A Genetic Screen for Genes Involved in the FGF Signalling Pathway in *Drosophila melanogaster*

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Table of contents

1 Introduction

Cell-cell communications are the fundamental bases of the involvement of multi-cellular organisms. Cells engage various ways to communicate. They send signals by ions, small organic molecules, and secreted proteins and also by membrane proteins. Signals can be detected by various receptors, which can be located at the membrane or within the cytoplasm. Upon binding to their ligands, receptors are capable of triggering a series of events, which can change cell behavior. A well-studied family of receptors are the receptor tyrosine kinases (RTK). Receptor tyrosine kinases respond to different signals, and have distinct consequences, although they share many common downstream components. How these processes are regulated presents a great challenge for biologists.

The embryonic development of *Drosophila melanogaster* provides a good model system to study the communication between cells. During the embryonic development, a fertilized egg forms a single layer of cells after 13 synchronized divisions. Morphogenesis movements start with ventral furrow invagination, followed by germband extension and posterior mid gut invagination. At mean time, invaginated mesoderm cells migrate along the lateral ectoderm to form a monolayer beneath the overlying ectoderm. Later on, specified tracheal placodes invaginate and branch out during germband retraction and dorsal closure, to form an interconnected tubule network of the larvae respiration system. RTK signals are required extensively during morphogenesis of the embryo development. Both mesoderm migration and trachea formation require fibroblast growth factor (FGF) signals. Compare to vertebrates, many downstream targets for RTK signals are conserved in Drosophila. The work presented in this thesis is an approach to identify other components involved in the FGF signalling pathway.

1.1 Receptor tyrosine kinase signals

RTKs are transmembrane glycoproteins that are activated by binding to their ligands. The ligand binding changes the conformation of the receptors, which leads to the receptor oligomerization and autophosphorylation. The phosphorylated tyrosine residues on the receptor provide binding sites for various signal molecules. By binding to and phosphorylating their substrates, receptor tyrosine kinases are able to trigger a series of signal events (Fig.1-1), such as Phospholipase Cγ (PLCγ) signal, Phospholinositide 3-Kinase (PI3K) pathway, Ras-mitogen-activated protein kinase (Ras-MAPK) pathway, and JAK-STATs pathway (Hubbard et al., 2000; Schlessinger, 2000)

1.1.1 The activation of the receptors

The protein family of RTKs includes receptors for insulin and growth factors, such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Platelet-Derived

Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF) and Nerve Growth Factor (NGF). All the RTKs consist of an extracellular portion that binds polypeptide ligands, a transmembrane helix, a cytoplasmic portion that possesses tyrosine kinase catalytic activity. The extracellular portion normally contains one or several of immunoglobulin-like domains, fibronectin type IV-like domain, Cystein-rich domains and EGF-like domains. The cytoplasmic region of the receptor can be subdivided to a juxtamembrane region, a tyrosine kinase catalytic region and a carboxy-terminal region.

