Identification of protein interaction between the Drosophila Runx1 transcription factor Lozenge and ETS-1 factor Pointed using site directed mutagenesis and yeast two-hybrid analysis

Shalini Singh

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Identification of protein interaction between the Drosophila Runx1 transcription factor Lozenge and ETS-1 factor Pointed using site directed mutagenesis and yeast two-hybrid analysis.

A Thesis
Presented to the Bayer School of Natural and Environmental Sciences
Department of Biological Sciences
Duquesne University

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For the Degree of Master of Science

by

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ABSTRACT
Many classes of transcriptional regulatory proteins are known to function in both cell proliferation and differentiation. Runx1 proteins one such family of transcription factors, plays critical roles in hematopoiesis, osteogenesis and leukemogenesis and act as promoter organizers that cooperate with other transcription factors such as Ets-1 in the regulation of gene activation or repression. Genes that are regulated by the Runx1-Ets1 complex, frequently have multiple, adjacent consensus binding sites in their promoters. I have investigated a similar interaction in developing fly eye. Lozenge (DmRunx1) and Pointed P2 (DmEts-1) cooperate to upregulate expression of prospero, which has multiple Lz and Ets binding sites. Prospero protein is essential for establishing R7 cell fate in the developing eye. Site directed mutagenesis and yeast two hybrid assay was employed to assess critical residues involved in the Lz-Pnt P2 interaction. Results unequivocally demonstrate that Lz-Pnt P2 interaction occurs independent of their DNA binding sites, implying that the interaction is not mediated by their mutual interaction with DNA. Site directed mutation reveals reduced Lz-Pnt P2 interaction, indicating the relevance of altered amino acids for the contact between the proteins. Interestingly, akin to AML1 (Runx1), Lz is also spliced over the domain important for interaction with Ets-1 proteins. Based on the results obtained in this study, we suggest that splicing produces variants that allow these proteins to either interact with Ets-1 and other proteins to transactivate other genes or to work independently in a divergent role in developmental process.
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INTRODUCTION

A fundamental regulatory problem for the cell biology of metazoan development and evolution is the balance between proliferation and differentiation. These processes are often thought of as antagonistic and must be tightly regulated to allow both processes to operate normally during development. The cellular mechanisms that underlie this regulation are fundamentally important in determining the size, shape, complexity and health of multicellular organisms (Coffman, 2003). Transcriptional regulatory proteins directly control the expression of genes in response to specific physiological/developmental signals, and thus lie at the core of every developmental regulatory mechanism. Many classes of transcriptional regulatory proteins are known to function in coordinating both cell proliferation and differentiation. Well known examples include homeobox proteins and CCAAT transcription factors like C/EBP and CBF (Ramji et al., 2002; Lekstrom-himes et al., 1998; Alam et al., 1992). A particularly intriguing family of transcription factors is defined by the runt domain (Runx), which is apparently a metazoan invention involved in controlling the proliferation and differentiation of cells during development (Rennert et al., 2003).

The runt box is a highly conserved DNA binding and protein-protein interaction domain that defines the Runx family of transcription factors. The runt domain derives its name from the Drosophila regulatory gene runt, which was discovered in genetic screen and named for its mutant phenotype, reflecting its role as a primary “pair rule gene” in establishing the pattern segments in the embryo (Gergen and Butler, 1988). Subsequently runt was shown to have
additional genetic functions in sex determination and neurogenesis (Duffy and Gergen, 1991; Duffy et al., 1991). A second Runx gene found in Drosophila is lozenge (lz). It was discovered genetically through its requirement in cell patterning in the eye and was shown to have additional roles in cell fate specification during hematopoiesis (Green et al., 1990; Rizki et al., 1981; Daga et al., 1996; Flores et al., 1998).

There are three Runx genes in mammals. Runx1 is required for definitive hematopoiesis and is a frequently mutated gene in human leukemia (Westendorf and Hiebert, 1999), Runx2 is required for osteogenesis and is associated with cleidocranial dysplasia (reviewed in Otto et al., 2002), and Runx3 controls neurogenesis in dorsal root ganglia and cell proliferation in gastric epithelium, and is frequently deleted or silenced in human gastric cancer (Li et al., 2002). In addition to those already mentioned, runx genes have been recently discovered and characterized in C. elegans (Nam et al., 2002), as well as in a variety of vertebrates including zebrafish (Kalev-Zylinska et al., 2002; Kataoka et al., 2000) and Xenopus (Tracey et al., 1998).

**Runx transcription factors and the developmental balance**

Studies using mammalian systems indicate that Runx proteins have essential functions in both cell proliferation and differentiation. As a result of these dual functions, Runx proteins are both proto-oncogenes and tumor suppressors. Thus, a central question concerning the cell biology of Runx proteins is how are the opposing functions of this class of transcription factors regulated during development? At least part of the solution to the above
predicament lies in the alternate splicing of the Runx proteins. The best evidence to date regarding the potential function of the different splice variants comes from experimental work of Tanaka et al., (1995). They showed that overexpression of the short Runx1/p26 isoform (AML1a), which lacks much of the C-terminus and hence, the transactivation domain present in longer isoform Runx1/p49 (AML1b), blocks differentiation and promotes proliferation of a murine cell line, whereas AML1b has the opposite effect of promoting differentiation and arresting proliferation (Tanaka et al., 1995). In another study, overexpression of isoform AML1d in embryonic stem cells inhibited the ability of these cells to form teratocarcinomas in vivo when transplanted into mice, while similar overexpression of AML1b failed do so (Ben Aziz-Aloya et al., 1998).

These studies suggest that differentially spliced Runx proteins play opposing roles. In addition, the cellular decision of whether to continue proliferating or to differentiate is affected by the relative levels of expression of the different variant. This has led to corollary hypothesis that acute myelogenous leukemia associated with runx1 translocations is fundamentally a disease of gene splicing, which results from an imbalance between the proliferation-specific and differentiation-specific Runx isoforms (Van der Reijden et al., 1996, Miyoshi et al., 1995).

It is therefore important to determine how phylogenetically widespread the differential splicing phenomenon is and how it is regulated during development.
**Runx1 proteins share common domains**

The signature of this protein family is a 128 amino acid motif called the runt domain. The runt domain typically lies near the N-terminus of the protein. Structural analysis revealed that this domain forms an Ig fold, which is also the 3-dimensional structure of the DNA-binding domain in transcription factors such as P53, NF-kB, NFAT, STAT, and the T-box proteins (Berardi et al., 1999). The sequence similarity between runt domains of distantly related species is remarkable. The domain in addition to playing a role in DNA binding also contains an ATP binding site (Crute et al., 1996, Kagoshima et al., 1993). The identity within the runt domain is 79% for AML1b and Lz and 96% for AML1b and mouse homolog PEBP2αB2. Additionally, amino acids at the C-terminal end of the runt domain along with a stretch of another 10 amino acids constitute a region responsible for nuclear localization of the proteins (Kanno et al., 1998). The C-terminal halves of Runx1 proteins tend to be rich in proline, serine and threonine and function in activation and repression of transcription. Finally every known *runx1* gene except one encodes a protein that terminated with the amino acid sequence VWRPY, which functions as a recruitment motif for the Groucho/TLE family of co-repressors. Derived from the sequence homology between the proteins, an illustrative figure indicating various functional regions is shown (Figure 1).

The mouse Runx1 protein PEBP2αB is also alternatively spliced and two isoforms have been identified and characterized by Bae et al., (1994). Their protein products are identical except for the deletion of the 64 amino acids long exon immediately C-terminal to the runt domain. The shortened isoform is
referred to as PEBP2\(\alpha\)B2. Electrophoretic mobility shift studies done in presence of CBF\(\beta\) (a subunit required for DNA binding of the Runx proteins) showed that the full length PEBP2\(\alpha\)B1 bound DNA with a different affinity than the shorter isoform. Both isoforms were able to transactivate a CAT reporter, but the shorter form had a decreased ability to do so (Bae et al., 1994).

**Interaction with Ets-1 factors**

The DNA binding activity of Runx1 is regulated in cis by amino acid sequences in the C-terminal of the protein that inhibit DNA binding by the runt domain. It was recently shown that interaction of Runx1 protein (Human AML1b and Mouse PEBP2\(\alpha\)B2) with ETS-1 transcription factor on adjacent sites of T-cell beta chain enhancer displaces the negative regulatory domains of both proteins, facilitating DNA binding (Kim et al., 1999; Gu et al., 2000). Ets-1 and AML1 cooperate in the regulation of T cell receptor and play critical roles in hematopoiesis and leukemogenesis (Wotton et al., 1994; Giese et al., 1995; Sun et al., 1995).

In mouse, the two PEBP2\(\alpha\)B isoforms interact with Ets-1 cooperatively to bind DNA in electrophoretic mobility shift assays, the shorter \(\alpha\)B2 showing a stronger cooperativity than \(\beta\)B1 in presence of increasing amounts of Ets-1 (Kim et al., 1999). Surprisingly, only the full-length form showed cooperative transactivation with Ets-1. The expression of both isoforms was increased in Ha-Ras transformed fibroblasts, suggesting that Ras acts upstream of PEBP2\(\alpha\)B (Bae et al., 1993).
The Ets family of transcription factors, named for the proto-oncogene E26 includes >20 proteins. Drosophila homologs of this family are YAN, Pointed (PNT P2) and ELG. The common feature of ETS proteins is a domain of 85 amino acid residues called the Ets domain, which is structured helix-turn-helix motif responsible for their binding to specific sequences and is 95% identical to PNT P2 protein (Klambt et al., 1993). Human c-Ets-1 protein and Drosophila Pointed P2 protein belong to a subset of Ets family of transcription factor called Ets-1, regions of similarity between the two proteins are illustrated in the figure 2.

The cooperative effect of the two protein families suggests that one function of the negative regulatory domains may be to favor DNA binding of the longer Runx1 variant to a specific subset of Runx1 target sequences. In fact, a common theme for runx gene regulation is context-dependency through functional, and, in this, case physical interaction with Ets-1 proteins. Several functional Runx1 binding sites in DNA flanked by Ets-1 site are known (Wotton et al., 1994; Sun et al., 1995). Depending on the specific context of the regulatory region with which they interact Runx1 proteins can function either as activators or repressors.

**Significance of interaction: Runx1 proteins and human health**

The significance of the protein binding domains found in Runx1 and their interaction with Ets-1 is further underscored by the fact that leukomogenic versions of  \( 	ext{\(B1\)} \) (AML1) and Ets-1 bear mutations that should affect the regulation of DNA binding and protein complex formation. Several translocations of the \( 	ext{\(B1\)} \) (AML1) gene generating chimeric proteins, such as AML1/ETO
(MTG8) and AML1/EV-1, have been found in human myeloid leukemia, all of which cause a deletion of the ‘negative regulatory DNA binding domain’ and Ets-1 interaction surface (Ito and Bae, 1997). Similarly, the viral version of Ets-1 contained in leukemogenic chicken virus E26 bears a mutation in the inhibitory C-terminal helix. It is also constitutively activated and does not require a cofactor for DNA binding (Hagman et al., 1992; Lim et al., 1992). The escape from the regulatory mechanisms that have evolved to tightly control the DNA binding activity of these transcription factors may thus be a major factor contributing to a leukemogenic potential of their mutant versions.

**Drosophila orthologs of Runx1 and Ets-1 are expressed during eye development**

Both *lz* and *pnt* P2 are required for normal eye development in Drosophila (Green and Green, 1956; Green, 1990; Brunner et al., 1994; O’Neill et al., 1994). This creates an advantage for studying Runx1/Ets-1 function in that the fly eye is a dispensable organ and that the developing fly eye is a well-studied model, affording a tissue-based system for analyzing these factors. Interestingly, like Runt1, the Lz protein is involved in several developmental processes, including eye, antennal (smell), and tarsal claw development (taste), hematopoiesis, and female fertility (Green and Green, 1956; Stocker et al., 1993; Batterham et al., 1996; Green et al., 1949).

