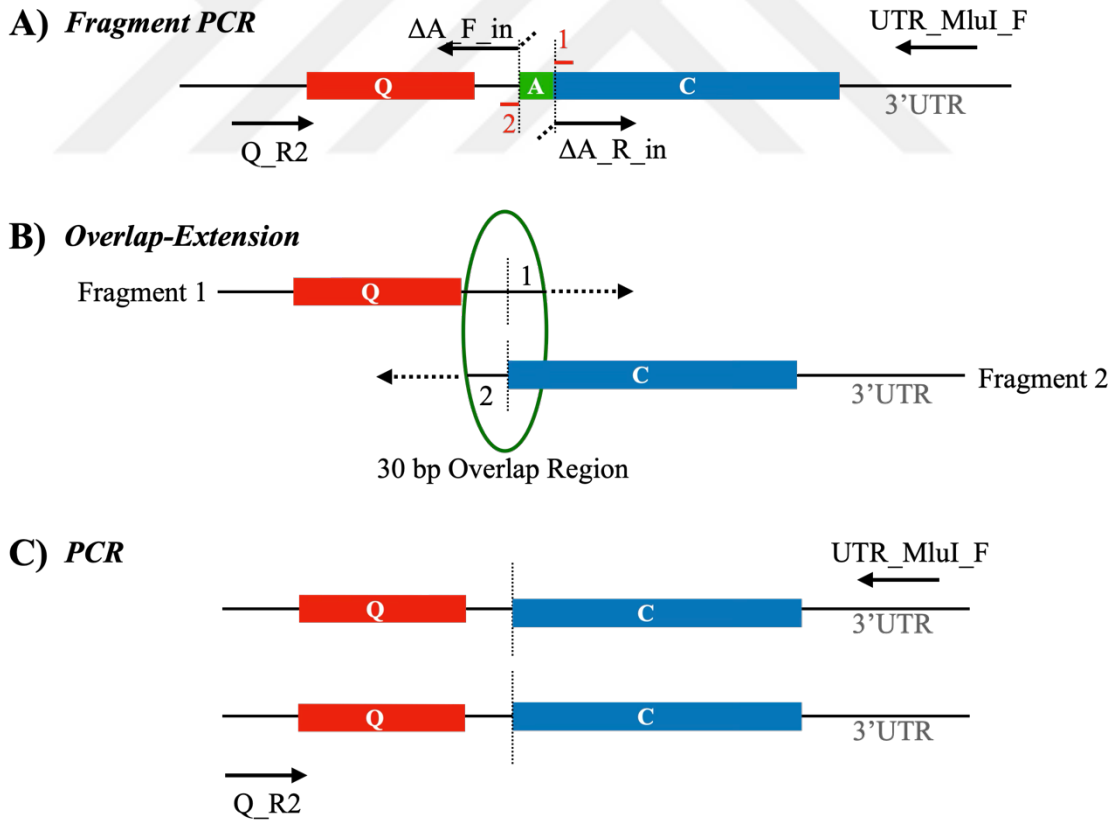

Figure 7: The SDM approach used for ΔA deletions. A) For the fragment PCR, the SDM primers were designed so that the deletion region (A) would not be included in the product and paired with primers that will cover the region of interest. Region 1 and 2 correspond to the first 15

bp 3' of the deletion region and last 15 bp 5' of the deletion region, respectively. **B)** The obtained fragments that had now contained a 30 bp overlap regions were put through an overlap-extension reaction for 1 to 10 cycles. **C)** Pairs for the SDM primers were added for a PCR reaction to amplify the now unified fragment.

The backbone and the template for the eGFP-tagged Bicoid injection plasmids was the piB-attB40-eGFP-bcd plasmid. This plasmid contains 2 ΦC31-specific attB40 recombination sites, the 1.9 kb *bcd* promoter followed by eGFP, a linker sequence, the *bcd* coding region, and the 0.8 kb 3'-UTR.

ΔQ-, ΔC-, and ΔQAC-eGFP-Bcd truncations were obtained by using a gene block from Gene Universal, Inc. The remaining truncations were done via SDM. ΔQ-, ΔA-, ΔC-, ΔQA-, ΔQC-, ΔAC-, and ΔQAC-eGFP-Bcd truncations were ligated through the BspEI and MluI sites to the piattB40-Bcd plasmid, while ΔST- and ΔSTQAC-eGFP-Bcd truncations were ligated through NheI and MluI sites to the piB-attB40-eGFP-bcd and piB-attB40-eGFP-ΔQAC-bcd plasmids, respectively.

After the ligation of fragments to the backbone, the reaction was used for transforming DH5α Ultra Competent E. coli cells prepared by the Inoue method (Green & Sambrook, 2020). The reaction was added to the bacteria tube and incubated for 20 minutes on ice. The bacteria were then heat-shocked by applying 42°C temperature for 45 seconds and immediately placed on ice. After incubation on ice for 5 minutes, LB broth was added to the bacteria and incubated for 45 minutes by shaking at 37°C. The culture was centrifuged at 4,000 rpm for 1 minute, and the LB broth excess was discarded. The bacteria pellet was resuspended in the remaining LB broth, spread onto an LB agar containing 100µg/mL ampicillin, and incubated overnight at 37°C.

The colonies formed the next day were picked by a pipette tip, resuspended in 5 µL ddH_2O, and stored at +4°C. Some of the resuspended colony was used in colony PCR reaction. The primers for the colony PCR were picked so that one primer would bind to the template while the other would bind to the insert. As the cloning was done using a full-length sequence containing plasmid as the backbone, the primer binding to the insert was

selected so that the size difference would show a successful deletion. The template plasmids were used as the positive control. Once a colony was screened positive for the insert, the remaining resuspension was streaked on an LB agar plate containing ampicillin and incubated overnight at 37°C. The next day, a single colony from the plate was selected and inoculated into 3 mL LB broth containing Ampicillin (100µg/mL) and cultured at 37°C by shaking. The plasmid isolation was done via the Alkaline Lysis method (see 4.8). The plasmids were digested with the cloning enzymes as a secondary control. The plasmids passing this confirmation were cleaned via PCR purification. Their sequence was then confirmed via Sanger sequencing by Macrogen Europe and analyzed via the Multiple Alignment using Fast Fourier Transform (MAFFT) alignment program on the Benchling electronic laboratory notebook platform. Sequence confirmed plasmids were prepared by Midiprep, following the producer's protocol, with 100 mL starting volume and stored at 1 µg/µL concentration at -20°C.

4.8 Plasmid DNA Isolation via Alkaline Lysis

Resuspension, lysis, and neutralization solutions were prepared before starting the protocol. Resuspension and neutralization solutions were kept on ice, while the lysis solution was kept at room temperature.

The bacteria culture was transferred into microcentrifuge tubes and centrifuged at 13,000 rpm for 1 minute at room temperature, and the supernatant was carefully discarded. This was repeated until all of the culture was centrifuged. The pellet was resuspended with resuspension solution by pipetting. Lysis buffer was added, and the tube was inverted several times. After incubating on ice for 5 minutes, neutralization buffer was added, the tube was inverted several times, and let incubate for 5 minutes on ice. The lysate was then centrifuged at 13,000 rpm for 5 minutes at +4°C. The supernatant was carefully transferred to a new tube, and 100% ethanol was added. The mixture was incubated at -80°C for at least 1 hour and centrifuged at 13,000 rpm for 15 minutes at +4°C. The supernatant was discarded, the pellet was washed with ice-cold 70% ethanol and centrifuged at 13,000 rpm for 5 minutes at +4°C. The supernatant was poured off, and the pellet was dried until it

became transparent. The pellet was then resuspended with ddH_2O containing 20 µg/mL RNase A. The plasmid was quantified by gel electrophoresis and stored at -20°C.

4.9 Microinjection of *Drosophila* Embryos

The injection plasmids were prepared by diluting to 200 ng/µL. The diluted plasmid was centrifuged at 5,000 rpm for 1 minute to settle all the big particles that could be clogging the needle. The cage was left to lay eggs for 30 minutes at 25°C. The embryos were then collected using sharp forceps and transferred into a dH2O-containing 40 µm cell strainer. Once sufficient embryos were collected, they were washed once with dH_2O, once with 95% ethanol, and twice with dH_2O. An 18 mm x 18 mm coverslip was placed on a slide. The embryos were transferred onto the coverslip with the help of a 0 number brush and aligned on the side of the coverslip so that the posterior of the embryo would face the micromanipulator. After drying the excess dH_2O with the help of the brush, the embryos were checked for their attachment onto the coverslip by pushing gently with the brush. Once dry enough, they were covered with 1:2 of Halocarbon 700 Oil: Halocarbon 27 Oil mixture and some of this mixture was let flow to the slide.

The borosilicate capillaries were pulled using Narishige PC-100 vertical puller with 2-step pulling setting. The capillary was first heated to 81°C and pulled with 93 grams weight, followed by heating to 72°C and pulling with 93 grams weight. The two needles formed through this process were carefully removed from the puller and placed in a petri dish with a play dough line to place the needles with an angle to protect the tip.

Narishige IM-400 pneumatic microinjector was used for microinjections. The micromanipulator was mounted onto the Nikon Eclipse Ti2 inverted microscope. A needle was filled with plasmid DNA from the back opening using a micro-loader tip. The needle was then attached to the micromanipulator, and its angle was adjusted so that the needle could enter the embryos as horizontally as possible. The embryo slide was placed on the stage, and the microscope was focused on the border of the coverslip and slide with the Halocarbon Oil mixture.