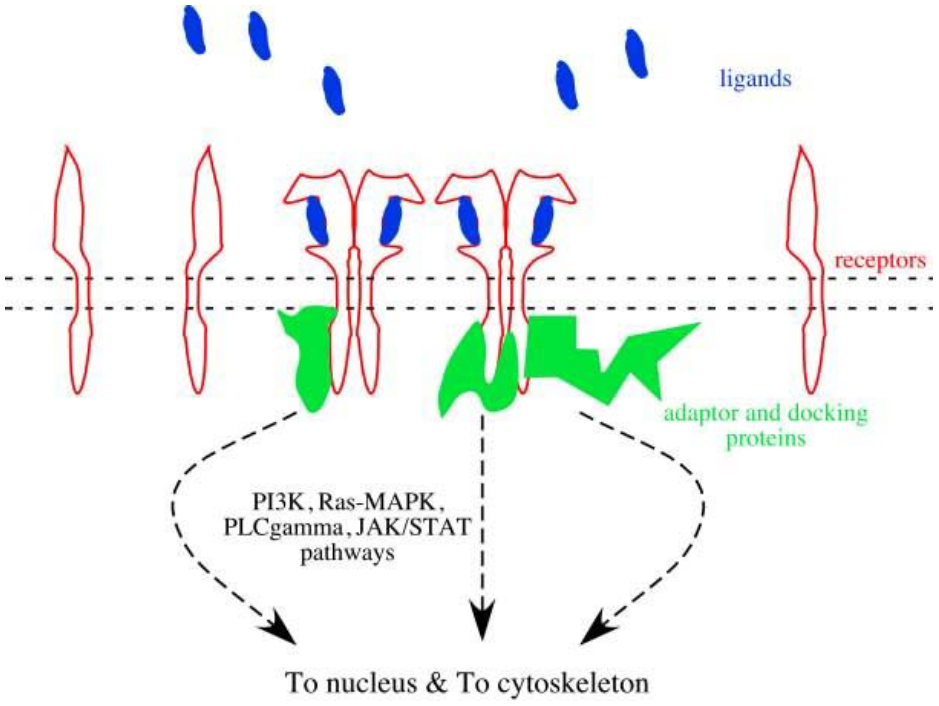

Fig.1-1. A simplified schematic representation of receptor tyrosine kinase signal (receptor activation after example of Epidermal Growth Factor Receptor). Signals are sent via ligands. Receptors exist mainly as monomers in their silent state on the membrane. The binding of the ligands to receptors leads to the activation of the receptors. Activated receptors recruit various adaptor and docking proteins, which triggers different pathways and therefore activates transcriptional factors or alters the cytoskeleton.

Except Insulin Receptor (IR), all known RTKs exist as monomers on the membrane in their silent state. They oligomerize upon ligand binding. Different ligands employ different strategies to activate the receptor. Recent solved structure of complex of human EGF (TGF-α) and the extracellular domains of its receptor (EGFR) revealed that EGF binds to its receptor in a 1:1 fashion. The binding causes the conformational change of the receptors, which leads to the direct interaction of the extracellular domains of the

receptors. This interaction is likely to be the key events of the dimerization and activation of the EGFRs (Garrett et al., 2002; Ogiso et al., 2002). The activation of FGF receptor (FGFR) requires heparin sulfate proteoglycan in addition of FGF. FGF binds to its receptor in a 1:1 manner similar to the binding of EGF to EGFR. In a crystallized complex, heparins make numerous contacts with both FGF and FGFR. These contacts are thought to stabilize the binding of FGF and its receptor. In addition, heparin also interacts with FGFRs in the region that two FGF-FGFR complexes adjoin, which is probably important for the dimerization of the receptors (Plotnikov et al., 1999). The oligomerization leads to the trans-phosphorylation of the receptors in their kinase domains and therefore activates the receptor. The activated receptors are thought to cis-phosphorylate their tyrosine residues in the juxtamembrane region and C-terminal region. These phosphorylated tyrosines provide binding sites for numerous signal molecules (Hubbard et al., 2000).

1.1.2 Bring the substrates close to the membrane

One important feature of the RTK signal transduction is that upon the activation of the receptor, numerous signal molecules are translocated to the membrane, either by direct interaction with receptor or the receptor binding proteins, or by interaction with the newly synthesized lipids such as phosphatidylinositol-3,4,5-trisphosphate or the proteins that bind to the lipids. This membrane translocation is important not only for the interaction of the molecules in the signal cascade, but also the increasing of local concentration of the downstream components, which is thought to ensure the specificity and efficiency of the signal transduction. For example, Ras is targeted to the inner leaflet of the plasma membrane through ER and Golgi by posttranslational modification of a C-terminal CAAX motif (Choy et al., 1999; Clarke et al., 1988), therefore to activated Ras would require the upstream molecules also localize to the membrane.

Proteins with different modules are able to recognize the phosphorylated tyrosine residues in different sequence motifs. The multiple tyrosine sites on the receptors are generally specific for binding of certain protein and therefore are responsible for specific signalling branches when phosphorylated. A well-studied case is PDGFRβ (Heldin et al., 1998). Activated PDGFRβs bind to signalling molecules at specific tyrosine residues. For instance, it binds PLCγ1 at tyrosine 1021, PI3K at tyrosine 740 and 751, the src class kinases at tyrosines 579 and 581. It also binds to proteins such as Grb2 (growth factor receptor-bound) and Shc (Src and collagen homolog). Proteins that are able to bind to receptors in RTK signal cascades are termed adaptor proteins and docking proteins.