**The developing eye**

The compound eye of the fruit fly is composed of about 800 unit eyes referred to as ommatidia (Ready et al., 1989). Each ommatidium contains an identical set of cells that include eight photoreceptor neurons (R1-R8), four lens-
secreting cone cells, pigmented supporting cells, and a mechanosensory hair nerve group. The compound eye is derived from the eye imaginal disc, a simple monolayer epithelium created during embryogenesis. The disc remains unpatterned until the third larval instar stage of development (approximately 4 days after hatching), at which time cell fate determination begins along posterior edge (Wolff and Ready, 1993). The onset of differentiation is marked by the passage of a morphogenetic furrow that initiates patterning of cell fates, first establishing photoreceptor neuron R8 (Baker and Rubin, 1989; Ma et al., 1993; Heberlein et al., 1993; Jarman et al., 1995; Brown et al., 1995). Cell R8 in turn recruits photoreceptor neurons R3 and R4, which together form the five-cell pre-cluster (Wolff and Ready 1993). Unfated cells posterior to the five-cell pre-cluster undergo a synchronous round of mitosis, repopulating the eye epithelium with undifferentiated cells that are needed recruitment of additional cells (Ready et al., 1976; Wolff and Ready, 1991). Photoreceptor cells R1, R6, and R7 then join the cluster, followed by the progressive recruitment of the four cone cells, and finally, the three types of pigment cells and the hair nerve group (reviewed by Wolff and Ready, 1993). A figure depicting this recruitment of cells is shown in figure 3.

The R1, R6, R7 and cone cells share a common developmental potential and are called the “R7 equivalence group”, as they all are competent to gain the R7 cell fate (Hiromi et al., 1993). Normally only one of them achieves R7 fate (reviewed in Dickson 1995). Cone cells are prevented from becoming R7-like because they do not contact R8, whose expression of BOSS protein leads to contact activation of the Sevenless tyr-K receptor protein in R7 precursors.
(Tomlinson and Ready, 1987; Zipursky and Rubin 1994). R1/R6 cells are normally prevented from becoming R7 by the expression of *seven-up (sev)* (Mlodzik et al., 1990). In turn, activation of *Bar*, which encodes homeobox proteins expressed in R1/6 cells, works to guide the differentiation of those cells (Higashijima et al., 1992). In addition, *Prospero (Pros)*, a homeodomain protein for R7 and cone cells (Kauffman et al., 1996; Flores et al., 1998) and *Dpax-2*, the *Drosophila Pax-2* homolog is required for development of cone cells (Flores et al., 2000).

In the developing retina, Lz is expressed in a subset of cells during late third larval instar (Crew et al., 1997; Flores et al., 1998). Transcriptional expression is seen in undifferentiated cells behind the morphogenetic furrow, a developmental marker for retinal differentiation. A few hours later in development, *lozenge* expression is elevated in three neuronal precursor cells as they establish their fate as R1, R6, and R7. Later, elevated expression is detected in the four cone cells, and the pigment cells of each facet. Genetic analysis has revealed that Lz positively regulates *Bar* in R1 and R6, and *DPax-2* in cone cells, and *pros* in R7 and cone cells (Figure 4). Expression of these factors is lost in animals mutant for Lz (Daga et al., 1996; Crew et al., 1997). Conversely, Lz negatively regulates *seven-up* expression (Daga et al., 1996; Crew et al., 1997). Loss of function mutations in *lz* allow *svp* to be ectopically expressed in the cone cells precursors, causing them to adopt neuronal fates. Thus, Lz protein is expressed early and in undifferentiated cells and coordinatey regulates various transcription factors.
EGF receptor in eye development and MAP kinase activation

In eye development, Ras signaling is initiated by activation of Drosophila EGF receptor (DER) by its ligand Spitz (Freeman et al., 1997). Spitz is a local signal, sent from neighboring neurons in the process of differentiation. The R7 photoreceptor is unique in that it also requires Ras activation by a second receptor tyrosine kinase, Sevenless (Fortini et al., 1992). Boss is the cell surface membrane protein on the R8 photoreceptor that activates Sevenless and is internalized with Sev RTK into R7 cell (Cagan et al., 1992). Ras activation leads to changes in gene expression controlled by phosphorylation (Brunner et al., 1994; O’Neill et al., 1994; Rebay and Rubin 1995; Wasylyk et al., 1997).

Research on the Ras pathway in the eye has deduced a developmental switch consisting of two antagonist Ets transcription factors, Yan and Pointed (Pnt). Yan is expressed in undifferentiated cells behind the furrow and its expression is dramatically reduced as cells differentiate (Lai and Rubin, 1992). This reduced expression is a result of phosphorylation by MAP kinase. Yan directly represses Prospero expression in developing R7 (Xu et al., 2000) and cone cells and D-Pax2 expression in cone cells (Flores et al., 2000). Pnt P2 is a transcriptional activator, and competes with Yan for cognate binding sites on both prospero and D-Pax2 enhancers. Pointed is also expressed in the undifferentiated cells in and behind the furrow. Phosphorylation by MAP kinase allows the activation of isoform Pnt P2, hence it ability to transactivate its targets (refer to Figure 5)(Brunner et al., 1994; O’Neill et al., 1994). Ectopic expression of Yan inhibits Prospero expression (Xu et al., 2000), D-Pax2 expression (Flores
et al., 2000), and Lz expression (Behan et al., 2002) and thus prevents differentiation (Rebay et al., 1995).

**Lozenge as a survival factor**

Recently, Lz in addition to its known role in gene regulation of cell-specific transcription factors has been implicated in repression of cell death mechanisms (Siddall et al., 2003). The early expression of Lz protein in undifferentiated cells has been documented. These unfatted, G1 arrested cells would normally be instructed to undergo cell death (reviewed in Baker et al., 2001). However, in eye development, these cells must survive for several hours before other mechanisms that control fate determination, such as EGF receptor activation, Lz control over genes including *seven-up* and *Bar*, and activation of the *sevenless* signaling cascade can take over. During this time, the default pathway must be repressed. Studies done by Siddall and co-workers, show that initial function of Lz is to create a permissive environment, allowing cells to survive long enough to allow the R7 equivalence group cells to establish their fates.

**Lozenge is alternatively spliced**

The Lz gene has many similarities to AML1. Not only is the regulation of gene expression under the Ras/MAPK signaling pathway conserved from flies to vertebrates but the Lz protein also shows a strong homology to the conserved runt domain and other regions associated with function including a nuclear matrix signal, transactivation nuclear localization, ATP binding etc. Like the human AML 1 and mouse PEBP2αB, the spectrum of *lozenge* phenotypes includes sensory
neuron and blood cell defects. Also, in parallel to the mutually exclusive roles of cell proliferation and differentiation of Runx1 proteins, recent studies on \textit{lz} have shown that Lz protein too has more than one role to play in the development. Aside from its role in determining the fates of a number of neuronal and non-neuronal cells by regulating the expression of multiple fate-determining transcription factors, it is required to contribute to the repression of cell death mechanism. Thus, in the undifferentiated cells it also acts as a survival factor allowing the undifferentiated cells to proliferate until they achieve their respective fates.

Interestingly, Flores and co-workers showed a partial rescue of \textit{lz} null mutant by expressing a full-length \textit{lz} cDNA behind its native promoter and eye-specific enhancer (Flores et al., 1998). The result was dramatic, yet paradoxical. While the adult eye appeared normal, the pattern of Lz expression in developing eye changed considerably. Lz protein was still found in the undifferentiated cells, but it could no longer be detected in the differentiated cells. These rescued flies where probed in John Pollock’s laboratory to examine the protein expression of Prospero, Runt and Bar showed expression pattern similar to that in wild type. The same flies when labeled with Lz antibody showed expression only in the undifferentiated cells. Contrary to expression in wild type, no expression was found in R1, R6, R7, cone and pigment cells (Flores et al., 1998). The success of the rescue led Flores and co-workers to conclude that Lz expression was only required in undifferentiated cells. However, experimental work from John Pollock’s laboratory indicated otherwise. In comparison with undifferentiated
cells, Lz protein expression was more pronounced in differentiated cells (Behan et al., 2001).

Drawing from the above study and similarities between Lz and other Runx1 protein, Charles Nichols in John Pollock laboratory worked on clones identified by the Banerjee laboratory and identified a splice variant that lacked exon V of the full length transcript (Nichols, 1997). Further, Kris Behan in the same lab identified the same two transcripts by doing RT-PCR on RNA derived from eye-imaginal disc. The full-length lozenge transcript is 3471 nucleotides, encodes six exons and is referred as lz-c 3.5. The difference in size was found to be about 360 nucleotides, the expected size of exon V. Sequencing of the products revealed exon V was precisely removed in alternative isoform, with an amino acid change form S to T at the splice interface. It is referred to as lz\[5].

A plausible explanation then for anomalies observed for Flores rescued flies is that since the antibody used in the above studies is made to a peptide sequence in exon VI, the protein detected in wild type was Lz\[5] (Behan, 2001).

**Functional homology between lz\[5] and PEBP2\[B2. Exon V encodes a putative interaction domain**

Two functions have been assigned to the shorter isoform of murine Runx1 gene (PEBP2\[B). PEBP2\[B2 lacks the exon C-terminal to runt domain. Runx1/AML1/PEBP2\[B auto-inhibits DNA binding by virtue of a negative regulatory region for DNA binding (NRDB) (Ito, 1999), a region that lies C-terminal to the Ets-1 interaction domain and extends beyond the alternatively spliced exon boundary. Auto-inhibition is relieved by cooperative binding Ets-1
(Gu et al., 2000; Kim et al., 1999). Also, Runx1/AML1/PEBP2β heterodimerizes with a beta partner CBFβ/PEBPα to form core binding factor (CBF). The beta partner does not contact the DNA directly, but changes the conformation of the Runx1 proteins to increase its affinity for target DNA (Berardi et al., 1999; Golling et al., 1996). When Runx1 is not associated with its beta protein it is rapidly degraded (Huang et al., 2001).

It would be consistent with the Runx1 paradigm that Lz also auto-inhibits DNA binding. Inhibition can be relieved by two mechanisms. First, splicing out this exon would alleviate inhibition and result in stable Lz protein. Second, autoinhibition would be relieved by cooperative binding and stabilizing with Pnt P2. Pnt P2 is expressed in undifferentiated cells behind the morphogenetic furrow, but expression ceases about six hours later (Brunner et al., 1994). This severely restricts the timing interval for Lz-Pnt P2 cooperativity. If full-length Lz binds in absence of Pnt P2, it would be degraded. This model helps to explain the presence of Lz protein in undifferentiated cells and loss of antibody staining in differentiating cells of rescued flies (Behan, 2001). It is also reasonable to deduce that full-length isoform is able to substitute for LzΔ5, if it ectopically expressed as both forms share the canonical domains. However, in accordance with the Runx1 paradigm the ectopically expressed binds DNA with less affinity (Behan, 2001).

Further, sequence comparison between Lz and Runx1 proteins points to a strong homology between Ets-1 interacting region in the Runx1/AML1/PEBP2αβ and exon V in Lozenge.
Lz-Pnt P2 interaction recapitulate Runx1-Ets-1 interactions

*Prospero* enhancer contains multiple binding sites for Lz and Ets factors (Xu et al., 2000); the Lz-1 site and Ets-A site are seven base pairs apart representing a potential Runt1-Ets composite motif. Prospero protein, expressed in R7 and cone cells shows increased expression in R7 relative to cone cells as the cells mature (Kauffmann et al., 1996). Previous in vitro studies indicate Yan outcompetes Pnt P2 for binding sites on the prospero enhancer by 100-fold (Xu et al., 2000). This work was recapitulated in vivo by Kris Behan in her doctoral work by expressing a hyperstable form of Yan\(^{ACT}\) via *sevenless* enhancer. This allowed Yan expression to be targeted directly to subset of cells, including R7 and cone cells (Rebay and Rubin, 1995). Prospero expression was found to be aberrant in R7 cells: the expression disappeared or decreased as the cells matured. Lz expression was also found to be decreased in these flies (Behan et al., 2002). Significantly, they also showed that ectopic expression of Lz tipped the competition in favor of Pnt P2. This shift in the Yan/Pnt P2 competition is strikingly similar to change in affinity that Ets-1 has for Mo-MLV enhancer in the presence of Runx1/AML1/PEBP2\(\alpha\)B (Goetz et al., 2000) and is consistent with the model of Lz-Pnt P2 cooperativity.