The needle was adjusted to be in the frame of vision, the internal pressure balance was set to 5 kPa, and the needle was gently lowered to the oil. The flow from the needle was observed via injecting with high pressure to the oil, and if there was no flow, the tip was opened by gently touching the edge of the coverslip. The injection pressure was set so that a volume of no more than 1/3 of the posterior of the embryos was injected. Injection was performed by moving the embryos to the needle, entering the embryos slightly under the most posterior point, and pulling back after turbidity was observed inside the embryos. Embryos that had pole cells formed were not injected and discarded by crushing with a blunt injection needle after the injection was completed. The number of successful injections with low leakage from the posterior was counted under the stereo microscope, and the coverslip was transferred into a food vial such that the posterior of the embryos would touch the food surface. The embryos were then incubated in a 25°C humid chamber until second instar larvae were observed.

The surviving flies were collected and crossed with balancer lines to screen for transformant flies by the color and fluorescence of their eyes.

4.10 Genomic DNA Isolation from a Single Fly

The fly was selected and placed in a 500 µL tube. It was then crushed with a 200 µL tip containing 200 µg/mL Proteinase K containing Squishing Buffer without releasing the buffer onto the fly. Once it was crushed enough, the buffer was released onto the fly and incubated at room temperature for 30 minutes. Proteinase K in the buffer was deactivated by incubating the tube at 95°C for 2 minutes. 2 µL of the obtained isolate was directly used in PCR reactions for screening and sequencing purposes.

4.11 Phylogenetic Tree Generation and Analysis

The phylogenetic tree was formed by combining the two previously published trees in Liu et al., 2018 and Clark et al., 2007. Bicoid protein amino acid sequences of 12 *Drosophila* species and 7 other fly species were downloaded from the National Center for Biotechnology Information (NCBI) Protein Database.

Annotation of the IDR domains was done manually, covering the repeat regions from the first amino acid to the last one. All sequences were aligned to the *D.melanogaster* Bicoid sequence using the MAFFT alignment program on the Benchling electronic laboratory notebook platform, and their sequence conservation was measured. Changes in the lengths and identities of the IDR domains were analyzed manually.

5. RESULTS

5.1 Bicoid Is Not Well Conserved Across Species Except for Its Homeodomain

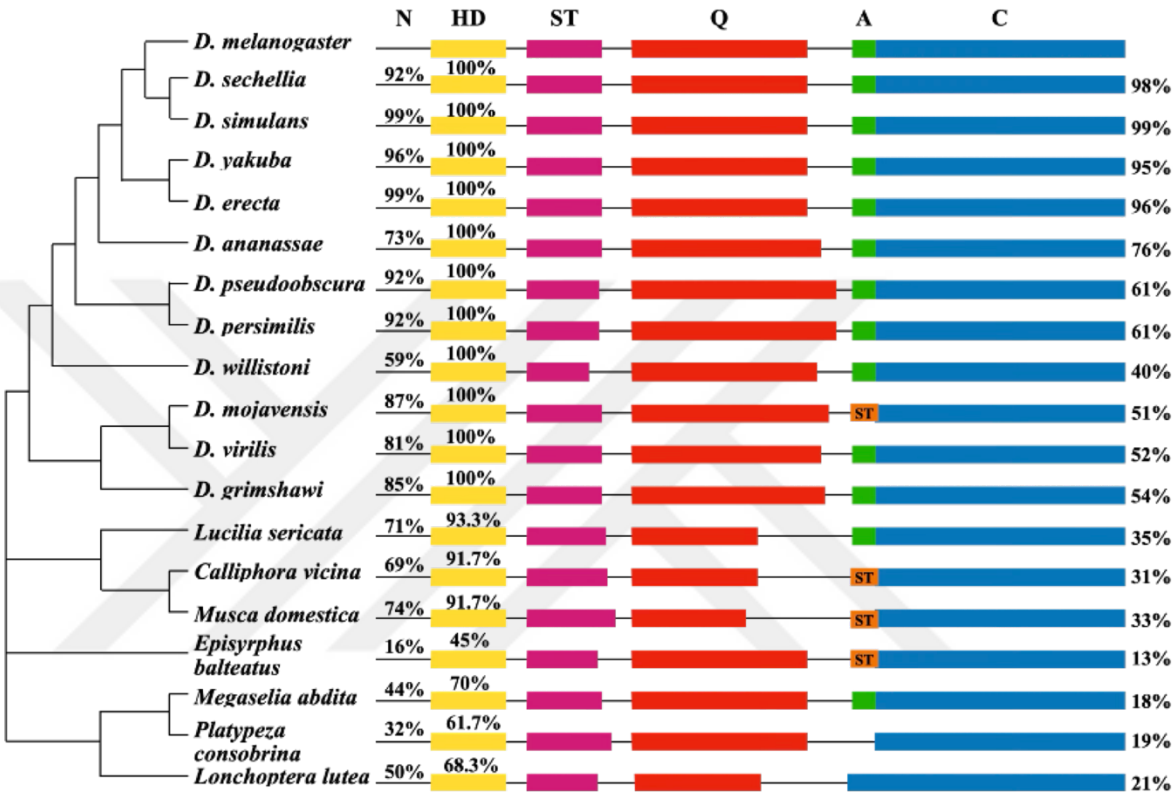

Figure 8: Phylogenetic tree showing the conservation of Bicoid N-terminal, Homeodomain, and C-terminal domains. The Homeodomain (HD), ST-rich region (ST), Q-rich region (Q), A-rich region (A), and acidic C-terminal region (C) are annotated by yellow, pink, red, green, and blue boxes, respectively. Orange boxes annotate the ST-rich regions found instead of the A-rich region in some species. The length of each region is proportional to the length of the boxes. Similarity of the region N-terminal to HD, HD, and region C-terminal to HD of each species' Bicoid to *D.melanogaster* Bicoid are given above the N-terminal region, above the HD region, and next to the C-terminal, respectively.

Bicoid plays a crucial role in embryonic development. However, it is not ubiquitous in the Dipterans. It is important to know how crucial each domain is for its activity. One way to determine the important domains is via phylogenetic analysis (see Methods for the phylogenetic tree construction). When the Bicoid aa sequences of 12 *Drosophila* species and 7 other species were annotated (see Appendix III) and compared, as can be seen in

Figure 8, it was clearly seen that the HD was the most conserved region of the protein. Although they were not highly conserved and their lengths varied, the presence of ST-rich and Q-rich domains and the acidic nature of the C-terminal were also conserved. On some occasions, the A-rich region was replaced with ST repeats, not only in other flies like *Calliphora vicina* (*Cv*), *Musca domestica* (*Md*), and *Episyrphus balteatus* (*Eb*) but also in *Drosophila mojavensis*.

5.2 Bicoid Homeodomain Alone Is Not Sufficient for Its Developmental Activity

Despite high conservation of the HDs the rest of the Bcd protein is not conserved outside of the Drosophilids (Figure 8). Although we know that a HD swap between Bcd and Zen Homologs with highly diverged sequences (<40 % similarity) confer most of Bcd functions it does not fully rescue all the Bcd functions (Q. Liu et al., 2018). To test whether Bicoid homologs with diverged effector domains can rescue *bcd*-null (bcd^{E1}) *D.melanogaster* (*Dm*) embryos, we used Bicoid swap flies that carried either *Ls* or *Cv* Bicoid sequences with the constructs in Figures 9B and 9C to see if they could rescue the activity in *bcd*-null (bcd^{E1}) background *D.melanogaster*. Interestingly, *Cv* Bicoid did not rescue the Bicoid activity, and the *bcd*-null larval phenotype with duplicated telson persisted (Figure 9B). On the other hand, *Ls* Bicoid also did not rescue the activity completely; however, it rescued the anterior polarity and allowed the thoracic segment development (Figure 9C).

This phenotype was confirmed by the expression patterns of Bicoid target genes *hb* and *eve*. In *Cv* Bicoid, the anterior *hb* expression pattern showed the duplication of the posterior expression (Figure 9B). The same observation can be made with the disruption of the *eve* pattern and the anterior shift of the first stripe (Figure 9B). The partial rescue observed with *Ls* Bicoid had also confirmed by the gene expression patterns and showed an anterior shift of the expression borders instead of posterior duplication (Figure 9C).