Adapter proteins and docking proteins
Adapter proteins in RTK pathways generally contain modules that can recognize and bind to phosphorylated receptors. They are the direct targets of the receptor tyrosine kinases. They can be classified to two groups. One group includes proteins also having enzymatic function, such as Src kinases, PLCγ and PI3K. The other group includes proteins mainly function to recruit other proteins, such as Grb and Shc.

SH2 domains and PTB domains are the domains that help adapter proteins recognize and bind to specific phosphorylated tyrosine residues. SH2 domain is the abbreviation for src homology 2 domain which consists of about 100 residues that binds phosphotyrosine residues in a specific sequence motif. It is first identified in Src kinase. PTB domain is the abbreviation of phosphotyrosine binding domain which consists of about 60 residues that recognize phosphorylated tyrosine in a distinct sequence context (Forman-Kay et al., 1999; Shoelson, 1997).

In addition to the domains that are required for the binding of receptor, adaptor proteins normally also contain other protein-protein interaction modules such as SH3 domain (src homologue 3) domain, WW domain, PH (pleckstrin homology) domain, PDZ (post synaptic density/disc-large/zo-1) domain and PYVE domain. SH3 domain and WW domain recognize similar but distinct proline rich motifs (Bedford et al., 2000). PH domain has affinity for phosphoinositides or their soluble head groups (Lemmon et al., 1996). PDZ domain binds specifically to hydrophobic residues at the C termini of their target proteins (Sudol, 1998). PYVE domains specifically recognize PtdIns-3-P (Misra et al., 1999).

There are also a group of proteins called docking protein that are associated with the cell membrane by either a myristyl anchor or a transmembrane domain. Examples are FRS2, which contains a myristyl anchor (Kouhara et al., 1997), and LAT, which has a transmembrane domain (Zhang et al., 1998). Most of the docking proteins have a PH domain at their N-termini, which also confers them abilities to bind to phosphoinositides on the membrane. Docking proteins contain specific domains such as PTB domains that enable them bind different receptors. Docking proteins are thought to function as platforms for the recruitment of other signalling proteins in response to receptor stimulation. One example for docking protein is FRS2. It contains a consensus myristylation sequence and a PTB domain. In addition it has several tyrosine sites that are the potential target for phosphorylation. It is myristylated and targeted to the cell membrane. It binds to activated FGFR and being phosphorylated. The phosphorylated FRS2 are able to recruit Grb2 to the cell membrane and therefore transduce the signal (Kouhara et al., 1997; Rabin et al., 1993).

Function of the lipids during signal transduction

One of the major consequences of the RTK signal is the change of the lipid environment on the membrane. The major targets for the RTK signals are the phosphatidylinositol-4,5-phosphotate (PtdIns(4,5)P_2). Of particular interest is the phosphorylation of the D-3 position of the inositol ring in the PtdIns(4,5)P_2 molecule. This phosphorylation generates a structure that can be recognized and bound by particular protein modules, such as PH domain and FYVE domain. Many signal molecules are translocated to the membrane via their PH domains binding to the PrdIns(3,4,5)P_3. Examples include serine-threonine kinases Atk (or protein kinase B, PKB), Phosphoinositide-Dependent Kinase 1 (PDK1), the Arf exchange factor Grp1, the docking protein Gab1, and PLCγ1 (Czech, 2000; Rameh

et al., 1999). The association of Atk and PDK1 to the membrane leads to the phosphorylation of Atk by PDK1 (Lawlor et al., 2001).

The model that some lipids are capable of aggregating on the membrane has emerged during the last decade. This model proposes the existence in biological membranes of lipid microdomains or rafts that have a high sphinogolipid and cholesterol content, which makes the rafts more ordered and less fluid than else where in the plasma membrane. As a consequence, these membranes are resistant to solubilization with nonionic detergents at low temperatures (Brown et al., 1998; Brown et al., 1992; Moldovan et al., 1995). Rafts are abundant at the plasma membrane but are also found in exocytic and endocytic compartments, such as Golgi apparatus and caveolae (Alonso et al., 2001; Caroni, 2001; Puertollano et al., 2001). GPI-anchored proteins have been first reported to associate with those detergent resistant membrane complexes in 1983 (Hoessli et al., 1983). Later, src family tyrosine kinases are also reported to be associated with GPI-anchored proteins in the rafts (Cinek et al., 1992; Stefanova et al., 1991). It was proposed that the rafts may act as platforms for conducting a variety of cellular functions such as vesicular trafficking and signal transduction (Simons et al., 1997). The reports that heterotrimeric G proteins, Ras and PKC can bind to caveolae and be inactivated further supported the proposal by Simon and Ikonen (Okamoto et al., 1998). The fact that PtdIns(4,5)P_2 also accumulates in the membrane rafts (Pike et al., 1998) suggests that rafts may play an important role in regulating RTK signal.