Further support that sequences in exon V are important for *prospero* regulation is supplied by mutant *lz*\(^9\). This allele represents a large deletion in *lz* locus, removing 254 amino acids of the C-terminal sequence, but retaining the entire exon V. The adult eye was severely perturbed, resembling a *lz* null. Surprisingly, at the cellular level, Prospero expression in *lz*\(^9\) is normal in
developing R7 cell. RT-PCR verified that the truncated \(lz\) transcript was expressed in the tissue, and the entire exon V was included in the transcript.

**Expression of both PEBP2\[B and Lozenge are altered in a Ras background**

Studies on Runx1 proteins in mammals also show that the cellular decision of whether to continue proliferating or to differentiate is affected by the relative levels of expression of the different variants of the protein (Coffman, 2003). There is compelling evidence that, similar to mammalian Runx1 proteins, expression of both \(lozenge\) isoforms are sensitive to changes in Ras expression (Behan, 2001). Because \(Lz\) acts downstream of the Ras signaling cascade during eye development (Behan et al., 2002), experiments were done to see whether a change in Ras activity alters \(Lz\) protein expression. This was achieved by ectopically expressing the dominant negative Ras\[^{N17}\]\(1\), which competes with native Ras product (Feig et al., 1988), resulting in cell fate alterations (Karim et al., 1996). Ras\[^{N17}\]\(1\) was targeted to subset of cells in the developing eye using the \textit{sevenless} enhancer system. Ras pathway is responsible for activating the Yan/Pnt P2 switch (O’Neill et al., 1994; Brunner et al., 1994). The dominant negative Ras\[^{N17}\] limits this switching mechanism. Quantitative RT-PCR revealed that both isoforms were downregulated in this Ras background.

The change in \(lz\) ratios is interpreted in two ways. One explanation suggested is that in a given subset of cells, a change in regulation of splicing occurred, which subsequently led to change in cell fate. Alternatively, it is possible that \(lz\) isoform ratio is cell type specific and the proportion of these cells changed. The cells that overlap Ras\[^{N17}\]\(1\) and \(Lz\) are the R7 and cone cells and a
~25% and 25-75% developmental failure was seen in these cells (Karim et al., 1996; Matsuo et al., 1997; Behan, 2001). This model is consistent with findings that ratios of Runx/AML isoforms are tissue/cell line specific.

**Research goals**

Runx proteins are evolutionarily well-conserved transcription factors that are involved in essential aspects of the development of metazoan animals ranging from fruit flies to humans. Genetic defects in any one of the three Runx proteins in humans can cause severe diseases. Although much is known about the functions and signaling pathways of the Runx proteins in mammals, there are still gaps in our knowledge with regards to functions of Runx proteins in normal development, diseases state and their regulation. The Drosophila genome encodes two Runx proteins, Runt and Lozenge that share similar expression patterns and biological functions with mammalian homologs, thus offering a promising model for studying the functions and regulation of Runt proteins.

Clearly, the data reviewed in this chapter indicates that Lozenge, the Drosophila homolog of Runx1/AML1, shares some of the functional features of the Runx1 proteins. Both proteins have pleiotropic functions and can act as activators or repressors of transcription through recruitment of additional transcriptional modulators. Additionally, like Runx1 proteins, alternate splicing of lozenge and relative levels of expression of isoforms may enable it to play opposing roles in promoting proliferation and/ or differentiation. From Kris Behan’s thesis it is also evident that the Runx1 paradigm of cooperation with Ets-1 protein is recapitulated in developing fly eye (Behan, 2001). Her studies
provide genetic data to show that Lz and Pointed P2, the Drosophila homolog of Ets-1 protein, cooperate to regulate expression of prospero, which in turn establishes R7 cell in ommatidium. However, evidence indicating a physical interaction between Lz and Pnt P2 protein is lacking.

The goal of this research, therefore, is to provide molecular data that corroborates this interaction. Furthermore, an effort is made to show that a putative site of interaction exists in the alternatively spliced exon of lozenge, which is essential for stabilizing this interaction. In order to identify protein-protein interaction, a yeast two-hybrid system was used as it provided an appropriate biological system for evaluating a physical interaction and its subsequent functional characterization. In addition, the system aids us to overcome the problem of getting pure proteins that would otherwise be an issue for lozenge, as it is known to be toxic to numerous cell lines (personal communication with Dr. John A. Pollock). However, in order to establish such a functional analyses, the isolation of mutant protein specifically altered in their ability to interact with a potential partner was critical. Thus, site directed mutagenesis experiments were designed to generate lozenge protein with altered sites in exon V. These mutant constructs also enable us to look more closely at the functionality of alternatively spliced exon V and its role interaction. Verification of proteins by Western analysis was done to strengthen the results. Furthermore, during the course of this research, an attempt was made to delve deeper in to analyzing the secondary structure of Lozenge and Pointed P2 proteins.
**Figure 1:** Diagrammatic representation of functional regions in PEBP2\(^{aB}\) and Lz isoforms.

The defining feature of the Runx1 protein, the Runt domain (yellow), functions in DNA binding. ATP binding region lies in this domain represented by a red line. Nuclear localization signals are also located in this domain. DNA binding ability of proteins is regulated by a region adjacent to Runt domain termed the negative regulatory region of DNA binding (NRDB/green) and tends to be rich in proline, serine, threonine (PST). Region adjoining the NRDB is called the activation domain (AD/pink) (Kanno et al., 1998). All proteins in this family (Lz and PEBP2\(^{aB}\) isoforms) terminate with amino acid sequence VWRPY (blue) that functions as a recruitment motif for the Groucho/TLE family of co-repressors. Region that helps in association with the nuclear matrix, distinct from nuclear localization is represented by pink line. Blue lines represent the regions that are responsible for interacting with Ets-1 factors. Lz\(^{5}\) and Pepb2\(^{aB2}\) (shown as Pepb2aB2) are two isoforms of the Dm-Runx1, Lz and Mm-Runx1, Pebp2\(^{aB}\) respectively. Splicing in the proteins removes 64 amino acid exon immediately C-terminal to the Runt domain in Peb2\(^{aB}\) and 118 amino acids or exon V in Lz. Splicing removes one of the Ets-1 interaction. Amino acids number and boxes on the illustration represent the functional domain and not the exon boundaries.
Figure 2: Schematic illustration of the functional regions in Ets-1 proteins.

A functional domain alignment between Dm-Pointed P2 and human Ets-1 family factor, c-Ets-1 is shown. The PNT P2 domain (pink) distinguishes this subset of Ets family and is contained within the regulatory domain of the Ets protein. DNA binding Ets domain (yellow) is flanked by autoinhibitory domains (green). MAPK phosphorylation site is marked (blue star). Blue lines represent three regions of Ets-1 responsible for interacting with Runx1 family of proteins. Amino acids numbers and regions do not represent exon boundaries.
Differentiation of the eye imaginal disc begins during the third larval instar at the posterior of the disc (bottom), and sweeps toward the anterior in a wave. The front wave is referred to as morphogenetic furrow. Each row represents a passage of approximately 1.5 hours time. Cells labeled yellow express Lozenge.

1. Cells in white are anterior to furrow, and are differentiated
2. Cells in the furrow express a number of markers. The first cell to specify a fate is R8 neuron, represented here as a single blue circle. One row later (approximately 1.5 hours later) the R2/R5 neuron pair has joined the R8. The next row shows the photoreceptor pair R3/R4 joining the others, marking the 5-cell precluster.
3. Undifferentiated cells enter S phase in a synchronous round, depicted here as mitotic cells in pink. These cells repopulate the epithelium
4. Photoreceptors R1/R6 join the precluster, followed by R7
5. Cone cells join groups of two, completing the cluster.

Blow up view of one ommatidium CC: cone cells. 1, 6, 7: Photoreceptor neurons are shown in their stereotypical position.
Figure 4: Lz regulatory pathway in eye development.

Illustation of many known steps in the Lz regulatory pathway as it is involved in photoreceptor neuron development and in development of accessory cells. Once Lz is activated, it in turn effects the expression of genes essential for correct differentiation of cells in the such as Bar, svp, prospero, D-Pax2, Runt and others (Daga et al., 1996; Crew et al., 1997; Flores et al., 2000; Xu et al., 2000; Behan et al., 2002).
**Figure 5: Ras MAPK activation in R7 cell.**

**Left panel:** In the undifferentiated cells, Yan (red octagons) represses *prospero* by directly binding to Ets sites. The transcriptional activator Pointed P2 (green squares) competes for the same DNA but with much less affinity. Lozenge (blue circles) transcription is tempered by Yan, but not entirely repressed.

**Right panel:** DER and Sev are activated by their respective ligands Spitz and BOSS, resulting in Ras1 stimulation. Ultimately, Yan and Pnt P2P2 are phosphorylated but with opposite effects. Phosphorylated Yan is targeted for degradation. Phosphorylated Pointed P2 binds to DNA with a higher affinity. Yan repression of Lz is alleviated, and upregulation occurs by some other mechanism. Upregulated Lz binds with Pnt P2 to mediate *prospero* transcription.
METHODS AND MATERIALS

Overview

This chapter was written to clearly state the procedural steps of the experiments and would serve as a resource to anyone attempting to investigate protein–protein interactions. Generation of the mutant construct of lozenge will be described first. This is followed by an outline of steps used for the construction of fusion genes and detection of protein interactions by the Yeast Gal4 two hybrid is elucidated. Finally, verification of proteins by Western blotting is outlined. The solutions and media used for the experiments are described at the end of the chapter.

Site directed Mutagenesis

In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships and gene expression, and for carrying out vector modification. Several approaches that have been used before require single-stranded DNA (ssDNA) as the template and are labor intensive or technically difficult. Stratagene’s QuikChange site-directed mutagenesis kit (Catalog #200516) allows site-specific mutation in virtually any double-stranded plasmid, thus eliminating the need for subcloning into M13-based bacteriophage vectors and for ssDNA rescue.
Figure 6: Overview of the Quick Change XL site-directed mutagenesis method

1. Plasmid containing the gene
   X represents the target site for Mutation

2. STEP 1: Denature and Anneal mutagenic primers

3. STEP 2: Temperature cycling with Pfu

4. STEP 3: Digestion with DpnI digarides
   Methylated nonmuted Parental strand

5. STEP 4: Transformation into XL-10 Gold ultracompetent cells
   Cells repair the nick in the PCR generated mutated strand

Gene containing mutation
Design of Primers

Mutagenic primers were designed for the lozenge gene that altered two contiguous conserved amino acids between AML-1 and exon V. Exon V is implicated in Ets interaction. The mutation was designed to alter the biochemical properties of helix, the strong secondary structure present in Exon V. The arginines were replaced by glutamic acid to reverse the charge completely keeping the helix structure intact. Primers guidelines used were as follows

1. Both oligos contained the same mutation and anneal to the same sequence on the opposite strand.
2. The desired mutation was flanked by unmodified sequence on either side by 10-15 complementary bases.
3. Length of the primers was 36 nucleotides and the Tm was approximately 10°C above extension temperature of 68°C.
4. Primers were PAGE purified and ordered from Integrated DNA technology.