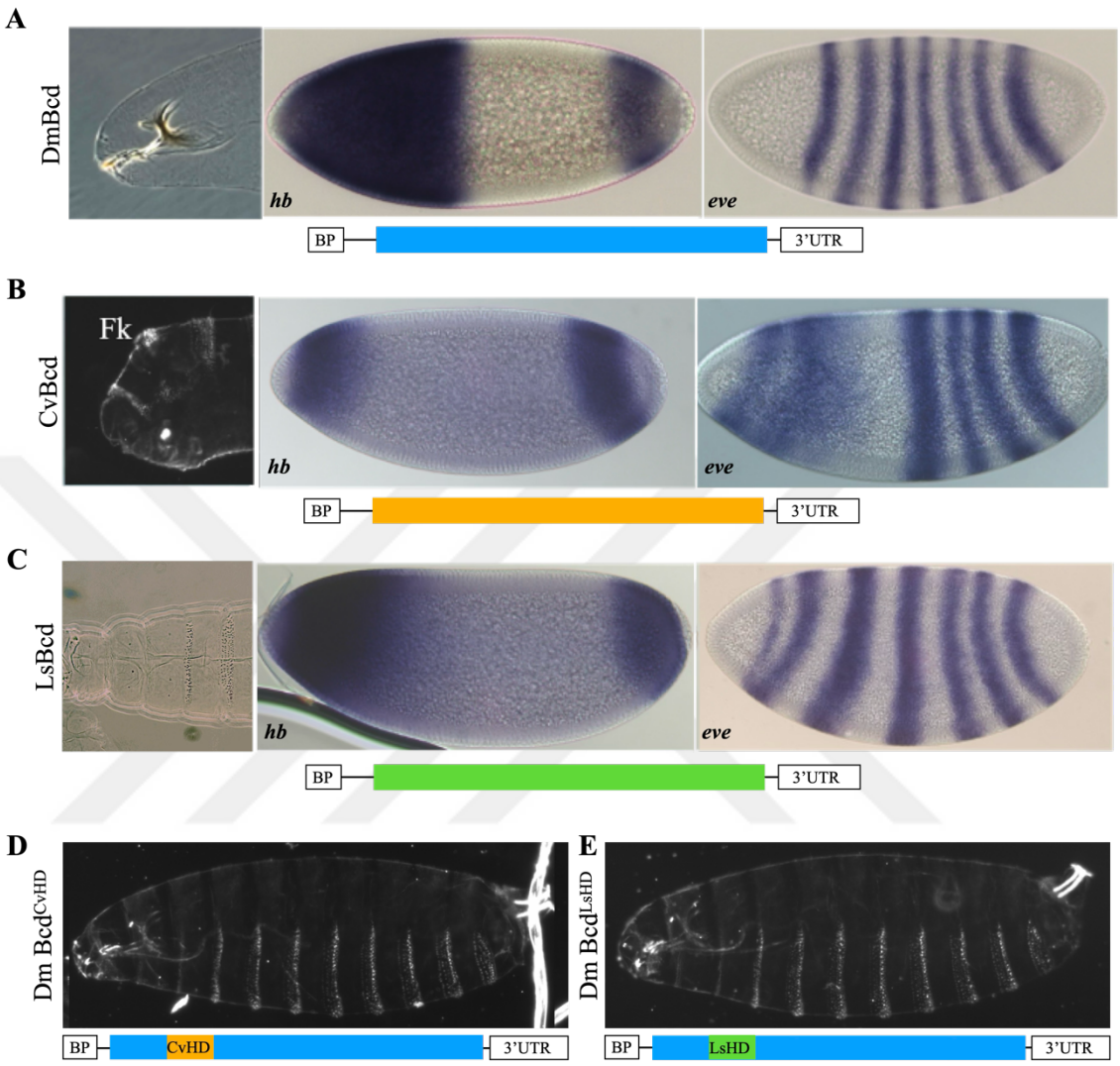

Figure 9: Bicoid and Bicoid HD swap experiments. The constructs and the larval phenotypes of L1 larvae (first column) and gene expression patterns (middle and right column) of Bicoid rescue lines carrying either *D.melanogaster* (*Dm*) Bicoid **(A)**, *Lucilia sericata* (*Ls*) Bicoid **(B)**, *Calliphora vicina* (*Cv*) Bicoid **(C)**, or *Dm* Bicoid with CvHD **(D)**, and LsHD **(E)**. Anterior is on the left and dorsal is up. Fk: Filz korper.

Although these two species (*Ls* and *Cv*) had a very similar sequence to each other, they do not have the exact same HD as *Dm*. *Cv* HD differs by 5 aa residues and Ls HD differs by 4 aa residues from Dm Bicoid. To understand if the failure to rescue Bicoid activity was due to the minute differences in HD, HD swap of *Dm* Bicoid with *Cv* Bicoid HD (*Cv* HD) and *Ls* Bicoid HD (*Ls* HD) was used (Figures 9D and 9E). Both *Cv* HD (Figure 9D)

and *Ls* HD (Figure 9E), replacing the *Dm* HD, rescued the Bicoid developmental activity and led to the development of a complete anterior pole with cervical and thoracic structures. Thus, we conclude that the domain responsible for the failure of rescue in *Cv* Bicoid and *Ls* Bicoid was not the HD but the remaining domains.

5.3 The Role of Effector Domains on Bicoid's Activity Can Be Uncovered with Effector Domain Truncations

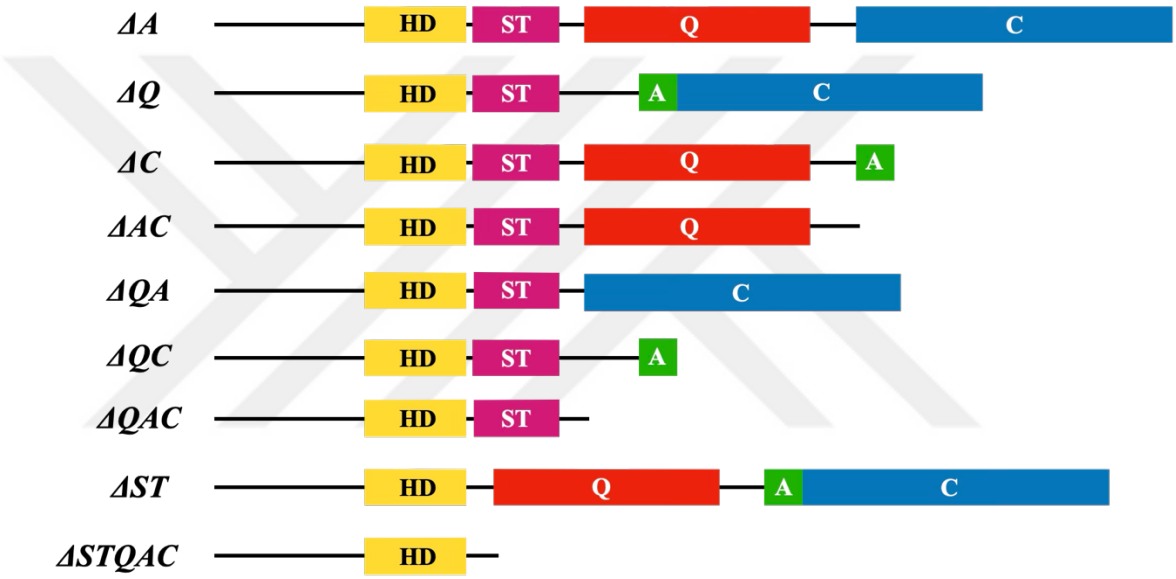

Figure 10: Construct designs for Bicoid effector domain truncations. The truncation constructs and the domain(s) that they contain.

The observation that HD was not responsible for the failure to rescue Bicoid activity, we decided to examine other differences between *Ls* Bicoid and *Cv* Bicoid to see what led to the partial rescue observed in *Ls* Bcd. The only other difference is found to be the A-rich region that is present in *Ls* but replaced with an ST-rich region in *Cv* (Figure 8). Thus, we concluded that the responsible region for the rescue failure lied in the effector domain on the C-terminal of HD and not the HD itself.

In order to understand the role of effector domains in Bicoid activity, we designed 9 different constructs lacking either one or multiple effector domains and cloned them into

maternal expression construct between the Bicoid promoter and 3'UTR to ensure maternal expression and anterior localization of our transgenes (Figure 10). The plasmid also contains 2 attB sites for RMCE. We also added ST-rich region deletions to better understand the ST-rich region that is already known to be important for Bicoid activity and stability (Janody et al., 2000). The whole effector domain deletion mutant (ΔSTQAC) was designed to understand the *bcd*^{E1} allele better and shed light on its loss of activity (see Discussion).

5.4 Generating the Bicoid Effector Domain Mutant Flies

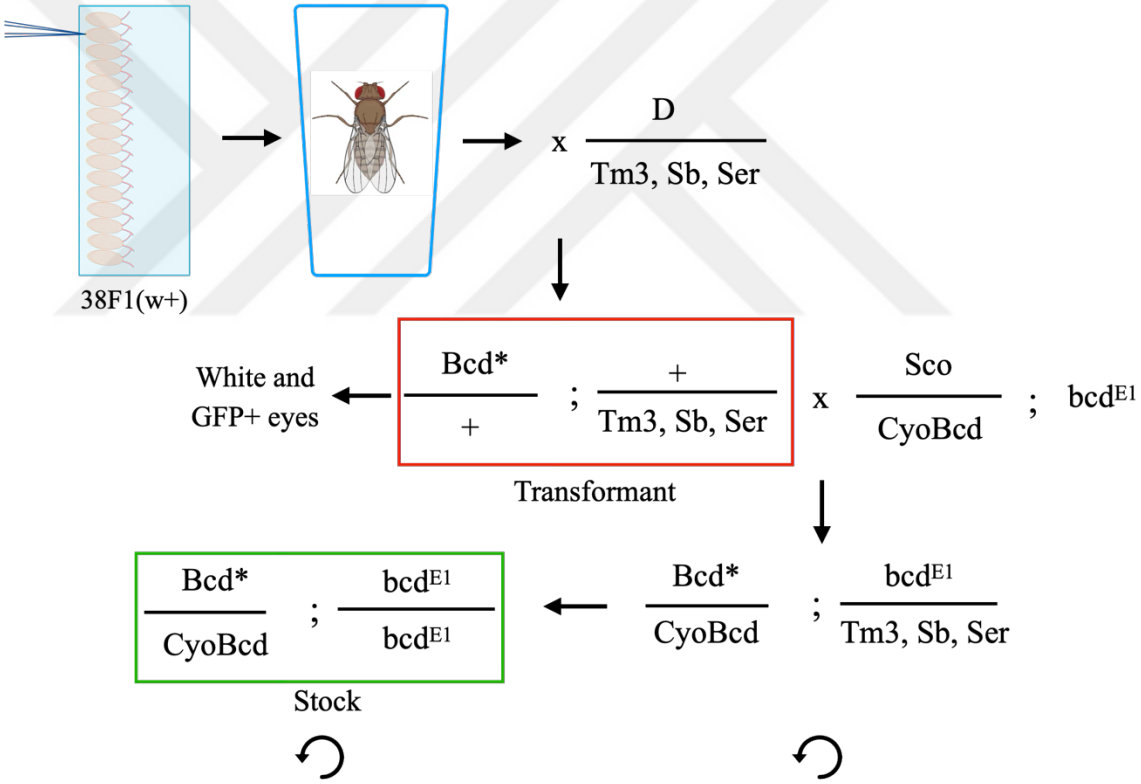

Figure 11: Bicoid mutant transgenic fly generation. The microinjection and balancer chromosome crosses planned for generating the Bicoid rescue lines.