1.1.3 Different downstream pathways

PLCγ pathway

PLCγ (phospholipase Cγ) contains one PH domain, one EF hand, two catalytic domains, two SH2 and one SH3 domains, and one C2 domain (Schlessinger, 2000). PLCγ is phosphorylated and recruited to membrane by binding to the activated receptor via its SH2 domains. It hydrolyzes PtdIns(4,5)P_2 to form two second messengers, diacylglycerol and Ins(1,4,5)P_3. By binding to specific intracellular receptors, Ins(1,4,5)P_3 stimulates the release of Ca^{2+} from intracellular stores. Ca^{2+} then binds to calmodulin, which in turn activates a family of Ca^{2+}/calmodulin-dependent protein kinases. In addition, both diacylglycerol and Ca^{2+} activate members of PKC family of protein kinases. These and other events generated by the activation of PLCγ finally lead to the changes in both transcription and cytoskeleton.

PI3K pathway

To date, all the PTKs can activate the phospholipid kinase PI3 kinase (Cantley, 2002; Katso et al., 2001; Schlessinger, 2000). Although multiple forms of PI3 kinases exist in higher eukaryotes, the class Ia enzymes are the primary targets of growth factors (Fruman et al., 1998). Group Ia PI3 kinases are heterodimers composed of a regulatory subunit (p85 in mammalian and p60 in Drosophila) and a catalytic subunit p110. The regulatory subunit which contains two SH2 and one SH3 domains, is responsible for binding to phosphorylated tyrosine sites on activated receptors or with tyrosine phosphorylated

docking proteins such as IRS (insulin receptor substrates) and Gab1 via its SH2 domains. Activated PI3 kinase phosphorylates PtdIns(4)P and PtdIns(4,5)P_2 to generate the second messengers PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. The latter induce the translocation of various signal molecules to the membrane, through which signal transduces to Akt and other components such as Arf6, Cdc42 and Rac, finally results in transcription and cytoskeleton changes.

Ras-MAPK pathway
All known RTKs and many other cell surface receptors stimulate the exchange of GTP for GDP on the small G protein Ras (Schlessinger, 2000). The guanine nucleotide exchange factor for this process is Sos (son of sevenless) protein. The adaptor protein Grb2 forms a complex with Sos via its SH3 domains. In order to activate Ras, Sos has to be recruited to the membrane where Ras is concentrated. There are several ways to achieve this. One way is that the complex binds to an activated RTK by the SH2 domain of Grb2, and thus brings the Sos to the membrane. Alternatively, the complex can bind to another adaptor protein such as Shc or a membrane linked docking protein such as IRS1 or FRS2α, which then can recruit the complex to the membrane. Vertebrate Grb cannot bind to FGFR directly. In the case of FGF signal, Grb/Sos complex is brought to the membrane through FRS2α/SNT1 (Kouhara et al., 1997; Wang et al., 1996).

Activated Ras can activate Raf and PI3 kinase. The activated Raf phosphorylates MAP-kinase kinase (MAPKK or MEK), which consequently activates MAPK. Activated MAPK phosphorylates a variety of cytoplasmic and membrane linked substrates. In addition MAPK is rapidly translocated into the nucleus where it phosphorylates transcription factors. This signal cascades from Ras to MAPK is highly conserved in yeast, invertebrates and vertebrates.

1.1.4 Signals to nucleus

Upon activation MAPK is translocated into nucleus. The major targets of MAPK are Ets domain transcription factors. In flies, Pointed and Yan are both directly phosphorylated by activated MAPK upon EGF signalling in the embryonic ventral ectoderm. This phosphorylation stimulates the activity of Pointed, a transcriptional activator, but inhibits Yan, a transcriptional repressor. Both proteins have similar binding preferences. Therefore the activation of MAPK is thought to promote transcription by causing a switch of activated Pointed for Yan at Ets sites (Gabay et al., 1996). C-Jun is another important target of MAPK. Its Drosophila homologue is Jra (Jun-related antigen) (Perkins et al., 1990; Zhang et al., 1990).