<table>
<thead>
<tr>
<th>420 RPYIDGFPLKALHESES</th>
<th>RrrRrrSaKvAvTTTAAAATAAANAAVAAAAAVAvTPTGG 480</th>
</tr>
</thead>
<tbody>
<tr>
<td>EESA</td>
<td>LOZENGE: 4 conserved sites are represented in red.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FORWARD PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDMF= CTG GAG TCG CTT GAG GAG TCC GCC AAG GTG GCA GCG</td>
</tr>
<tr>
<td>Tm=75.6, length=36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>REVERSE PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDMR= CGC TGC CAC CTT GGC GGA CTC CTC AAG CGA CTC CAG</td>
</tr>
<tr>
<td>Tm=75.6, length=36</td>
</tr>
</tbody>
</table>

Table 1: Primers for Site directed Mutagenesis
**Mutant Strand Synthesis Reaction**

*Lozenge* in pBluescript and *Lozenge* Exon V in pCRBlunt were used as dsDNA template. Control and sample reaction of 50 µl were assembled as follows:

<table>
<thead>
<tr>
<th>Control Reaction</th>
<th>Sample Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl of 10x reaction buffer</td>
<td>5 µl of 10x reaction buffer</td>
</tr>
<tr>
<td>2 µl (10 ng) of pWhitescript control template</td>
<td>2 µl (10 ng) of pBluescript-lz/pCR Blunt ExonV</td>
</tr>
<tr>
<td>1.25 µl (125 ng) of control primer#1</td>
<td>1.25 µl (125 ng) of SDMF</td>
</tr>
<tr>
<td>1.25 µl (125 ng) of control primer#2</td>
<td>1.25 µl (125 ng) of SDMR</td>
</tr>
<tr>
<td>1 µl of dNTP mix (10 mM)</td>
<td>1 µl of dNTP mix (10 mM)</td>
</tr>
<tr>
<td>3 µl of QuikSolution</td>
<td>3 µl of QuikSolution</td>
</tr>
<tr>
<td>36.5 µl ddH2O</td>
<td>35.5 µl ddH2O</td>
</tr>
</tbody>
</table>

1 µl of *PfuTurbo* DNA polymerase was then added and following cycling parameters were used:

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95 °C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>95 °C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68 °C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68 °C</td>
<td>1 minutes/Kb</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68 °C</td>
<td>7 minutes</td>
</tr>
</tbody>
</table>

**Table 2: Cycling parameters for Site directed Mutagenesis.**

Following temperature cycling, the tubes were immediately placed on ice for 2 minutes. Amplification of the product was checked by electrophoresis of 10 µl of the product on a 1% agarose gel.
Dpn I Digestion of the Amplification Products

1 μl of Dpn I restriction enzyme (10 U/μl) was then added and mixed gently and thoroughly by pipetting. The tubes were incubated at 37°C for 1 hour to allow digestion of the parental supercoiled dsDNA.

Transformation of XL 10 Gold Ultracompetent Cells

Cells were thawed on ice and 45 μl was aliquoted in prechilled Falcon 2059 polystyrene tubes. 2 μl of β-ME was then added and the contents were swirled gently. The tubes were incubated on ice for 10 minutes and swirled every 2 minutes. 2 μl of Dpn-I treated DNA was added to the cells and kept on ice for 30 minutes. Treatment with β-ME was followed by heat pulsing, which permeablized the cells. Tubes were heat pulsed in a 42°C water bath for 40 seconds and transferred on ice for 2 minutes. 0.5 ml of preheated NYZ broth was then added and the tubes were incubated at 37°C for 2-3 hrs with shaking at 250 rpm. 200 μl of the transformation reaction was plated on the LB-kanamycin for lozenge and LB-Ampicillin for Exon V containing 80 μg/ml X-gal and 20 mM IPTG and incubated at 37°C for 18 hours. Transformation efficiency was calculated for all the transformations.

Recovery of the Mutant Constructs

5 ml of LB with appropriate antibiotic was inoculated with a single colony and incubated overnight at 37°C with shaking at 250rpm. Colonies were harvested and DNA was isolated using Wizard plus SV Miniprep DNA
Purification System by Promega (Catalog #A1340). 1% agarose gels were run to check the product size.

**Sequencing**

Quantification of DNA was done by spectrophotometer and 250 ng of each mutant clone was sent for sequencing to the Molecular Medicine Institute, Pittsburgh (http://www.mmi.pitt.edu/dnaseq.html). Exhaustive sequencing of both the mutant clones was done to ensure that no mutation other than that desired was incorporated in the clones.

**Subcloning: Construction of Fusion gene**

Lozenge, pointed P2, lozenge Exon V and the mutants were prepared for cloning in the yeast plasmids by PCR with appropriate restriction sites incorporated into the primers. Each of the above listed candidates was cloned in both the yeast plasmids, i.e., yeast plasmid containing the GAL4 DNA binding domain (pGBKKT7) and plasmid containing the GAL4 activation domain (pGADT7).

**Primer Design and Amplification**

Forward and reverse primers were designed to introduce compatible restriction enzyme sites and 2 additional bases were added at the 5’ ends to allow for efficient digestion by restriction enzyme. In order to conserve the frame of the fusion genes extra bases were added for cloning of pointed P2 in to the yeast plasmid.
<table>
<thead>
<tr>
<th>Fusion gene</th>
<th>Yeast Plasmid</th>
<th>Primer sequence</th>
<th>Temperature</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lozenge (Lz)</strong></td>
<td><strong>pGBK7</strong></td>
<td><strong>GBK7LzF=GCGAATTTCATGCATTTGCATCTCCTGGCGGA</strong>&lt;br&gt;<strong>GBK7LzR=GCCCCGGGTCAATAGGGGTGCCACACCT</strong></td>
<td><strong>Tm 69.9°C</strong>&lt;br&gt;<strong>Tm 72.2°C</strong></td>
<td><strong>Length 32</strong>&lt;br&gt;<strong>Length 28</strong></td>
</tr>
<tr>
<td><strong>Lozenge Mutant (LzSDM)</strong></td>
<td><strong>pGADT7</strong></td>
<td><strong>GADT7LzF=GCGAATTTCATGCATTTGCATCTCCTGGCGGA</strong>&lt;br&gt;<strong>GADT7LzR=GCCCCGGGTCAATAGGGGTGCCACACCT</strong></td>
<td><strong>Tm 69.9°C</strong>&lt;br&gt;<strong>Tm 72.2°C</strong></td>
<td><strong>Length 32</strong>&lt;br&gt;<strong>Length 28</strong></td>
</tr>
<tr>
<td><strong>Lozenge ExonV (LzE5)</strong></td>
<td><strong>pGBK7</strong></td>
<td><strong>GBK7LzE5F=GCGGATCCGTCCGACCGGTGGTCC</strong>&lt;br&gt;<strong>GBK7LzE5R=GCCTGCAGTGCTTGTATGTGCGGCGC</strong></td>
<td><strong>Tm 70.5°C</strong>&lt;br&gt;<strong>Tm 70.1°C</strong></td>
<td><strong>Length 24</strong>&lt;br&gt;<strong>Length 26</strong></td>
</tr>
<tr>
<td><strong>Lozenge ExonV Mutant (LzE5SDM)</strong></td>
<td><strong>pGADT7</strong></td>
<td><strong>GADT7LzE5F=GCCCCGGGTAGTCCGACCGGTGGTCC</strong>&lt;br&gt;<strong>GADT7LzE5R=GCGGATCTCCTGGCTTGTATGTGCGGCGT</strong></td>
<td><strong>Tm 74.4°C</strong>&lt;br&gt;<strong>Tm 70.2°C</strong></td>
<td><strong>Length 27</strong>&lt;br&gt;<strong>Length 29</strong></td>
</tr>
<tr>
<td><strong>Pointed P2</strong></td>
<td><strong>pGBK7</strong></td>
<td><strong>GBK7PF=GCGGATCCGTATGGAATTGGCGATTTGTAA</strong>&lt;br&gt;<strong>GBK7PR=GCCTGCAGCTAATCCACATCTTTTTTCTCAAC</strong></td>
<td><strong>Tm 64.6°C</strong>&lt;br&gt;<strong>Tm 63.5°C</strong></td>
<td><strong>Length 30</strong>&lt;br&gt;<strong>Length 33</strong></td>
</tr>
<tr>
<td></td>
<td><strong>pGADT7</strong></td>
<td><strong>GADT7PF=GCCCCGGGTATGGAATTGGCGATTTGTAA</strong>&lt;br&gt;<strong>GADT7PR=GCGGATCCCTAATCCACATCTTTTTTCTCA</strong></td>
<td><strong>Tm 66.5°C</strong>&lt;br&gt;<strong>Tm 62.6°C</strong></td>
<td><strong>Length 30ntd</strong>&lt;br&gt;<strong>Length 31ntd</strong></td>
</tr>
</tbody>
</table>

**Table 3: List of Primers designed for Subcloning experiment.**

**Amplification**

Gene fragments were generated by PCR utilizing the Stratagene PCR kit (cat #200516). Thin walled 0.5 ml PCR tubes were used and each reaction was set in volume of 50 µl as follows:

- **Reaction buffer**: 5 µl
- **Quick Solution**: 3 µl
- **Forward Primer**: 1.25 µl (100 ng/µl)
- **Reverse Primer**: 1.25 µl (100 ng/µl)
d NTPs 1 μl
Plasmid 2-3 μl (200-300 ng)
ddH₂O up to 50 μl
Pfu Turbo DNA polymerase 1 μl (2.5 U/μl)

Denaturation was done at 95°C for 1 minute to start, then 50 seconds per cycle. Annealing for 50 seconds at 60°C followed by extension for 1 minute/kb of the gene or gene fragment at 68°C. This was repeated for 18 cycles and finally terminated with an extension step at 68°C for 7 minutes for all samples. All products obtained were analyzed by gel electrophoresis.

**Gel Clean up**

PCR products were purified to remove unincorporated nucleotides. Samples were run on 1% low melting agarose to allow isolation of proper fragments. Correct size bands were excised with a clean fresh razor blade and Wizard SV gel and PCR clean-up system (Promega cat #A98280) was used for purification. About 95% product recovery was made and this was confirmed by running 1% agarose gel at 80 volts for 2 hours. The amount of DNA was estimated by spectrophotometer.

**Restriction Enzyme Digestion**

**Digestion by Xma I and Eco R I**

*Lozenge* in pBluescript/ pCR blunt, mutant *Lozenge* in pBluescript and yeast plasmids pGBK7 & pGAD7 were digested with the above two restriction enzymes.
enzymes. Buffer compatibility was taken into consideration in doing the double digests hence digestion by Xma I (New England Biolabs cat #R0180S) was done first. The digests were then cleaned by Wizard SV PCR clean up system. Digestion with EcoR I (New England Biolabs cat #R0101S) was done next and again the digests were purified. The reactions were assembled on ice in the order listed below. Buffers and BSA were always kept 1/10th and 1/100th of the reaction volume, respectively.

<table>
<thead>
<tr>
<th>Xma I</th>
<th>EcoR I</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA(Plasmids, Gene fragments)</td>
<td>45 µl of the purified Xma I digest</td>
</tr>
<tr>
<td>dH₂O to make 50 µl</td>
<td>dH₂O to make 50 µl</td>
</tr>
<tr>
<td>NEBuffer4 5 µl</td>
<td>NEBuffer EcoR I 5 µl</td>
</tr>
<tr>
<td>BSA 0.5 µl.</td>
<td>-</td>
</tr>
</tbody>
</table>

Gently mix by pipetting

Mix by pipetting
Kept in 37°C water bath for 90 minutes
Heat inactivated in 65°C water bath for 20 minutes

Table 4: Restriction Digests for Subcloning.

Digestion by Xma I and BamH I

*Pointed* P2, *lozenge* Exon V, mutant *lozenge* Exon V and yeast plasmid pGADT7 were digested by these two enzymes, assembled and purified according to the table presented above. 0.5 µl (10 U/µl) of BamH I (Fisher cat #BP3320-1) was used on purified Xma I digest and the reaction was incubated in 37°C for 2 hours.
Digestion by Pst I and BamH I

Pointed P2, lozenge Exon V, mutant lozenge Exon V and yeast plasmid pGBKT7 were first digested with Pst I followed by BamH I. 0.5 μl (10 U/μl) of Pst I (Fisher cat #BP3404-5) was used.