In order to examine the effect of these mutations *in vivo*, we generated Bicoid mutant flies via ΦC31-mediated RMCE. We transformed the germ line of the embryos carrying attP sites on the 38F1 locus on the II. chromosome via microinjection. The fly line used for injection also expresses the ΦC31-integrase driven by the *nanos* promoter in the posterior of the embryo.

To be able to screen the transformant flies and track the crosses to be made on the III. chromosome, we crossed the surviving flies with the III. chromosome balancer line, D/Tm3, Sb, Ser (Figure 11). We screened the transformant flies for the loss of *mini-white* and GFP autofluorescence in the eyes and crossed those flies with the II. chromosome balancer line that has the bcd^{E1} background (Sco/CyOBcd; bcd^{E1}) (Figure 11). In order to minimize the risk of cross-over, we refrained from using transformants with D wing phenotype and used the ones with Sb bristles and Ser wings (Tm3) as much as possible ($bcd^{truncated}$/+;Tm3, Sb, Ser/+). We chose the flies with curly wings (CyOBcd), Sb bristles and Ser wings (Tm3) and GFP-autofluorescent eyes for self-cross (Figure 11). From this cross, we used the flies with curly wings (CyOBcd), normal wings, and normal bristles for self-cross so that the stock line can be obtained (Figure 11). This way, we were able to place our deletion mutants on the bcd^{E1} background while balancing it with the CyO balancer chromosome expressing wild-type Bicoid. Homozygous mutant flies can be selected for the loss of curly phenotype (normal wing phenotype) to be used in experiments.

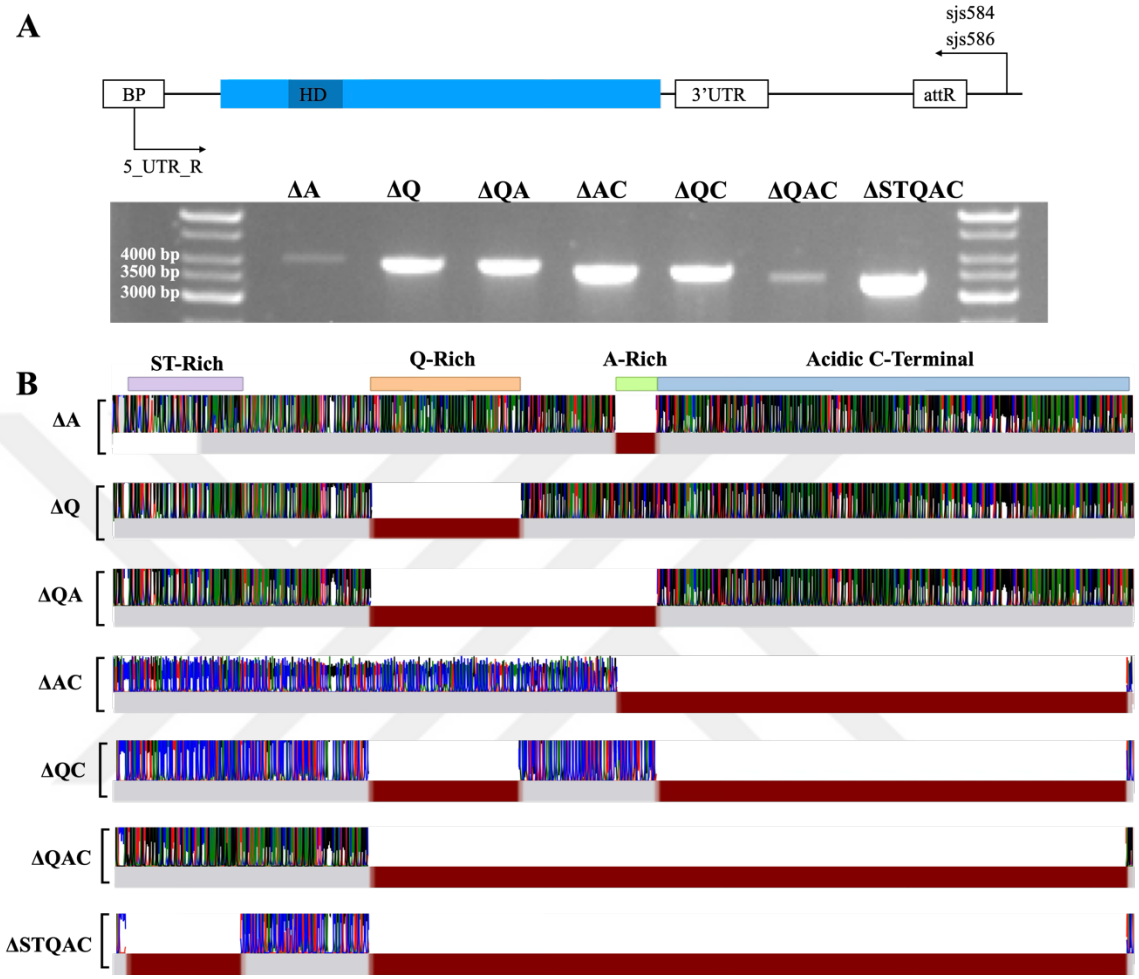

Figure 12: Confirmation of inserts in transformants. A) Primer binding sites and the PCR products obtained from the genomic DNA of the transformant flies for PCR confirmation. BP: Bicoid promoter. **B)** Sequencing result of all 7 transformants aligned to *Dm* Bcd cDNA sequence (domains are annotated at the top). The red bars at the bottom represent deletion, while the grey bars represent correctly aligned sequences.

In order to confirm a successful recombination into the recombination site, we were able to use the loss of the marker gene (*mini-white*) and gain of GFP expression in the eye from the insert (see Methods for the details on the construct). However, the sequence integrity should also be confirmed. To confirm that the sequences were intact and no mutations were observed during RMCE, we first used primers binding to the 5' and 3' of the attP sites on the II. chromosome and one binding to the Bicoid promoter to confirm the direction of the insert. Then, we used the direction information to amplify the inserted

region to confirm its integrity by Sanger sequencing. The bands from the amplified regions can be seen in Figure 12A. Sanger sequencing results aligned to wild-type Bicoid show that the correct regions have been deleted without any issues (Figure 12B). We were able to successfully generate 7 Bicoid mutant lines: ΔA, ΔQ, ΔQA, ΔAC, ΔQC, ΔQAC, and ΔSTQAC. Their transgenic stock line and Sanger sequencing confirmation status can be seen in Table 11. Unfortunately, due to the low number of surviving adults from the microinjections, we could not generate ΔC-Bicoid and ΔST-Bicoid lines.

Construct	Transgenic Line	Sequence Confirmation
ΔC-Bicoid	✗	N/A
ΔA-Bicoid	✓	✓
ΔQ-Bicoid	✓	✓
ΔQA-Bicoid	✓	✓
ΔQC-Bicoid	✓	✓
ΔAC-Bicoid	✓	✓
ΔQAC-Bicoid	✓	✓
ΔST-Bicoid	✗	N/A
ΔSTQAC-Bicoid	✓	✓

Table 11: Bicoid rescue fly lines generated in this study and their current confirmation status. The status of Bicoid mutant transgenic stock lines and their confirmation via Sanger sequencing is given.

6. DISCUSSION

In this study, we first tried to identify the transregulatory modules of maternal transcription factor Bicoid. Comparisons of aa sequences of 12 *Drosophila* species and 7 other Cyclorrhaphan flies (Figure 8) revealed that Bicoid is not very well conserved except for its HD. Bicoid swap experiments between *Dm*, *Ls*, and *Cv*, and HD swap experiments between DmBcd, LsBcd, CvBcd, and Zen revealed that although it is important, HD is not sufficient alone for Bicoid activity (Q. Liu et al., 2018). In light of this finding, we moved our focus to the effector domains that are intrinsically disordered. IDRs have been identified as regions without a known or predictable stable 3D structure due to their flexible and dynamic nature (J. Liu et al., 2006). They mediate low affinity, high specificity multivalent molecular interactions (J. Liu et al., 2006). Transcription factors regulate different target genes via diverse molecular interactions, most of which are mediated through disordered effector domains. Bicoid regulates hundreds of genes and Bicoid transcription factor effector domains could also be mediating its transcriptional and developmental activity. Indeed, Schaeffer and colleagues generated effector domain mutant flies via P-element transposon system and observed that the larval phenotypes and Bicoid target gene expression (*hb*) were disturbed, although not completely lost (Figures 5 and 13) (Schaeffer et al., 1999). They saw larval phenotype and gene expression pattern of *hb* were only disturbed in the ΔC, ΔAC, and ΔQC mutants but not in ΔQAC mutant, which was explained by the ST-rich region providing the required negative charge in the C-terminal and rescuing the absence of the acidic C-terminal domain (Schaeffer et al., 1999). This hypothesis has not yet been tested, and the exact regulatory mechanisms of the IDRs on Bicoid activity is still unknown.