RTKs can also induce gene transcription by activating the JAK/STAT signalling pathway. Activated RTKs can activate JAK which subsequently tyrosine phosphorylates STATs. STATs then are able to form homodimers or heterodimers. The dimeric STATs translocate to the nucleus to activate transcription of targeted DNA sequence (Darnell et al., 1994; Ihle, 1995).

1.1.5 Signals to cytoplasm

The cytosolic events include cytoskeleton rearrangement, changes in vesicle transport and metabolism. How exactly these events are achieved are largely unknown. Many components involved in the signal network have potential to regulate cytoskeleton. Raucher et al. have shown that PtdIns(4,5)P_2 regulates the adhesion energy between the cytoskeleton and the plasma membrane. They overexpressed PtdIns(4,5)P_2 –specific PH domain in cells, and measure the interaction force between plasma membrane and the cytoskeleton by pulling membrane tethers using optical tweezers (Raucher et al., 2000). They found that when they sequenster PtdIns(4,5)P_2 by overexpression of PH domain, the adhesion force between plasma membrane and cytoskeleton is decreased. They obtained same affect with overexpression of membrane targeted 5'-specific PtdIns(4,5)P_2 phosphotase. Further more, they showed that by simulate the activation of PLCγ they could reduce the adhesion force between the cytoskeleton and cell membrane. Their results imply a direct way that RTK signals affect cytoskeleton. Although PtdIns(4,5)P_2 can bind to many actin regulatory proteins *in vitro*, there is no direct evidence on how membrane associated PtdIns(4,5)P_2 can regulate the cytoskeleton. One possible answer comes from the recent studies on GAP43-like proteins. PtdIns(4,5)P_2 is enriched together with GAP43-like proteins in the membrane rafts (Laux et al., 2000). GAP43-like proteins are capable of binding to PtdIns(4,5)P_2, calcium/calmodulin, PKC and actin filament. They can induce filopodia and microspikes at the periphery of cells in the overexpression experiment. Studies have pointed to raft association as critical determinant of the protein function (Caroni, 2001).

Small GTPase such as Rho1, Rac and Cdc42 are important for cytoskeleton rearrangement. In Swiss 3T3 cells, the activation of Rho1 results in the formation of stress fibers (Ridley et al., 1992a), while the activation of Rac leads to polymerization of actin at the plasma membrane, producing lamellipodia and membrane ruffles (Ridley et al., 1992b). Cdc42 can bind to WASP family proteins, which are adaptor molecules that bind multiple signalling and cytoskeletal proteins such as Arp2/3 complex (actin related protein 2/3 complex). These three molecules form a complex to regulate the actin skeleton (Carlier et al., 1999; Mullins, 2000). Recent studies by Fukata et al. have shown that activated Rac1 and Cdc42 bind to IQGAP and CLIP-170 to form a tripartite complex. The latter two proteins are microtubule binding proteins. The forming of tripartite complex attaches the microtubule to actin network, which is essential for cell polarization (Fukata et al., 2002). This paper presents a direct evidence of how RhoGTPase regulates the microtubule network.

PI3K upon the activation by IR, can promote the glucose transport and metabolism, which is partially regulated through the cytoskeleton rearrangement caused by activating Rac (Katso et al., 2001). Activated Cdc42 interact with Sec3p, one of the eight components of exocyst, and is required for targeted secretion in yeast *S. cerevisiae* (Zhang et al., 2001).

1.1.6 Signal termination

The RTK signals have to be tightly controlled. The attenuation and termination of the signal events is as important as the initiation of the events. All the signal pathways engaged negative feedback to ensure the signals are terminated efficiently.