Dephosphorylation and Ligation

Calf Intestinal Alkaline Phosphatase (CIAP Promega cat #M1821) was used to catalyze the hydrolysis of the 5’ end and 3’ end phosphate from the plasmid to prevent vector religation, which reduces the number of background colonies. Just prior to use CIAP was diluted in CIAP 1X reaction buffer to a final concentration of 0.01 U/μl. Amount of CIAP was calculated according to the pmol of DNA ends. For dephosphorylation, the vector digest, 5 μl CIAP buffer and 0.5 μl calf intestinal phosphatase was added to an end volume of 50 μl and incubated in 37°C water bath for 30 minutes. An additional batch of enzyme (0.5 μl) was added and incubated for further 30 minutes. 300 μl of CIAP stop buffer was added to terminate the reaction. The dephosphorylation was followed by purification step to remove any phosphatase left in the solution.

Molar ratio of 3:1 of insert: vector was used for ligation. Conversion of molar ratios to mass ratios was done using the formulae:

\[
\frac{\text{ng of vector} \times \frac{\text{kb size of Insert}}{\text{kb size of vector}}} \times \text{molar ratio of Insert} = \text{ng of Insert}
\]
The reaction was assembled in sterile microcentrifuge tube as shown:

- Vector DNA: 2 µl (40 ng)
- Insert DNA: 6 µl (120 ng)
- Ligase 10x Buffer: 1 µl
- T4 DNA ligase: 0.5 µl
- ddH₂O: 2.5 µl

The tubes were incubated at 4°C overnight.

**Transformation and Miniprep**

E.coli JM109 cells were used for transformation. 3 µl of the ligase reaction mix was used and blue/white screening was done for selecting transformed colonies. A single colony was used to inoculate 5 ml of LB broth containing appropriate antibiotic. Following overnight culture, DNA was isolated using the Wizard plus SV minipreps DNA purification system (Promega cat #A1330).

**Sequencing**

DNA was quantified by spectrophotometer. All clones were thoroughly screened by designing appropriate primers. Each sequencing reaction gave 800-900bp of readable sequences. Sequencing was done at the Molecular Medicine Institute, Pittsburgh ([http://www.mmi.pitt.edu/dnaseq.html](http://www.mmi.pitt.edu/dnaseq.html)). Frame and point mutations were exhaustively searched for.
Figure 7: Location of Primers designed for sequencing.

### Primers for lozenge

<table>
<thead>
<tr>
<th>BT7</th>
<th>TCGCGAGAGAGGAGTAGTAACAAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADT7</td>
<td>TTATCGTAGAGAGATACCCCA</td>
</tr>
<tr>
<td>T7</td>
<td>TAAATCGTTCAACTATAGGGGC</td>
</tr>
<tr>
<td>LZ F3</td>
<td>AGCAAGCTAACAATAAACAC</td>
</tr>
<tr>
<td>LZ F4</td>
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</tr>
<tr>
<td>LZ F6</td>
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<tr>
<td>LZ F7</td>
<td>GACAAATCGCTCTCCGATTT</td>
</tr>
<tr>
<td>AD</td>
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<tr>
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</tr>
<tr>
<td>RBT7</td>
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</tr>
<tr>
<td>LZ R3</td>
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</tr>
<tr>
<td>LZ R4</td>
<td>GAAACCGCATCATTGGAACT</td>
</tr>
<tr>
<td>LZ R6</td>
<td>CGAGGCTGAGATCTATTCG</td>
</tr>
<tr>
<td>LZ R7</td>
<td>AACTCGAGGATCTCATTTCG</td>
</tr>
</tbody>
</table>

### Primers for pointed P2

<table>
<thead>
<tr>
<th>BT7</th>
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</tr>
</thead>
<tbody>
<tr>
<td>ADT7</td>
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</tr>
<tr>
<td>T7</td>
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<tr>
<td>PF1</td>
<td>AGCGAGGTACTGAAGGCATCC</td>
</tr>
<tr>
<td>PF2</td>
<td>AGCAAGCTAACAATAAACAC</td>
</tr>
<tr>
<td>PF3</td>
<td>GATGCTCAATCCTCCAGTCC</td>
</tr>
<tr>
<td>PF4</td>
<td>TACTATTTAGACAAAAACA</td>
</tr>
<tr>
<td>AD</td>
<td>AGATGCTCTAGTCACAGAGCAGAG</td>
</tr>
<tr>
<td>BD</td>
<td>TTTTCCGTTTTAAAAACCTAAGAGT</td>
</tr>
<tr>
<td>PR1</td>
<td>GGATGCTCTAGTCACAGAGCAGAG</td>
</tr>
<tr>
<td>PR2</td>
<td>GTTATTTTCTGCTGTTTCTTGCTT</td>
</tr>
<tr>
<td>PR3</td>
<td>CGACTGGAAGGATGACATTC</td>
</tr>
</tbody>
</table>

Table 5: Primers designed for Sequencing
Yeast Two–Hybrid System

Matchmaker Gal4 Two-Hybrid by Clontech (cat #K1261-1) was used. It is an advanced Gal4-based two-hybrid system that provides a transcriptional assay for detecting protein interaction in vivo in yeast. In addition, the Matchmaker System 3 DNA-BD and AD fusion vectors, pGBK7 and pGADT7 have been designed for high level protein expression. The “bait” and “prey” inserts are expressed as GAL4 fusion with c-myc and HA epitope tags respectively which eliminates the need for generating specific antibodies allowing convenient identification of the fusion proteins.

Figure 8: Yeast two hybrid system

(A) Protein X (the bait protein is fused to DNA-binding domain (DBD) and protein Y is fused to transcription activation domain (AD). Both hybrid proteins are expressed in yeast cells from plasmid. Interaction of X and Y leads to activation of reporter gene that allow the yeast to grow on a defined medium. (B) Protein X and Y do not interact; consequently, the reporter gene is not expressed.
Yeast Strains

Two yeast strains were provided with the kit. Y187, which uses a lacZ reporter encoding β-galactosidase, and AH109, which in addition to lacZ has two other reporter genes HIS3 and ADE2. These two genes provide strong nutritional selection for positive clones. Both of the strains were recovered from frozen stocks by streaking on YPDA plates. Both the strains exhibit an ade2-101 phenotype and turn pink when grown on the YPDA plates at 30°C for 3-5 days or until the colonies are about 2-3mm in diameter. Several such colonies were used to inoculate 5 ml YPDA liquid media to prepare liquid cultures for transformation. The tubes were vortexed vigorously to disperse any clumps and were incubated at 30°C for 16-18 hrs with shaking at 230-270rpm.

Checking Reporter Activity for Individual Plasmid Constructs

The overnight cultures were grown to stationary phase (OD$_{600}>1.5$). This culture was used to inoculate another 50 ml culture to produce an OD$_{600}$ of 0.2-0.3. The flask was incubated at 30°C for 5 hours with shaking (230-270 rpm) to get a mid-log phase measuring an OD$_{600}$ of 0.5± 0.1. This culture is sufficient for 10 transformations. The culture was harvested in sterile 50 ml centrifuge tube at 3000xg (2500 rpm) for 5 minutes. The medium was poured off and 25 ml of sterile dH$_2$O was added to resuspend the cells. The tubes were centrifuged again to remove the water. 1.0 ml of 100 mM lithium acetate (LiAc) was added to the pellet and vortexed to resuspend the cells. This allows permeabilization of the yeast cells. The suspension was transferred to sterile microcentrifuge tubes and cells were centrifuged at top speed for 5 seconds. LiAc was removed carefully
with a pipette. 400 µl of 100 mM LiAc was then added and pellet vortexed to get a final volume of 500 µl. 50 µl of this was transferred to new microcentrifuge tube for each transformation. The cells were centrifuged again to remove LiAc. The transformation mix was then added to tubes in the order shown below:

- 240 µl of PEG (50% w/v)
- 36 µl of 1.0 M LiAc
- 25 µl of single stranded carrier DNA (2.0 mg/ml)
- 50 µl of H₂O and plasmid DNA (1 µg)

Each tube was vortexed until the pellet completely dissolved. The tubes were incubated at 30°C for 45 minutes followed by heat shock at 42°C for 25 minutes. Cells were centrifuged at 6000 rpm for 20 seconds to remove the transformation mix. 500 µl of sterile dH₂O was added to the tubes and the pellet was resuspended by pipetting gently. 200 µl of it was used for plating onto appropriate selective plates.

Detection of Reporter Activity: Colony–lift Filter Assay

Colony–lift filter assay was used to detect reporter lacZ gene activity. It measured the β-galactosidase activity encoded by lacZ. It was used to screen the large number of transformants that survived the nutritional selection.

Plates were incubated at 30°C until colonies appear and grew to size of 1-2 mm, this usually took 4-5 days. For each plate of transformants assayed, a sterile Whatman #5 or VWR grade 410 filter was used. It was placed in 2.5-5.0 ml of Z buffer/X-gal solution in a clean 150 mm plate. Using clean forceps, a clean dry filter was placed over the surface of the plate of colonies and gently
rubbed with the side of the forceps, which helped the colonies to cling to the filter. After the filter was evenly wetted, it was carefully lifted off the agar plate and transferred to a pool of liquid nitrogen (colonies facing up). Liquid nitrogen lysed the yeast cell thus allowing detection of the reporter gene. The filters were submerged for 10 seconds after which they were completely thawed at room temperature. The filter colony side facing up was then cautiously placed on the presoaked filter careful not to trap any bubbles between the two filters. Filters were incubated at 30°C and checked periodically for the appearance of blue/white color. White colored colonies were indicative that all clones by themselves were not activating the reporter lacZ gene activity.

**Co- transformation of Yeast strains for Protein-Protein Interaction**

Yeast colonies were grown in 5 ml YPDA media overnight at 30°C (shaking at 250 rpm) and were used to inoculate 50 ml of YPDA media. The colonies were grown until mid log phase and then prepared by transformation as described earlier. In the DNA mix a 2:1 molar ratio of bait to prey DNA was used for co-transformation. The transformation mix was plated on an SD minimal media -Leu/-Trp. The plated were incubated at 30°C and as soon as the colonies appeared were tested for β-galactosidase activity. Colony filter lift assay were performed as already discussed. Positive clones were identified as blue colonies and restreaked on fresh plates to confirm interaction.
Verification of Protein Expression: Western blotting

Preparation of Protein Extract

For each transformed yeast strain assayed, a 5 ml SD minimal media –Leu/-Trp culture was started and incubated for 7-8 hours at 30°C. It was used to start a 25 ml culture at an OD₆₀₀ of 0.05 and incubated overnight at 30°C with shaking. The cultures were centrifuged at 3000 rpm for 5 minutes and the supernatant discarded. The pellet was resuspended by vortexing in 0.5 ml of sterile dH₂O and transferred to sterile microcentrifuge tube. Tubes were centrifuged at 9000 rpm for 1 minute to remove water and then resuspended in 200 μl ice-cold extraction buffer and lysed with glass beads with 8 cycles of 30 seconds vortexing with 30 seconds pausing on ice. Unbroken cells and debris were removed by centrifugation at 10,000 rpm for 10 seconds. The supernatant was removed and kept on ice henceforth.