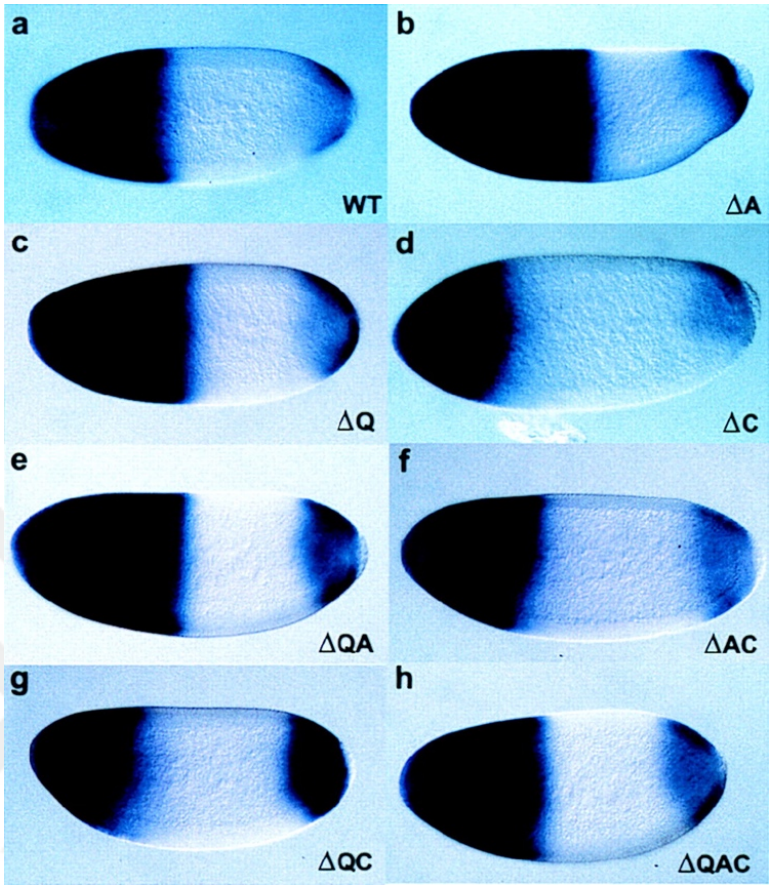

Figure 13: *hb* gene expression patterns of Bicoid IDR truncation flies. Anterior is on the left and dorsal is up. Adapted from Schaeffer et al., 1999. Copyright © 1999, The National Academy of Sciences.

In order to elucidate the role of IDRs in Bicoid activity in more detail, we cloned Bicoid truncation mutants lacking single or multiple IDR domains using standard molecular cloning techniques and SDM into a plasmid containing 2 attB recombination sites used for ΦC31-integrase mediated RMCE. In addition to Q-rich, A-rich, and acidic regions that were deleted in Schaeffer et al., 1999, we also truncated the ST-rich region, which is known to regulate Bicoid activity through phosphorylations (Janody et al., 2000). By truncating the complete C-terminal region (ΔSTQAC), we aim to understand the mechanism behind the rescue observed in the ΔQAC mutant flies, and by truncating only the ST-rich region (ΔST) we can observe if the effect of the ΔSTQAC mutant would be due to the loss of the ST-rich region alone. We were able to generate 7 out of 9 transgenic lines containing Bicoid truncation mutations by utilizing germ line transformation via microinjection and inserted Bicoid truncations into the 38F1 locus on II. chromosome.

After crossing into a bcd^{E1} allele background on the III. chromosome, to make our transgenes the only maternal *bcd* alleles with possible functionality, we generated Bicoid-rescue lines. The wild-type Bicoid expressed on this chromosome has previously been shown to be rescuing the embryonic development in Q. Liu et al., 2018 and many more studies. This gives an advantage over the flies generated in Schaeffer et al., 1999 where the P-element transposon system was used, inserting the construct to an uncharacterized region, and making the mutant constructs vulnerable to position effect.

Unfortunately, ΔST and ΔC mutant flies could not be generated due to the low number of injected embryos and the low survival rate of those embryos. Before continuing with further experiments, these lines should be generated by focusing on more injections with these constructs. From the previous study, neither the variation in larval phenotypes nor the expression patterns of Bicoid-target genes except for *hb* is known. After all of the mutant lines are generated, the effect of the loss of effector domains can be studied firstly by utilizing these flies in phenotypic analysis via cuticle preparation and gene expression analyses of Bicoid target genes such as *eve*, *kr*, *otd*, and *gt* in addition to *hb*.

Although the recombination to 38F1 system is a well-characterized system, it relies heavily on the rescue from the bcd^{E1} allele. The effect of the mutant *bcd* allele expressed in the background cannot be ignored. The mutant mRNA expressed from this allele can be detected but no protein product has been detected so far. This could be due to the changes in stability of the mutant protein or failure of antibodies to recognize, making it hard to characterize at which point the mutation implements its affect. Especially if any technique requiring pull-down such as chromatin immunoprecipitation-sequencing (ChIP-seq), immunoprecipitation (IP) and Mass-Spectrometry is to be utilized, any undetectable protein expressed in the background could interfere with the interaction of the mutant protein with DNA and proteins. This is why, we decided to take it a step further and utilize CRISPR-Cas9 system to generate the transgenic flies carrying mutant alleles on the endogenous *bcd* locus (Figure 14). For this purpose, we followed a 2-step approach, CRISPR-Cas9 followed by RMCE, in which the endogenous *bcd* locus was replaced with dsRED via CRISPR-Cas9 and 2 attP sites were introduced to be utilized for ΦC31-

integrase mediated RMCE (Figure 14). The constructs designed for RMCE contained mutant Bicoid genomic DNA sequences. In additions, these constructs were tagged with eGFP in the N-terminal (Figure 15A). The reason for this tag was to have a target for quantitative protein detection that would not be possible in the non-tagged Bicoid since the truncations could interfere with anti-Bicoid antibody recognition.

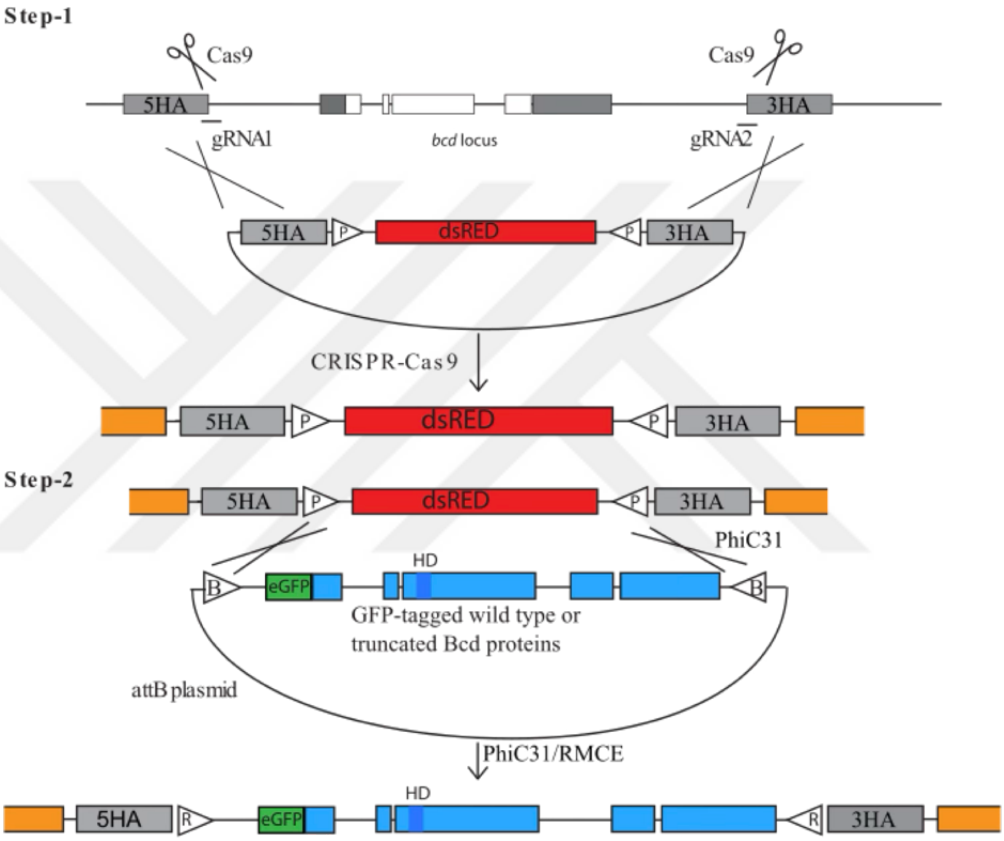

Figure 14: 2-Step CRISPR-Cas9 system for generating transgenic flies. In the first step the 7 kb *bcd* locus is targeted with guide RNAs (gRNA). dsRED construct flanked by attP sites, containing homology arms (HA) was introduced to trigger HDR mechanism and replace the locus. Utilizing ΦC31-mediated RMCE, either full length or truncated *bcd* gDNA sequences can be inserted into the locus, and the transformants can be screened via the loss of dsRED.

The survivors of microinjection are to be crossed with III. chromosome balancer line D/Tm3, Sb, Ser to screen transformants for the loss of dsRED autofluorescence in the eye (Figure 15B). The transformants are to be crossed once again with the same balancer line to obtain progeny with Tm3 balancer chromosome so that they can be self-crossed to obtain a stock line (Figure 15B). Mutant flies can be selected for their wing (normal) and

bristle (normal) phenotypes to be used in the experiments. The eGFP-tag does not interfere with the Bicoid activity, as can be seen with the previously generated eGFP-Bcd line that conserves the Bicoid gradient and target gene (*hb* and *eve*) expression (Figure 16).