Some feedbacks function at receptor level. For instance, the activation of EGFR leads to the expression of a secreted EGF-like protein, such as Argos in Drosophila. Argos competes the binding domain for Spitz on the receptors but cannot activate the receptor, and therefore attenuate the EGF signalling (Jin et al., 2000a). Receptor endocytosis and degradation provide a more direct and efficient way of terminating the signal. Growth factor stimulation results in rapid endocytosis and degradation of both the receptor and the ligand. Ligand binding induces receptor clustering in coated pits on the cell surface, followed by endocytosis, migration to multivesicular bodies and eventual degradation by lysosomal enzymes. The kinase domain of the receptor is important for the process (Ullrich et al., 1990). It is not clear how this process is regulated. In case of EGFR and PDGFR, they are ubiquitinated by Cbl and degraded (Joazeiro et al., 1999). Recently identified cell adhesion molecule-echinoid is a negative regulator of EGFR signal during the formation of Drosophila eyes. Echinoid is localized to the cell membrane of every cell through out the eye disc. Its expression does not dependant on EGFR signalling (Bai et al., 2001).

Sprouty has been found to be a negative regulator of both EGF and FGF signals (Hacohen et al., 1998; Taguchi et al., 2000). It functions probably downstream of the receptors and upstream of Ras (Casci et al., 1999). The SH2-containing phosphatases (SHIP1 and SHIP2) dephosphorylate the 5 position of the inositol ring to produce PtdIns(3,4)P_2 and therefore reduce the amount of PtdIns(3,4,5)P_3 on the cell membrane. This dephosphorylation is important for insulin signals (Clement et al., 2001). The phosphatase PTEN dephosphorylates the 3 position of PtdIns(3,4,5)P_3 to produce PtdIns(4,5)P_2 and therefore terminate the signal (Goberdhan et al., 1999; Maehama et al., 1999).

1.2 Retain the specificity of different receptor tyrosine kinase signals

Fambrough et al. in 1999 tried to address the question of if the activated RTKs lead to similar transcription response by analyzing the RNA expression induced by PDGFRβ using DNA microarray technique. They mutated the specific tyrosine binding sites for different signal molecules in PDGFRβ, and tested the ability of the mutated receptor to induce transcription of a set of 'immediate early genes' (IEG). They found that although signals from the same receptor divert, these divert signal pathways virtually induce a set of largely overlapping IEGs in NIH3T3 cells if they provide saturating amount of the ligand, M-CSF. Even more, they found when they stimulated NIH3T3 cells with saturating amount of PDGF or FGF for 1 hour, both PDGFRβ and FGFR induce almost the same set of IEGs. In contrast, EGF stimulation results in a similar, but clearly distinct,

transcriptional response. These results lead to their conclusion that different downstream pathways of RTKs induce a set of overlapping, rather than independent genes *in vitro* (Fambrough et al., 1999). However, their experimental settings have intrinsic differences in comparison to the *in vivo* situation. Firstly, they provided saturating amount of the ligand stimuli, which is unlikely to be always the case *in vivo*. Secondly, they have only studied the response in one cell type.

The studies in *C. elegans* provide good examples of how the regulation of RTK signals happens *in vivo*. The Let-23 signal (EGFR signal) in *C. elegans* is required in multiple tissues for specific responses. Lesa and Sternberg found that different tyrosine containing domains in the cytoplasmic part of the receptor are required differently for Let-23 function in viability and vulval induction in comparison to the development of hermaphrodite gonad, which suggests that different downstream pathway is activated in different processes (Lesa et al., 1997). The significance of this finding is that, activation of different downstream pathways of EGFR signal is important for different outcome *in vivo*.

In an *in vivo* system cells with different developmental history are intrinsically different. Therefore even if they receive same signals, they interpret the signals differently. A good example comes from studies of *C.elegant* vulva development. A Hox gene *lin-39* is expressed low level in the vulval precursor cells (VPCs) before the vulval induction occurs. The induction of vulva requires EGF signal stimulated Ras activity as well as a later notch signal. The activation of Ras increases *lin-39* expression in the VPCs. The increased expression of *lin-39* is crucial for vulval induction. However, without the presence of low level fully functional Lin-39 at first place, activated Ras alone can not increase the *lin-39* expression (Maloof et al., 1998).

These examples and others (Pawson et al., 1999; Simon, 2000), suggest that RTKs are likely to activate different combination of downstream molecules *in vivo* and the signals that RTKs send also interpreted by the receiving cells according to their developmental states. To fully understand the biological function of RTK signals, we have to study signal events in an *in vivo* system. The studies of FGF signal in vertebrate development provide a good example.