Western Analysis

Protein concentrations were determined using Bicinchoninic Acid Protein Assay Kit from Sigma (cat #BCA-1). Samples were heated at 37°C for 10 min with Tris–Gly SDS sample buffer 2X (Novex LC2676). A total of 40-50 μg was loaded per lane into 4-20% Tris-Gly Gels 1.5 mmx10 well (Invitrogen EC6028) and Benchmark Prestained Protein Ladder (Invitrogen LC2005) and MagicMark Western Standard (Invitrogen LC5600) were used as standards. Gels were sandwiched with PVDF transfer memberane (Invitrolon PVDF, Invitrogen LC2005) and samples were transferred on membrane using the Xcell II blot
Module from Invitrogen (E19051) at 20V for 2 hours. Membranes were stained using Ponceau 0.5 % in 1% acetic acid for 5 minutes and then thoroughly rinsed in deionized water to check the efficiency of the transfer. The membrane was dried and scanned for records. Membrane was treated for 5 minutes in HPLC grade methanol rinsed with deionized water and transferred in 1% BSA in PBS-Tween for blocking. It was kept in blocking solution overnight at 4°C in with shaking. Equilibration of membrane was done for 15 minutes at room temperature. Primary antibody (Monoclonal Antibody HA-11, Covance cat# MMS-101P and Monoclonal antibody c-Myc, Clontech cat #3800-1) was added to solution at 1:5000 dilution ratio and incubated at room temperature with shaking for 90 minutes. Primary antibody solutions were removed and the membrane was washed with PBS-T for 30 minutes changing the solution every 5-6 minutes. Membrane was incubated in secondary antibody (Peroxidase-labeled affinity purified antibody to mouse IgG (H+L), KPL cat# 074-1806) in 1% BSA/PBS-T at 1:75000 dilution for 1 hour at room temperature. Membrane was washed for 30 minutes in PBS-T, with changing the solution every 5-6 minutes. It was incubated with SuperSignal Dura Extended Duration Substrate (PIERCE cat #34057) for 5 minutes at room temperature and sealed using thin plastic. Membrane was exposed to Kodak Biomax MR film for 5-10 minutes and developed.
Preparation of Media and Reagents

Site Directed Mutagenesis and Subcloning

LB Agar (per liter)

10 g NaCl
10 g Tryptone
5 g Yeast extract
20 g Agar

Added deionized water to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclaved
Cooled to 55°C

Added 50 mg of filter-sterilized ampicillin/kanamycin
Poured into Petri dishes

NYZ⁺ Broth (per liter)

10 g NZ amine
5 g Yeast extract
5 g NaCl

Added deionized water to a final volume of 1 liter
Adjusted pH to 7.5
Autoclaved

Added the following filter-sterilized supplements prior to use

12.5 ml of 1M MgCl₂
12.5 ml of 1M MgSO$_4$

20 ml of 20% (w/v) glucose

**SOB Medium (per liter)**

20.0 g Tryptone

5.0 g of NaCl

Autoclaved

Added 10 ml of 1 M MgCl$_2$ and 10ml of 1 M MgSO$_4$/liter of SOB medium

**SOC Medium (per liter)**

SOB medium

Added 1 ml of a 2 M filter-sterilized glucose solution and filter sterilized.

**CIAP Stop Buffer**

10 mM Tris-HCL (pH 7.5)

1 mM EDTA

200 mM NaCl

0.5% SDS

**Yeast Two Hybrid**

**YPDA (per liter)**

960 ml d H$_2$O

50 g YPD
15 ml of 0.2% adenine hemisulfate
20 g agar added for plates
Deionized water to make 1L
Adjust pH to 6.5
Autoclaved for 30 minutes
Pour plates

**SD Medium (per liter)**

- 6.7 g Yeast nitrogen base without amino acids
- 20 g Agar (only for plates)
- 5 g Ammonium sulfate
- 20 g dextrose
- 100 ml (6.4 g/100 ml) of 10X Dropout solution (all amino acids except –Leu/-Trp)
- Deionized water to make 1000 ml
- Autoclaved for 30 minutes
- Poured plates

For single transformation the amino acid not carried by plasmid was also added to media. (200 mg/L for Trp and 1000 mg/L for Leu)

\[\beta\text{-galactosidase Filter Assay}\]

**Z Buffer**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O})</td>
<td>16.1 g/L</td>
</tr>
<tr>
<td>(\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O})</td>
<td>5.50 g/L</td>
</tr>
</tbody>
</table>
KCL 0.75 g/L
MgSO$_4$.7H$_2$O 0.246 g/L

Adjusted the pH to 7.0 and autoclaved

**X-gal Solution**
20 mg /ml in DMF

**Z Buffer/ X-gal solution**
100 ml Z-buffer
0.27 ml $\beta$-mercaptoethanol
1.67 ml X-gal stock solution

**Western Analysis**

**Extraction Buffer (200 ml)**

1760 ml Buffer “Western”
200 ml Triton X-100
20 ml 100 mM PMSF
20 ml 10% SDS

**Primary Antibody (1:5000)**
150 ml PBST
1.5 g BSA
30 μl primary antibody

**Secondary Antibody (1:75000)**

150 ml PBS-T
1.5 g BSA
2 μl secondary antibody
RESULTS

Overview

The vertebrate transcription factors Ets-1 and AML-1 (the Runx family B1 subunit of murine PEBP2α) play critical roles in hematopoiesis and leukemogenesis, and cooperate in the transactivation of the T-cell receptor β chain enhancer. Kim and coworkers (1999) reported a direct interaction between Ets-1 and PEBP2α. They also showed that this interaction leads to a reciprocal stimulation of their DNA binding activity and activation of their transactivation activity.

Pointed (Ets-1) and Lozenge (Runx1) represent Drosophila homologs of the two families of transcription factors. Interestingly, both genes are involved in eye development, and binding sites for both have been found in close proximity in the enhancers of several genes, including prospero (Flores et al, 2000).

Similar to the AML-1 factor our laboratory has previously shown that Lz is alternatively spliced (Behan, 2001; Siddall et al., 2003). We have predicted distinct roles for the two isoforms, the full-length lozenge (lz c3.5) and lozenge lacking the exon V (lz V5) in eye development. Drawing from the similarities between the vertebrate and Drosophila proteins, it is proposed that the RUNX-Ets paradigm previously characterized (Kim et al., 1999) is recapitulated in Drosophila eye development. We suggest that Lz-Pnt P2 cooperate to upregulate Prospero expression for specifying R7 cell during development. Genetic evidence for this observation has been presented by John Pollock’s prior
graduate student Kristina Behan. However, evidence for a physical interaction between the Lz and Pnt P2 proteins was lacking.

Experiments were thus designed and performed in the course of this current study to search for a biochemical interaction between the Lz and Pnt P2 proteins that corroborate our findings. These involved site directed mutagenesis of Lz in the alternatively spliced exon. Next, yeast two-hybrid analysis was carried out to directly assess the biological interaction of the Lz proteins and Ets-1 protein Pointed P2. This included the construction of yeast vector containing Gal4 DNA binding domain fused to lozenge and the second yeast vector carrying the activation domain fused to pointed P2. Western analysis of yeast cells containing the two vectors confirmed the expression of the corresponding fusion proteins. Lastly, extensive analysis of Lz (Runx-1) and Pnt P2P2 (Ets-1) protein families was performed using a range proteomics databases and tools.

**Lozenge and Pointed represent RUNX1 and Ets-1 by sequence and functional homology**

Lozenge and Pointed sequences were analyzed to investigate secondary structure and protein domains. A figure illustrating the results derived for Lozenge protein in comparison to mouse homolog is shown (Figure 10).
Site Directed Mutagenesis: Modulating the putative Ets interacting domain in Lz

On the basis of protein analysis by various automated databases and sequence analysis, it was deduced that Exon V of lozenge encodes a putative Ets interaction domain. Site directed mutagenesis was used to create a mutant clone in which two contiguous amino acids, in the core of a highly conserved domain in Exon V were altered. Using the PCR technique, primers containing the desired sequence change, introduced the mutation. This was performed on both full-length Lz sequence and a gene fragment encoding just exon V. The mutation switched the charge of two amino acid residues from positively charged arginines to negatively charged glutamic acid. The substitution was checked for secondary structure and was found to keep the alpha helix intact. A 1% agarose gel was run to check amplification and revealed the correct size band for both templates including the control provided with the mutagenesis kit (Figure 11). Digestion of the PCR product with Dpn I endonuclease assured the removal of methylated parental DNA template. This results in minimizing the background and selection of mutation containing synthesized DNA. The next step involved the transformation of competent cells and identification positive clones by blue/white screening of LacZ induction. To demonstrate the effectiveness of the method, the pWhitescript 4.5 kb containing a stop codon (TAA) at a position where glutamine normally appears in the β-galactosidase gene was used as a control. The oligonucleotide control primers created a point mutation that reversed the change. Using 250 µl of the transformation reaction for each of the conditions
including the pBluescript-lozenge and pCR Blunt–Exon V and pWhitsecript 4.5 kb control plasmid were plated on LB-antibiotic plate containing X-gal and IPTG. As control, pUC 18 plasmid was used to verify transformation efficiency. Following transformation, colonies containing the mutated control plasmid appeared blue, as did the colonies transformed with mutated plasmids. Mutagenesis efficiency for each was calculated and is represented in the table given below:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No. of blue colonies</th>
<th>No. of white colonies</th>
<th>Mutagenesis efficiency (ME) = No.of blue colony forming units x100 / No.of total colony forming units</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript-lozenge</td>
<td>62</td>
<td>20</td>
<td>75.0%</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>18</td>
<td>82.6%</td>
</tr>
<tr>
<td>pCRBlunt–Exon V</td>
<td>58</td>
<td>12</td>
<td>82.8%</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>23</td>
<td>76.2%</td>
</tr>
<tr>
<td>pWhitsecript 4.5 kb</td>
<td>128</td>
<td>21</td>
<td>85.9%</td>
</tr>
<tr>
<td>control</td>
<td>180</td>
<td>10</td>
<td>94.7%</td>
</tr>
</tbody>
</table>

**Table 6: Mutagenesis efficiencies for experimental and control transformations**

Plasmid was recovered from isolated colonies grown in 5ml of LB-antibiotic media. (Figure 11). Incorporation of mutant alters the recognition site of restriction enzyme RsrII. The position 1549 CGCGGTCCGCC represents that site. Results confirm that the recovered plasmids contain the insert.

Gel purified plasmids were sequenced. Primers were designed such that overlapping sequences were obtained. Each sequencing reaction yielded reading lengths of about 800 bases. DNA Strider™ 1.3f13 program was used to align and examine the sequences obtained from *lozenge* mutant clone and from Exon V clone. No ambiguities in terms of any insertions/deletions or base pair changes.
except the ones introduced by primes provided evidence for the success of experiments. Sequencing was done for the opposite strand also and results obtained corroborated the presence of mutated sequence. Mutant clones were named Lz-SDM for full-length lozenge mutant and E5-SDM for Exon V mutant.

Interaction between the two proteins: Yeast Two Hybrid Assay

The yeast two-hybrid technique identifies the interaction between two proteins (X and Y) by reconstituting a transcription factor that activates a reporter gene driven by promoter containing the relevant binding site for transcription factor. The system used for my experiments is Gal4 based. Two shuttle vectors pGADT7 and pGBK7T carry the activation domain and DNA binding domain of Gal4 respectively. Genes of interests, *prt P2, lz*, and mutant constructs were fused to these domains. The reporter gene used in these experiments was the bacterial *lacZ*. Also, growth selection markers such as *LEU* and *HIS* on the two plasmids allowed powerful growth selections for successful transformants.
Construction of fusion genes: pGADT7-Iz and pGBK7-pointed

In order to ensure success with yeast two-hybrid system the most critical step involved the precise cloning of gene and gene fragments into appropriate vectors. Careful design of primers ascertained appropriate reading-frame and orientation for expression of the fusion proteins. Lozenge, lozenge Exon V, pointed P2 and mutant clones were PCR amplified to introduce compatible restriction sites. Amplification was checked by agarose gel electrophoresis and the product was gel purified (Figure 12). Sequential restriction enzyme digests
were done and the digested products were purified to remove traces of restriction enzymes and buffers. Repeated purification ensured minimal interference with optimal enzyme activity. Vectors were treated with a phosphatase enzyme and purified. Dephosphorylation prevented the religation of vector during cloning and also reduced number of background colonies. 1:3 ration of vector and insert was used in ligation reaction. Ligation mix was used to transform *E. coli* competent cells. More than 80% efficiency was achieved for all transformation. After the plasmid isolation, products were checked electrophoretically and quantified spectrophotometerically (Figure 12). Sequencing data established that cloned inserts were in frame for translation with no base changes for all clones constructed.