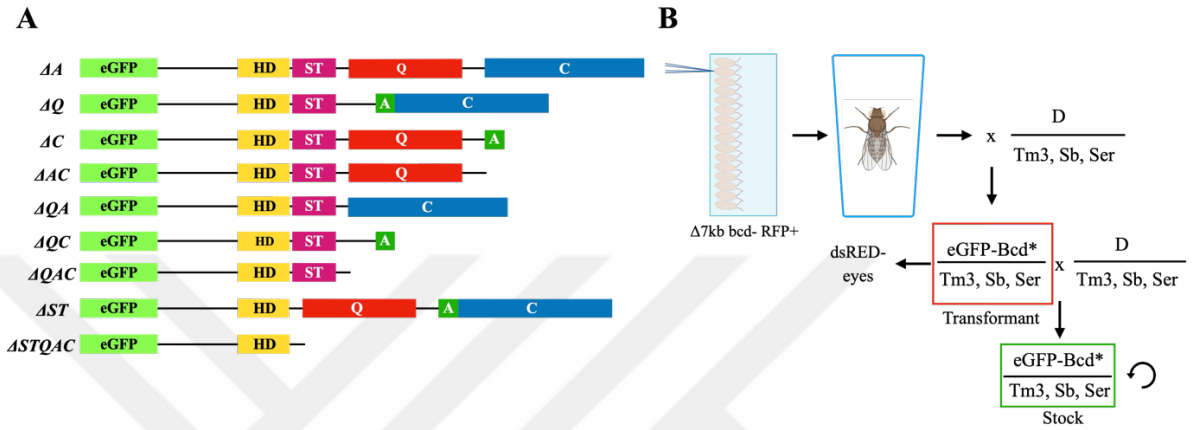

Figure 15: Generation of eGFP-tagged Bicoid mutant flies using 2-step CRISPR-Cas9 system. A) The construct designs for recombination into the endogenous *bcd* locus. **B)** The crossing plan to obtain Bicoid mutant lines.

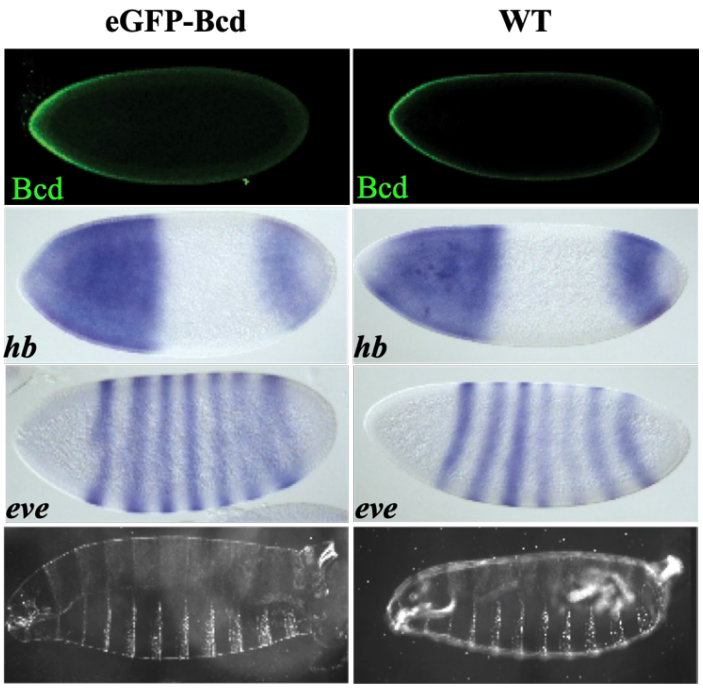

Figure 16: The eGFP tag does not interfere with Bicoid activity. The eGFP-tagged Bicoid line generated via 2-Step CRISPR-Cas9 acts the same as wild-type Bicoid. The larval phenotypes of L1 larvae, Bicoid gradient, and target gene expressions are preserved. Anterior is on the left and dorsal is up.

Once these lines are generated, they can be utilized for uncovering the molecular interactions of Bicoid with proteins, DNA, and even with small molecules via ChIP-seq and Mass-Spec. The eGFP tag can also be utilized for live imaging of mutant embryos for the changes in Bicoid gradient formation and its effect on the progression of embryonic development. Another advantage the eGFP-tag will provide is that we can characterize the bcd^{E1} mutant allele further and see if any protein is generated from the mutant mRNA. The ΔSTQAC mutant partly is a parallel mutant to bcd^{E1} since both consist of the loss of the whole C-terminal region. We have already started working on the generation of these eGFP-tagged Bicoid mutant flies and there are currently 2 constructs with transformants (ΔSTQAC and ΔST) that are waiting for confirmation through PCR and sequencing.

7. CONCLUSION AND FUTURE DIRECTION

IDRs are regulatory regions found all over the proteome that are yet to be fully characterized. They are involved in regulation of many proteins including transcription factors that tightly regulate gene expression. Understanding their exact regulatory roles would give important insights on protein functionality in fundamental biological processes. *D.melanogaster* is a powerful genetic tool developed over 100 years. It is easy to care for with a short lifespan, allowing researchers with bigger sample sizes in a shorter time span and a lot cheaper than other models. Utilizing *Drosophila* genetic manipulation tools such as RMCE and CRISPR-Cas9, one can generate IDR truncated protein expressing lines to examine the regulatory roles of IDRs *in vivo*.

In this study, we aimed to generate Bicoid truncated transgenic flies to study the regulation of Bicoid by its intrinsically disordered effector domains. Utilizing these flies, we can first examine the physiological effects on embryonic development via cuticle preparations of L1 larvae. Later, we can analyze the Bicoid target gene expression to uncover the roles of these effector domains in the transcriptional activity of Bicoid. So far, only 7 out of 9 planned lines have been generated; however, we will be generating the missing ones in the future by focusing on increasing the number of injected embryos for each construct.

To characterize the regulatory roles of IDRs on Bicoid's interactions with DNA and proteins, we are also going to generate eGFP-tagged versions of these lines via 2-step CRISPR-Cas9. Utilizing these flies, we can uncover how IDRs regulate Bicoid-protein and Bicoid-DNA interactions via Mass-spectrometry and ChIP-seq by pulling down via the eGFP-tag. Utilizing this tag, we can also do live imaging of embryos. The truncation mutants can also be crossed with target gene mutant lines to uncover more regulatory mechanisms. In addition to the in vivo studies, truncated Bicoid alleles can be cloned into bacterial expression vectors to study their interactions in vitro. In order to characterize the crucial and redundant domains, as well as the control of functionality of each domain IDR shuffle and IDR swap mutants can be generated by changing the positions of each domain, their repeat contents and replacing them with other domains.

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> Molecular Biology and Genetics
> Bilkent University
> Ankara/TURKEY

Appendix III: Annotated Bicoid Amino Acid Sequences

The amino acid sequences used for phylogenetic comparison and the annotations of the sequences are given below. (HD: yellow, ST-Rich Region: Pink, Q-Rich Region: Red, A-Rich Region: Green, Acidic C-Terminal: Turquoise, ST-Rich Region replacing A-Rich Region: Dark Green)

Lonchoptera lutea

MAQPPDQNFYHHPQLQQLQLPTQFRNPFDLLFDERTGGLNYNYIRPYIPTQPVV
PDVRNEAVRADPLVMRRPRRTRTTFTSAQISKLEQYFNESKYVNASRLAELSGK
LNLGNAQVKIWFKNRRRRLRIEQLKLKELNGSNDTTPAVSVSKDLCLALPLTPT
TLTPSPSLTPTSTPNISDQYSENYTYNPYTYNPYVQQHAYEQQVRAQPMATQYY
QQPSAISQQLTRDFLTSIKTEPDFNYNSTPYMRMPAAETMVNYTKIPTKNCYLPE
LSPNSEVYEPLTPKTEGRGSPKMANTSDEISNTHLVDAKPEVSSDTASQIYEMTK
SVPEGGYQCTMDSILQAYNQHRNTNTNNGYNTQFAFCFN

Platypeza consobrina

MAQHPDQNFYTHQQQYGFNNNHQQMQFPPHFRTPYDFVKMFDERAVALNYN
YMRPYMAHQMQQMQMQQMQQQMQQGYHDMNNSMHDMLSESLVMRRTRR
LRTTFTQQQLQELEQEFQINKYVTALRLADITSRLNLANAQVKIWFKNRRRKHK
IEEARMKELKGTLPLGLNVSIPNLNGSLTSNSLDSSLSESAPPSETKSESPPLPLTP
NPLTPSPTPSATSTPSASDKQSDNSNYGNQFYYNNNNNQMPQYYQTPPATSNQQ
QFEFPTKVQQQNETRYNNNNNNFSQQQQFNRLASQEKLAEFAKQLKIKSEMAD
FNSAELSPNSEVYEPLTPRTDTSPHSGHSDEIDETLKSNHAHTPTAAELNGDEPQP
DAASAAYQGQPMYNNNSNRRCGDEQMFGYRYN

Calliphora vicina

MAQPPPDQNFYHPHPHPHAHPHPQLQLPPQFRNPFDLLFDERTGVINYNYIRPYI
PNQLQKPDDLSDSLVMRRPRRTRTTFTSAQIAELEQHFLQGRYLTSSRLAELSAK
LALGTAQVKIWFKNRRRRHKIQADQQKDYSCDSMPLSPAASNSSKSETNGSASS
CGSSSSSGSSTSSSGPPSLQSLSLNGSGGSTPNPLTPSPTPTTPTANLMEHYGEAAF
NPYYYNNHHASHPPHHHQAHHHTHASLTHPYAAAGTQYYPPPTGSLQHHQHQ
HQQQYHAHHPHQFQMQHKPQASSIKEDPEYSYDNPYYMRMPTSLPETTATTTV
QPSTAMSPNSDVYEPLTPKNDECNGVGGGNGDAPEDLNETKTTIRELVTNNAN

GNDDACSNGNPIGSEGSGTPAINIMEDCTGAFAKFQKMSPDTTDPNYQCTMDTL
MHAYNNHRNTSANTQQFATYCFN

Lucilia sericata

MAQPPPDQNFYHPHPHPHAHPHPQLQLPPQFRNPFDLLFDERTGAINYNYIRPYI
PNQLPKPDDLSDSLVMRRPRRTRTTFTSAQIAELEQHFLQGRYLTSSRLAELSAK
LALGTAQVKIWFKNRRRRHKIQSDQQKDFSCDGMPLSPTVSNSVKSDSNGSASS
CSSSGSSASSAGPPSLQSLSLNGSGGSTPNALTPSPTPTTPTANLMEHYGESAFNP
YYYNNHHASHPPHHHHQAHHHTHASLTHPYAAAGTQYYPPPPPPGSLQHHQH
QHQQQYHAPHPHQFQMQHKAQSTSIKDDPDYNFNNPYYMRMPTSLAGNAAAT
AVQPSSAMSPNSEVYEPLTPKNDENSSLCNGVGGSDAPEDLNETKTKLRVLVTN
NANGNDDTCSNGNPIGSESSGTPINIMEDCTGAFAKFQKMSTDTSDPNYQCTMD
TLMHAYNNHRNTSANTQQFATYCFN

Megaselia abdita

MAQPPPPLCDTSAYFHPVHHAPAHPHPPPPPHPQMQIPSQFLNPFEMLYDDRTGT
LNYNYMRPYIPSQIQLPDSGLSDSFVMRRRRTRTTFTSSQIAELEEYFRQGKYLN
NIRLSELTGRLNLGQAQVKIWFKNRRRRFKIEQTKLNDSASFDMPLQLKDVKVP
VGELTPSSTPSSAASSPAPPTTTTSSYIGNEIPSQPDTPNCFASGYFFNHNFPSHYP
YPTPPTDPAFDLSTHHGFSYGSNPLWRIAPQTPSSTSSEPSPTTVADVYEPLTPKN
EDSSPKIRAPDEIEDKSSLLKVDCSPKVTVEPVQSTVDTILQAYSTHRATNAGGQ
FAYCFN

Musca domestica

MAQPPPDQNFYHPHPHPHAHPHPHPQLQLPPQFRNPFDLLFDERTGAINYNYIRP
YIPNQLPKPDDLSDSLVMRRPRRTRTTFTSAQIAELEQHFLQGRYLTSSRLAELS
AKLTLGTAQVKIWFKNRRRRHKIQSDQQKEFSCDGMPLSPSLSTTIKSEPQGSAS
SCGSNNSNGSTSSSSSSGGPPSLQSLSLNGNGGSTPNPLTPSPTPTTPTTNLMDHY
SEPAFNPYYYNNHHSTHHHHHQPPHHATLTHPYGCSAGATGGQYYPPPPPPSSL
QHHHSQHQQQYHSPHPHQFQMEHKPHAAVIKEDPDYNFNNPYYMRMPPTAGS
NPSGVTTVEPSSAMSPNSEVYEPLTPKNDDNSSLCNGAGGNVDVGDNLDETKA
KLRVIVSSNANRTDDTCSNANAIGNEGSGTPAINIMEECTGAFAKYQKMTTADP
NDPNYQCTMDTIMHAYNNHRNTSANNQQFAYCFN

Episyprus balteatus

MAEEPCVTYLNLDSIKHPPMTRIYDSRYISLDEQYPPRVRETRLDKSSNSLVMRR
PRRSRTVFTPDQVAELKYHFMQCNYIRFEKAEQIAAKFSMPVGPVKVWFKNQR

RKLKIKENENHILGIGDTTIEPPNYTRHDLILPTLTPRPLTPLSTPSPSISPIEIKFSYD
PMYLNSTAYNVKQEPSYNFKHETAYQWLQNLPALTPSQLVYNSASPHHVPQMP
SFHDTVINFNRLPYYTLPSPASTTSGDGNHITTNSDTSDYFSQMSPNSEITEPLTPI
SNHGETESVFNGILDSLKTPETEPLIETVFEELTNAPEQTKIKSKPYIDPISSEHANT
LEIV

Drosophila melanogaster

MAQPPPDQNFYHHPLPHTHTHPHPHSHPHPHSHPHPHHQHPQLQLPPQFRNPFD
LLFDERTGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTFTSSQIAELEQH
FLQGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYEGMP
LSPGMKQSDGDPPSLQTLSLGGGATPNALTPSPTPSTPTAHMTEHYSESFNAYY
NYNGGHNHAQANRHMHMQYPSGGGPGPGSTNVNGGQFFQQQQVHNHQQQL
HHQGNHVPHQMQQQQQQAQQQQYHHFDFQQKQASACRVLVKDEPEADYNFN
SSYYMRSGMSGATASASAVARGAASPGSEVYEPLTPKNDESPSLCGIGIGGPCAI
AVGETEAADDMDDGTSKKTTLQILEPLKGLDKSCDDGSSDDMSTGIRALAGTG
NRGAAFAKFGKPSPPQGPQPPLGMGGVAMGESNQYQCTMDTIMQAYNPHRNA
AGNSQFAYCFN

Drosophila ananassae

MAQPPPDQNFYHHPLPHTHTHPHSHPHPHSHPHPHPHPHPHPHPHPHQHPQLQL
PPQFRNPFDLVSSSLGFSGASVTYDPPKLFDERTGAINYNYIRPYLPNQMAKPEE
LPDSLVMRRPRRTRTTFTSSQIAELEQHFLQGRYLTAPRLADLSAKLALGTAQV
KIWFKNRRRRHKIQSDQHKDQSYEGMPLSPGMKQADGDPPSLQTLSLGGGATP
NALTPSPTPSTPTANMVEHYSESFNAYYNYNGTHVHGQGQASGHRHMHAQYQ
NAANGGAPNGGQFFSQQHQHQAQQQQHHLHQQQLHHHTNHGPLHMQQQQQ
QQQQQQQQQTAAQQQQYQHFDFPQKPASACRVLVKDEPDADYNFNSSYYMRS
ALSGATAVAVARGAASPGSGSEVYEPLTPKNDESPSLCGIGIGGPCATAVGETEA
ADDMDDGTSKKTTLQILEPLKGLDKSCDDGSSDELSPGLRALSGTGIRGTAFAK
FGKLSPAQAPPPPPLGMGMGVTMSESNQYQCTMDTIMQAYNPHRNAAGNSQF
AYCFN

Drosophila erecta

MAQPPPDQNFYHHPLPHAHTHPHPHSHPHPHSHPHPHHQHPQLQLPPQFRNPFD
LLFDERTGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTFTSSQIAELEQH
FLQGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYEGMP
LSPGMKQSDGDPPSLQTLSLGGGATPNALTPSPTPSTPTAHMTEHYSESFNAYY
NYNAGHNHAQANRHVHMQYTSGGGPGPGSTNANGGQFFQQQQVHHHHQQLH
HQNNHVPHQMQQQQQQAQQQQYHHFDFQQKQASACRVLVKDEPEADYNFN

SSYYMRSAMSGATASASAVARGASSPGSEVYEPLTPKNDESPSLCGIGIGGPCAI
AVGETEAADDMDDGTSKKTTLQILEPLKGLDKSCDDGSSDDMSTGIRALAGTG
NRGAAFAKFGKPSPPQGPQLPLGMGGVAMAESNQYQCTMDTIMQAYNPHRNA
AGNSQFAYCFN

Drosophila grimshawi

MAQPPPDQNFYHHTLPHPHTHPHPHTHPHTHAQHPQLQLPPQFRNPFDLLFDER
TGAINYNYIRPYLPNQMPKPDELSESLVMRRPRRTRTTFTSSQIAELEQHFLQGR
YLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYEGMPLSPSM
PNAPAKTADADPPSLQALSLSGSAATPNALTPSPTPSTPTAHLVEHYSESFSAYY
NYNGNQHTQAATGQQRIVTQMPGQYSNASASNSAAQFFPGAAAMHQQHGNA
HQQVHHQQQQPQQLHHPHAIAQQQQQQQQQQQQQQYHHFDFQQKPGNGCRV
PLIKCENEDDYNFNGSYYMRTAALPLSGVAASGMPAVEAVTSALSPGSEVYEPL
TPKNDESPSLCGGGGPAGGGVGVGGPCATAVGDADVTDDMDNGSNKKTTTLQ
NLEPLKSNNNNNAVADKTCDNGSDILSQGLLAVARGNLIGCGGSSGSSTFGKFG
KLTVPSTQPPPHPGGSMEAMQESNQYQCTMDTIMQAYNPHRNAGGNTQFAYC
FN