<table>
<thead>
<tr>
<th>pGADT7−Activation Domain+ LEU2</th>
<th>pGBK7-DNA binding domain+TRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lozenge (Lz)</td>
<td>Lozenge (Lz)</td>
</tr>
<tr>
<td>Lozenge mutant (Lz SDM)</td>
<td>Lozenge mutant (Lz SDM)</td>
</tr>
<tr>
<td>Pointed P2</td>
<td>Pointed P2</td>
</tr>
<tr>
<td>Lozenge Exon V (E5)</td>
<td>Lozenge Exon V (E5)</td>
</tr>
<tr>
<td>Lozenge Exon V mutant (E5 SDM)</td>
<td>Lozenge Exon V mutant (E5 SDM)</td>
</tr>
</tbody>
</table>

**Table 7: Clones designed for Yeast Two-Hybrid.**

**Checking for auto activation**

Since the two-hybrid system is based on the reconstitution of a functional transcription factor, checking the auto-activation capacity of the target is crucial for the overall feasibility. Y187 and AH109 strains were grown from frozen stocks for 4-5 days at 30°C. Healthy colonies of 2-3 mm size appeared and were pink in color and these were used to inoculate 5 ml of YPDA media. The cultures were
allowed to grow until they reached the stationary phase and then transferred to fresh 50 ml of YPDA media. Lithium Acetate (LiAc) transformation method was used to transform the plasmids into yeast strains (auxotrophic for Trp and Leu). For all yeast strain that were transformed with pGADT7, SD-Leu media supplemented with Trp was used and vice versa for all strains transformed with pGBK7T7. The cells were plated in triplicate for each transformation. Colonies appeared from all transformation reactions within of 4-5 days. Six colonies from each plate were spread into patches on a fresh plate and incubated until colonies appeared. β-galactosidase colony lift filter assay was done to detect lacZ activity. LacZ is the reporter gene under the control of GAL4-responsive sequences. Filters were checked periodically for color development. The pCL1 plasmid that encodes the full-length wild type GAL4 protein was used as a positive control for β-galactosidase assay. None of the single transformants were blue. The above experiment verified that the constructs by themselves were unable to activate the reporter genes. A schematic showing the procedure and the result obtained for one such experiment is shown in figure 13.

**Simultaneous vs. sequential transformation**

In my next set of experiments I picked all single transformants colonies and prepared them for a sequential transformation. A table depicting all possible combination used for experiments is shown. Both positive and negative controls comprising plasmids pGADT7-T + pGBK7T7-53 and pGADT7-T + pGBK7T7-Lam were included in the experiments. 200 µl of each was plated on SD/-Leu/-Trp. It took the more than 5 days for the colonies to appear, indicating that colonies
grew far slower with both the plasmids. This was also done in triplicate and 6 colonies from each plate were patched on a fresh plate to be assayed for reporter gene activity. Single colonies from the positive control plates were also included to make one patch on all plates. After incubating the filters for 8 hours at 30°C, the experimental colonies showed no lacZ activity and were white colored. The positive and negative controls yielded expected blue and white colonies respectively, on the filters. The assay was repeated to screen all colonies and the same result was obtained.

<table>
<thead>
<tr>
<th>Single transformant</th>
<th>Plasmid for sequential transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGADT7</td>
<td>Pointed P2</td>
</tr>
<tr>
<td>Lozenge (Lz)</td>
<td>Pointed P2</td>
</tr>
<tr>
<td>Lozenge mutant (Lz SDM)</td>
<td>Pl pointed P2</td>
</tr>
<tr>
<td>Lozenge Exon V (E5)</td>
<td>pGADT7</td>
</tr>
<tr>
<td>Lozenge Exon V mutant (E5 SDM)</td>
<td>pGBKT7</td>
</tr>
<tr>
<td>Pointed P2</td>
<td>pGBKT7</td>
</tr>
<tr>
<td>Lozenge (Lz)</td>
<td>Pointed P2</td>
</tr>
<tr>
<td>Lozenge mutant (Lz SDM)</td>
<td>Pl pointed P2</td>
</tr>
<tr>
<td>Lozenge Exon V (E5)</td>
<td>pGADT7</td>
</tr>
<tr>
<td>Lozenge Exon V mutant (E5 SDM)</td>
<td>pGBKT7</td>
</tr>
</tbody>
</table>

**Table 8: Combinations used for Sequential transformation.**

Appearance of blue color for positive and white for negative controls indicated that transformation protocol worked even though all experimental colonies gave no reporter gene activity. Protein was extracted from experimental
colonies that failed to show positive interactions and a Western analysis was done. Figure 14 illustrating the number of times each experiment was tried and tested in shown at the end of the chapter.

**Results of Simultaneous co–transformation**

Given the failure to detect reporter gene activity in sequential transformants, a different transformation approach that involved a little tweaking form the earlier approach was used. Both yeast strains AH109 and Y187 were grown to stationary phase and transferred to a 50ml fresh YPDA media for a small-scale transformation. Bait to prey ratio of 2:1 was used and the incubation time with the transformation mix was increased by 15 minutes from the previous transformation. Colonies appeared after incubating for 2-3 days and were immediately screened for reporter gene activity. Incubation of 2 hours gave color development on the filters (Figure 16). All experimental colonies transformed with Lz and Pnt P2 fusion proteins except the mutated Exon V construct gave blue color for lacZ activity. Positive and negative controls were as expected. The positive colonies for all experimental candidates were re-streaked on fresh plates in patches and assayed for reporter gene activity. Appearance of blue color confirmed the fact that there was an interaction between the proteins. The experiments were repeated thrice to ascertain the interaction (Figure 15). Table 9 shows the percentage colonies that showed reporter gene activity in co-transformation experiments.
Detection of Fusion proteins

In addition to the fusion with the Gal4 domains, the two interacting proteins are also expressed as a fusion to a c-myc and hemagglutinin tags. The epitope tags allow easy detection of the proteins thus eliminating the need to generate specific antibodies. Hence, to verify the presence of proteins in the double transformants, protein was extracted and a Western blot was done. Controls for the experiments included protein extracted from untransformed yeast strains and positive controls from co-transformed colonies. Protein extract from a sequentially transformed colony was also included (Figure 17). Lozenge and Exon V, pointed P2 proteins were detected from colonies transformed with pGBK7-PNT P2 and pGAD7-Lz/LzSDM/E5/E5SDM. Untransformed yeast strains showed no bands. Interestingly, only one of the proteins was detected in sequential transformants explaining the failure to detect reporter gene activity. Also in my first Co-transformation experiment I did not detect reporter gene activity for p GBKT7-Pnt P2 and p GADT7-E5 as I did in my second experiment, which is explained by absence of partner PNT P2 protein evident in western blot.
Figure 10: Schematic representation of the secondary structure of Lz protein.

ClustalW alignment of Lz with murine Runx1 protein isoform, PEBP2β1 (represented as PEBP2a) is shown. The secondary structure determined for Lz protein is represented above the sequences. Alpha helices are shown in pink, beta strands are depicted in yellow arrows, Red and green curved arrows indicate the Beta hairpin and Beta turn respectively. Blue lines indicate random coils. Purple arrows show exon boundaries for Lz protein. Orange square on PEBP2β1 sequence marks the exon that is alternatively spliced.
Figure 11: Site directed mutagenesis.

Panel A: Site directed mutagenesis was performed on full-length lozenge. Lane 1 and 2: plasmids p Bluescript-lz (6.2 kb) pCR Blunt-lz (6.2 kb) respectively. Lane 3 to 5: PCR product of plasmids p Bluescript-lz, pCR Blunt-lz and pUC18 control plasmid (5 kb). Lane 6 and 7: Mutated lz in p Bluescript and pCR Blunt isolated from competent cells. Panel B: Site directed mutagenesis of lozenge exon V (E5) in pCR Blunt. Lane 1 and 2: template plasmids pCR Blunt-E5 (4 kb). Lane 3 and 4: Product obtained after Mutant strand synthesis by PCR. Lane 5: Isolation of mutated pCR Blunt E5 from competent cells.
**Panel A:** Compatible restriction sites were introduced by PCR and the product checked on 1%Agarose gel. Lane 1: *pointed p2*, Lane 2 and 3: PCR product from p Bluescript-lz and p Bluescript-lz SDM, Lane 4 and 5: PCR product from pCR Blunt-lz and pCR Blunt-lz-SDM, Lane 6 and 7: PCR product from pCR Blunt–lz E5, Lane 8 and 9: PCR product from pCR Blunt–lz E5 SDM. **Panel B:** Plasmids isolated from competent cells transformed with ligation mixture of the gene of interest and yeast plasmid. p GBKT7 (7.3 kb) containing Gal4 DNA binding domain. Lane 1: *Pointed p2* in p GBKT7, Lane 2 and 3: *Lozenge* and *lozenge* SDM in p GBKT7, Lane 4 and 5: *Lozenge* E5 in p GBKT7, Lane 6 and 7: *Lozenge* E5 SDM in p GBKT7. **Panel C:** p GADT7 (8.0 kb) containing Gal4 activation domain and gene of interest. Lane 1 and 2: *Pointed p2* in p GADT7, Lane 3 and 4: *Lozenge* in p GADT7, Lane 5 and 6: *Lozenge* SDM in p GADT7, Lane 7: *Lozenge* E5 in p GADT7, Lane 8: *Lozenge* E5 SDM in p GADT7
Figure 13: Checking for autoactivation.

Yeast were transformed with all plasmids constructed to check for autoactivation and assayed for LacZ activity. All experimental colonies show no LacZ activity. Positive control in the experiment was colonies that were transformed with pCL1 plasmid encoding full length Gal4 transcription factor.
Figure 14: Schematic showing the result obtained for all combinations utilizing Sequential transformation experiments.
Figure 15: Schematic showing the plating and re-streaking done for Lz + Pnt P2, Lz SDM + Pnt P2, Lz E5 + Pnt P2 combinations utilizing Co-transformation experiments.

Co-transformation mix:
Lz + Pnt P2
Lz SDM + Pnt P2
Lz E5 + Pnt P2

Plating done in triplicates from each transformation mix.
9 Colonies selected from each plate and restreaked in patches.
6 colonies were used to streak fresh plates.

Western Analysis.
Interaction of full-length Lz, Lz E5, Lz SDM, Lz E5 SDM with Pnt P2 protein. Plates were streaked with single colony of yeast transformants that contain plasmids expressing different GAL4 DNA binding Domain and GAL4 activation domain fusion proteins are shown. Cell growth on Leu/Trp medium demonstrates successful transformation. The colonies were lifted on a filter and assayed for LacZ activity. Positive interaction between proteins is indicated by development of blue color on the filters incubated with the X-gal substrate. The schematic in the bottom indicates the Gal4 fusion proteins expressed in transformants streaked in each sector of the plate.
Table 9: Percentage colonies that showed Reporter gene activity after Co-transformation. Results shown for co-transformation experiments only. All experiments done using sequential transformation gave no reporter gene activity. Positive and negative controls in the sequential transformation gave same results as shown here for co-transformation.* Because colony distribution was not uniform an area of high colony density was used for counting. ± Signifies the combinations that were used in western blots.
Soluble protein extracts were prepared from yeast strain AH109 and Y187, co-transformed with p GBKT7 encoding c-Myc tagged Gal4 DNA binding domain (DBD) fusion protein and p GADT7 encoding HA tagged Gal4 Activation domain (AD) fusion protein. Each well contains 50 µg of protein. Gels were run in duplicate and probed with different antibodies. **Panel A and D:** Samples probed with anti-c-Myc antibody and anti-HA antibody respectively. (Pnt P2~81kD; Gal4 DBD~25kD) (Lz/LzSDM~90kD; Gal4 AD~30kD) Lane 1: AH019 (p GBKT7-Pnt P2 and p GADT7-Lz), Lane 2: AH109 (p GBKT7-Pnt P2 and p GADT7-Lz SDM), Lane 3: Y187 (p GBKT7-Pnt P2 and p GADT7-Lz), Lane 4: Y187 (p GBKT7-Pnt P2 and p GADT7-Lz SDM). **Panel B and D:** Samples probed with anti-c-Myc antibody and anti-HA antibody respectively. (LzE5 ~7 Kd+ Activation Domain ~30kD), Lane 1: AH019 (p GBKT7-Pnt P2 and p GADT7-Lz E5), Lane 2: AH109 (p GBKT7-Pnt P2 and p GADT7-Lz E5 SDM). **Panel C and F:** Samples probed with anti-c-Myc antibody and anti-HA antibody respectively. Lane 1 and 2 represent sequential transformed experiment, no Lz is detected which explains the failure to detect reporter gene activity. Lane 3 represents the E5+PNT P2 combination that did not show reporter gene activity in first set of co-transformation experiment. Untransformed yeast strains were run as control and showed no specific bands.
DISCUSSION

Results presented in this study illustrate, for the first time that *lozenge* and *pointed*, homologs of Runt1 and Ets-1 family, interact at the protein level. In Drosophila, Lz and Pnt P2 cooperation is central to the specification of the R7 cell fate and establishing the structure of the eye (Behan et al., 2002; Xu et al., 2000). Evidence for their physical interaction is provided by employing the yeast two-hybrid system. Further, the data obtained, unequivocally demonstrate that the concert between the Lz and Pnt P2 occurs independent of their consensus binding sites, GGCCGCA for Lz (Kamachi et al., 1990; Xu et al., 2000) and ATATCCGC for Pnt P2 (Xu et al., 2000). Interaction in absence of binding site also establishes the fact that the interaction between the two proteins is not mediated by their DNA-binding properties (Figure 18). Additionally, our experiments indicate that an essential role in this interaction is played by the alternatively spliced exon V of Lz.