Drosophila mojavensis

MAQPPPDQNFYHHTLPHPHPHTHPHPHPHPHPHSHSHSQHPQLQLPPQFRNPFD
LLFDERTGAINYNYIRPYLPNQMTKPDELSDSLVMRRPRRTRTTFTSSQIAELEQ
HFLQGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYDG
MPLSPSMPNVPQKGADPDPPSLQALTLSGGAATPNALTPSPTPSTPTAQLVEHYS
ESFGAYYNYNQQQQQRHVAQLHGGQHGGQYGVSSASSATAQFFQHGNLHTQQ
QQLHQHPQQLHHHHVIAQQQQQQQQQQQQQQQQHQQQQHQQQQYHHFDFQ
QKLGNACRVPLIKCENEEEYNYNGSYYMRTTQTSALAMSGSSSSGSGSGSGSMP
AVESGVTSAMSPGSEVYEPLTPKNDESHSLCGVGPAGGGVSVGGPSAIPVGAAD
VNDDMDNGPNKKTTTLQNLEPLKTTSNVVADKSCDNGSDVLAAGLLTVARNT
LIGCGSNGSSAFGKFGKLTAPLATQTPPPPNAALEAMHEANQYQCTMDTIMQA
YNPHRNAGGNTQFAYCFN

Drosophila persimilis

MAQPPPDQNFYHHPLPHTHTHPPHPHPHPHPHHPHQHPQLQLPPQFRNPFDLLF
DERTGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTFTSSQIAELEQHFL
QGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYDGMPLS
PGLKTSEGDPPSLQNLTLGGGATPNALTPSPTPSATTAHLVEHYGETFNAYYNY
NHGHGQAQGQRHVGHVHGQYSGAPGPQNGAQFFQTQQQQQLHQQQQQQPPH
HHQNHQQQQQQHLHHQLPHTNHVPHQMQAQQQQQQQQEQQQQQQQLYHHF

DFQQKTASACRVVKDEPEADYNFNNSYYMRSALSGVGVAAAAAAAATAAPGT
ASSAVAAAVSAAGEVVTSALSPGSEVYEPLTPKNDESPSLCGIGGPCATAVGDT
DIADDMDDGTTNKKTTTLQNLEPLKSHTVVVGLDKSCDDGSSDDMSTGMRVL
SGRGAFAKFGKPSAGQAQPPPPPLGMMHDTNQYQCTMDTIMQAYNPHRNAGG
NTQFAYCFN

Drosophila pseudoobscura

MAQPPPDQNFYHHPLPHTHTHPPHPHPHPHPHHPHQHPQLQLPPQFRNPFDLLF
DERTGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTFTSSQIAELEQHFL
QGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYDGMPLS
PGLKTSEGDPPSLQNLTLGGGATPNALTPSPTPSATTAHLVEHYGETFNAYYNY
NHGHGQAQGQRHVGHVHGQYSGAPGPQNGAQFFQTQQQQQLHQQQQQQPPH
HHQNHQQQQQQHLHHQLPHTNHVPHQMQAQQQQQQQQEQQQQQQQLYHHF
DFQQKTASACRVVKDEPEADYNFNNSYYMRSALSGVGVAAAAAAAATAAPGT
ASSAVAAAVSAAGEVVTSALSPGSEVYEPLTPKNDESPSLCGIGGPCATAVGDT
DIADDMHDGTTNKKTTTLQNLEPLKSHTVVVGLDKSCDDGSSDDMSTGMRVL
SGRGAFAKFGKPSAGQAQPPPPPLGMMHDTNQYQCTMDTIMQAYNPHRNAGG
NTQFAYCFN

Drosophila sechellia

MAQPPPDQNFYHHPLPHSHTHPHPHSHPHPHHQHPQLQLPPQFRNPFDLLFDER
TGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTFTSSQIAELEQHFLQGR
YLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYEGMPLSPGM
KQSDGDPPSLQTLSLGGGATPNALTPSPTPSTPTAHMTEHYSESFNAYYNYNGG
HNHAQANRHMHMQYPSGGGPGPGSTNANGNQFFQQQQVHHHQQQLHHQSNH
VPHQMQQQQQQQAQQQQYHHFDFQQKQASACRVLVKDEPEADYNFNSSYYM
RSAMSGATASASAVARGAASPGSEVYEPLTPKNDESPSLCGIGIGGPCAIAVGET
EAADDMDDGTSKKTTLQILEPLKGLDKSCDDGSSDDMSTGIRALAGTGNRGAA
FAKFGKPSPPQGPQPPLGMGGVSMGESNQYQCTMDTIMQAYNPHRNAAGNSQ
FAYCFN

Drosophila simulans

MAQPPPDQNFYHHPLPHSHTHPHPHSHPHPHSHPHPHHQHPQLQLPPQFRNPFD
LLFDERTGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTFTSSQIAELEQH
FLQGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYEGMP
LSPGMKQSDGDPPSLQTLSLGGGATPNALTPSPTPSTPTAHMTEHYSESFNAYY
NYNGGHNHAQANRHMHMQYPSGGGPGPGSTNANGGQFFQQQQVHHHQQQL
HHQSNHVPHQMQQQQQQAQQQQYHHFDFQQKQASACRVLVKDEPEADYNFN

SSYYMRSAMSGATASASAVARGAASPGSEVYEPLTPKNDESPSLCGIGIGGPCAI
AVGETEAADDMDDGTSKKTTLQILEPLKGLDKSCDDGSSDDMSTGIRALAGTG
NRGAAFAKFGKPSPPQGPQPPLGMGGVSMGESNQYQCTMDTIMQAYNPHRNA
AGNSQFAYCFN

Drosophila virilis

MAQQPPDQNFYHHPLPHPHPHSHPHSHTHSQHPQLQLPPQFRNPFDLLFDERTG
AINYNYIRPYLPNQMPKPDELSDSLVMRRPRRTRTTFTSSQIAELEQHFLQGRYL
TAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYDGMPLSPSMPN
APMKTLEADPPSLQALSLSGGAATPNALTPSPTPSTPTAHLVEHYSESFSAYYNY
NNHHQQGQQRHVPQVHGQYGRSSASNAAAQFFQHGNPHAQQQLHQQGAPPPS
HHQQSQQLHHHHVMAQQQHQQQQQQQQQHQQQQQQQQQQQQQQQQQQQYH
HFDFQQKSGNACRVPTIKCENGDEYNFNGSYYMRTTALALSGVAASSMSGVEA
VTSALSPGSEVYEPLTPKNDESPSLCGGGPAGGGVGVGVGGPCATAVGAADVT
DDMDNGSNKKTTTLQNLEPLKSNNNVVTDKSCDNGSDVLGTGLLTVARGNLIG
CGSNSSSSAFGKFGKLSVPSTQTPPPPGGSLEAMHETNQYQCTMDTIMQAYNPH
RNAGGNTQFAYCFN

Drosophila willistoni

MLIFFNDFPISENAGKETNKINYWWNLGKMAQPPPDQNFYHHPLPHHQHPQLQ
LPPQFRNPFDLLFDERTGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTF
TSSQIAELEQHFLQGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQ
HKDQSYEGMPMSPSLKPTDSVHDPPSLQALSLTPSPTPSATTGGHHLMDHYGDT
FNGYYNYNNQNGHVHAQYAAANNWQHQQQQQQQQQQLLHQAAPHHLLQQQ
QQEQQQLHHHHHHTSHVPTHQQQQQQQQQHQEQHQQQEQYQHHFAVNSCR
GSMVKDEPDPDYNFNNSSYYMRSTTTTTTLQLSSGVGIGVGVSQRVAGGGSSSS
NSSGSTNASGSASVSAHAPGSGSGSATVSALEVTSALSPGSEVYEPLTPKNDESP
SLCGIGVGSIGGIGGPSATAVVVSGDNVADVNDTIDNGSTKKTTLQNLEPLKSSN
MVLDKSCDDGSNELAAGLRAVSRNCIRFGKLSPIQAPIAPPTLNVGGSPMDSTM
SAMHDANQYQCTMDTIMQAYNPHRNSAANTQFAYCFN

Drosophila yakuba

MAQPPPDQNFYHHPLPHTHTHPHTHPHSHPHSHPHSHHQHPQLQLPPQFRNPFD
LLFDERTGAINYNYIRPYLPNQMPKPEELPDSLVMRRPRRTRTTFTSSQIAELEQH
FLQGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQSDQHKDQSYEGMP
LSPGMKQSDGDPPSLQTLSLGGGATPNALTPSPTPSTPTAHMTEHYSESFNAYYT
YNGGPNHAQANRHVHMQYPSGGGPTPGPGSTNANGGQFFHQQQVHHHQQHL
HHQNNHVPHQMQQQQQTQQQQYHHFDFQQKQASACRVLVKDEPEADYNFNS

SYYMRSAMSGATASASAVARGASSPGSEVYEPLTPKNDESPSLCGIGIGGPCAIA
VGETEAADDMDDGTSKKTTLQILEPLKGLDKSCDDGSSDDMSTGIRALAGTGN
RGATFAKFGKPSPPQGPQPPLGMGGVAMAESNQYQCTMDTIMQAYNPHRNAA
GNSQFAYCFN

Appendix IV: Microinjection Survival and Transformation Efficiencies

Construct	Survival Rate	Transformation Efficiency
ΔA-Bicoid	6%	22%
ΔQ-Bicoid	20%	23%
ΔQA-Bicoid	12%	15%
ΔQC-Bicoid	14%	11%
ΔAC-Bicoid	12%	21%
ΔQAC-Bicoid	10%	17%
ΔSTQAC-Bicoid	15%	46%

The microinjection survival and transformation efficiencies. The survival rate was calculated as a ratio of total adults eclosed to the total number of injected embryos. The transformation efficiency was calculated as a ratio of the total crosses resulted in a transformant fly to the total number of crosses.