Designing the mutant

Proteomic analysis of the alternatively spliced exon of Lz revealed two important facts. First, a remarkable sequence identity among amino acids of the alternatively spliced exon existed that was found to be conserved across species. Second, structure prediction for this exon showed a prominent alpha helix extending almost the entire length. Presence of such a strong secondary structure for an alternatively spliced exon of a protein and conserved identity of few amino acids among species leads us to believe that the helix must be important in orchestrating the interaction with the Ets-1 protein. To test our
hypothesis, I generated site directed mutation of conserved amino acids in the alpha helix, creating a reversal of surface charge. The same mutation was also incorporated in clone of just exon V. These lozenge clones were tested in combination with Pnt P2.

The Lz-Pnt P2 concert: Utilizing the yeast

The yeast two-hybrid system first described by Fields and Song (1989) is based on the modular nature of transcription factors, and provides a convenient assay for studying protein-protein interactions. As it is performed in vivo it is not limited by artificial conditions of in vitro assays like the electrophoretic mobility shift assay (EMSA) and pull down experiments. In addition, sensitivity of the yeast two-hybrid simplifies mutational analysis allowing investigations such as mapping of motifs or residues required for interactions. Thus, in the present study it not only provided a relevant biological system for detection, but it also enabled us to evaluate the role of few crucial residues, and an alternatively spliced exon of lozenge.

The crucial steps essential for the feasibility of the yeast two hybrid involved a meticulous subcloning strategy. Lz and Pnt P2 were fused with Activation Domain (AD) and DNA Binding Domain (DBD) of Gal4 transcription factor carried by two shuttle vectors. Once generated, the clones were sequenced to ensure that no PCR errors were introduced in this process and the proteins were in-frame and in right orientation with its fusion partner.

Since the system is based on reconstitution of a functional factor, brought together by the interacting fusion proteins, checking the auto-activation capacity
of the target is crucial for overall success of the experiments. Single plasmid now encoding the fusion proteins was used for transformation of yeast strain carrying a UAS reporter lacZ gene responsive to the gal4 system. The yeast strain was also auxotrophic for two amino acids Trp and Leu. Successful transformation was evident by growth of colonies on media supplemented with the amino acid not carried by plasmid. No lacZ activity was seen, in any of the single transformants now carrying plasmids encoding fusion proteins-lozenge, pointed P2, lz exonV, full-length lz mutant and Exon V mutant.

Previous work using yeast two hybrids suggests that a sequential transformation strategy allows stable expression of the first protein, prior to transformation with the second plasmid (Criekeinge et al., 1999). This strategy was followed in my first attempt and the second plasmid was introduced in the single transformants in order to detect an interaction. These double transformants grew on plates lacking both amino acids, indicating that both the plasmids were present in the yeast. However, I failed to detect any reporter gene activity in these double transformants. Why did I not see any activity? The experiment introduces a runt homology-domain protein into yeast. Analysis of the yeast genome indicates that yeast has no homologs for Runt or runt related genes. (Adya et al., 2000) Presumably, by some undetermined mechanism, the Lozenge expression is shut off but the plasmid can still overcome the auxotrophic barrier and grow. Additionally, Lz shows some level of toxicity when expressed in cell lines derived from both eukaryotes and prokaryotes (Personal communication with Dr. Pollock). Evidentially, when yeast were transformed with
Lz protein it interfered with normal yeast metabolism that may have given rise to clones having spontaneous deletions in the plasmids that confers the yeast clone a growth advantage. This would explain the fact that the even though the colonies grew and looked normal they failed to show reporter gene activity. It made the selectable marker fine however, it did not mean that the interaction was occurring.

The failure of above experiments led me to try a different approach. Similar studies with toxic proteins and yeast two hybrid had shown that co-transformation works better than sequential transformation in these cases as it alleviate some of the toxicity problems even though it yields significantly less double-transformants (Criekinge et al., 1999). Utilizing this approach two distinct sets of colonies were observed after the transformations. One set of colonies grew similar in size and color to those obtained in sequential approach, while the other set grew slower, were smaller than those in sequential transformation. This observation corroborated the fact that Lz expression interferes with yeast metabolism and hampers their growth. The smaller colonies were checked for reporter gene activity and LacZ activity was detected in all double transformants except the ones transformed with mutated lz exon 5 and pnt P2. Another important difference noted from these different approaches is that an optimal protein expression is observed in the growth phase of the newly transformed yeast after which the spontaneous deletion process starts to occur, leading to negative results. In other words it was essential to assay the colonies before the toxicity starts to affect their growth and metabolism.
The results of the co-transformation clearly demonstrated an interaction between Lz and Pnt P2 proteins. Cell lysates from transformants were probed with antibodies against the epitope tags expressed in fusion with the chimeric proteins, confirming our results. The experiments also demonstrate that the alternatively spliced exon has a functional significance and is essential for Lz-Pnt P2 interaction. Interestingly, the full-length mutant lozenge also showed LacZ activation however, mutated exon V alone failed to do so.

The results presented in this study explicitly prove that there is a physical interaction between Lozenge and Pointed P2. Since this interaction occurs in the absence of their DNA consensus binding sites it helps us to reject the earlier held notion that interaction between the Runt-1 protein and Ets-1 protein occurs due to their DNA binding ability. Lz exon V protein by itself showed interaction suggesting that this spliced exon plays an essential role in this interaction.

In a related study on the mouse Runx1, PEBP protein that used EMSA, Kim et al., showed two Ets-1 interacting domains in regions that include the alternatively spliced exon. Results from this study add to their observation. Positive results obtained with exon V in yeast two hybrid corroborate, that alternatively spliced exon in Runt1 proteins have regions that are critical for their interaction with Ets-1 proteins. It is interesting to note that the shorter isoforms; Lz5 in Drosophila and PEBP 2B2 in mouse still has only one of the two Ets-1 interacting regions identified in Kim et al. study. In their study, shift in electrophoretic mobility of the DNA-protein combination was evident, but significantly reduced, indicating the single Ets interacting domain is insufficient.
The data I obtained from the Lz exon V mutated clone, which has an altered charge residue on the helix, adds to this story. This alteration did not overlap with the deduced Ets-1 interacting region contained in spliced exon V. Mutation in full-length lozenge showed an interaction equivalent to non-mutated Lz protein however, the same in just exon V failed to do so. This establishes the fact that exon V and its secondary helix structure are essential for interaction, and the two putative Ets-1 interacting regions help in stabilizing this interaction.

The fact the site of alternate splicing is found conserved from flies to humans also raises the question of why nature would want to conserve this so strictly, over several million years. A possible explanation can be drawn from research conducted here. Given that the alternate splicing of exon V creates a protein that is responsive to Ras MAPK activation of the Ets-1 factors. Eliminating the Ets–1 interaction domain found in exon V of lz, creates a protein that is responsive to the Ras MAPK activation of the Ets factors. Giving the Runx1 family of proteins this capacity to enhances their capacity to function within the complex roles of differentiation and proliferation. In case of Lozenge, its early expression, which supports the survival of undifferentiated cells, is independent of Ras MAPK activation of the Ets-1 factors. This expression of Lz may employ the LzD5 splice variant. A few hours later in eye development, the increased expression of Lz is dependent on the Ras MAPK activation of the Ets-1 factors. Here it is expected that the full length Lz is expressed, permitting the subsequent interaction of Lz with its Ets-1 transcription partner, Pnt P2. Future experiments can support this hypothesis.
Conclusions and Future studies

Lozenge proteins are clearly critical for cell differentiation during development, and recent evidence shows that they also play important role in cell survival (Siddall et al., 2003). Depending on the gene target, they can act as activators or repressors (Crew et al., 1997). Previous research done in Pollock lab showed that in parallel to Runx1 proteins, lz is alternatively spliced. This finding helps to provide some insight into how these multiple roles may be controlled during development. My own research strengthens the notion that alternate splicing allows lz to contribute to these roles during differentiation and proliferation.

Interestingly, AML1, Runx1 protein in humans has 11 isoforms and some of them are known to have distinct roles in development. To this date only two isoforms of lz have been identified but given the fact that Lz like AML1, is also pleotropic hints that there may be other isoforms of lz.

My own mutant studies clearly show that lozenge exon V is essential for interaction with Ets-1 protein. The next logical step is to generate new mutants to enable us to identify whether the helical structure found in exon V is important for this interaction or not. Inserting prolines in helix would produce a kink and completely disturb the secondary structure of the exon. Generation of this mutant will allow us to evaluate its role in the interacting with Ets-1. A second mutant, currently begin developed in the Lab involves the truncation of amino acids that make up the helix. The significance for probing the interaction regions for proteins is underscored by the fact that the leukemogenic versions of AML1
(humans) and Ets-1 bear mutations that should affect the regulation of DNA binding and protein complex formation (Kim et al., 1999). Several translocation of AML1 gene generating chimeric proteins, such as AML1/ETO and AML1/EVI-1 have been found in human myeloid leukemia, all of which cause a deletion of the Negative regulatory domain and EII interaction surface (Ets interacting region, in alternatively spliced exon) (Ito and Bae, 1997). Similarly, the viral version of Ets-1 contained in leukomogenic chicken virus E26 bears a mutation in inhibitory C-terminal helix. It is also constitutively activated and does not require a cofactor for DNA binding (Hagman et al., 1992; Lim et al., 1992). The escape from the regulatory mechanisms that have evolved to tightly control the DNA binding activity of these transcription factors may thus be major factor contributing to the leukemogenic potential of their mutant versions. Thus generation of the mutant along with the rich developmental genetic framework available in Drosophila would makes an attractive system for furthering our knowledge on the structure, function and developmental regulation of Runx1 family of proteins.

Surely, interaction of Lz protein is not restricted to Ets-1 protein. This is also evident from evolutionarily conserved feature of the Runx family, namely, their wide expression. This strongly suggests that Lz proteins are likely to interact with other factors. It is therefore important that further studies on this aspect are carried out by utilizing the existing Lz-yeast plasmid to screen a library prepared from a tissue in which Lz protein is known to be biologically relevant like the antenna (smell), tarsal claw (taste), spermathecae etc. It will enable us to identify and isolate novel associating proteins, thus elaborating our existing knowledge.
Yeast two hybrid analysis shows a physical interaction between Lz (Runx1) and Pointed P2 (Ets-1) protein. In R7 photoreceptor Ras MAPK activation leads to increased expression of Lz and phosphorylation of Pointed P2 proteins, permitting their subsequent interaction. Lz and Pnt P2 then bind to the *prospero* enhancer and mediate its transcription.
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