1.3 Fibroblast growth factor signalling in vertebrate development

FGF signals are involved in many aspects of animal development. During the development of Xenopus embryos, FGF signals are required in controlling mesoderm production and maintenance as well as morphogenetic movements during gastrulation. This mesoderm production has been shown to dependent on Ras1-MAPK signal (Umbhauer et al., 1995; Whitman et al., 1992), and PLCγ does not appear to affect mesoderm induction in Xenopus ectodermal explants (Muslin et al., 1994). The association of Grb/Sos complex to receptor requires the docking protein FRS2, which activates Ras1 (Ong et al., 2000). In addition, a member of the src-related gene family-

Laloo appears to act specifically downstream of FGF signal during mesoderm formation of Xenopus embryos, although it was not tested if Laloo could also proceed signals from other RTKs (Weinstein et al., 1998). The Low-Molecular-Weight Protein Tyrosine Phosphatase1 (LMW-PTP1) found in Xenopus also involves in FGF signal during mesoderm formation. Injection of morpholino antisense specific for LMW-PTP1 inhibits Ras1-MAPK signal and therefore blocks FGF signal during mesoderm formation. Additional phenotypes were also observed which suggests the gene does not function merely in FGF signalling pathway (Park et al., 2002). However, it is still very little that we know about how the signal is transduced.

1.4 Drosophila development and signal transduction

Receptor tyrosine signals have been studied in Drosophila extensively. They are involved in many aspects of Drosophila development. Here I will mainly focus on signals required during Drosophila eye development and FGF signals during embryo development.

1.4.1 Signals required during Drosophila eye development

Drosophila eye development
The Drosophila eye is composed of approximately 800 ommatidia, each of which comprises eight photoreceptor cells (R1-R8), four lens secreting cone cells and eight accessory cells including three classes of pigment cells and a bristle complex. The eye morphogenesis initiates during the third larval instar of development as a morphogenetic furrow moves across the disc from posterior to anterior (Ready et al., 1976). Cell differentiation starts at the posterior edge of the morphogenetic furrow. R8 is first differentiated, which in turn recruits R2/R5 and R3/R4 in pairs. R1/R6 are differentiated afterwards, and the R7 is the last to differentiate. The differentiation of the cone cells and accessory cells occurs almost simultaneously with R1/R6 and R7 (Wolff et al., 1993). The photoreceptor cells in each ommatidium are organized into a polarized pattern. In a cross section of a mature ommatidium, R8 lies beneath the R7, the R1 to R6 surround the R7 (or R8) to form a trapezoid shape (Fig.1-2). The relative position of R3 and R4 determines the polarity of the ommatidium. Ommatidia are arranged in mirror-symmetry across the dorsoventral midline, the equator. This chirality of the ommatidia arrangement is termed epithelial planar polarity (EPP) in Drosophila eye (Mlodzik, 1999).

The specification of photoreceptor cells
EGF signals are required for the differentiation of all cell types in the eye. In addition, it is also essential for proper furrow initiation, proliferation, spacing, recruitment and survival of cells in the eye discs. These functions are achieved mainly through MAPK pathway to inhibit the ETS domain repressor Yan and activate the activator Pointed.

The Sevenless (Sev) receptor tyrosine signal was first identified to be involved in the determination of a single cell type R7. Later it has also been found to be important for the

determination of R2/R5 fate (Zipursky et al., 1994). The ligand for Sev is Boss (Bride of sevenless). The activated Sev binds to Drk (Downstream of receptor kinase), the Drosophila homologue of Grb2, which recruits Sos and in turn activates Ras1-MAPK pathway. Corkscrew, a protein-tyrosine phosphotase and Dos, a docking protein, have also been shown to be able to transduce the signal to Ras85D. The Drosophila MAPK pathway consists of Raf, Dsor1 (MEK), and Rolled (Rl, MAPK). Kinase Suppressor of Ras (KSR) is genetically function downstream of Ras85D and upstream of Raf (Raabe, 1998). Recently, Roy et al. have reported that KSR can bind independently to Raf and MEK in Schneider cells and function as a scaffold protein to promote the signal transduction from Raf to MEK (Roy et al., 2002).

It has been shown that the function of Sev for the fate determination of R7 can be replaced by an activated version of EGFR (Freeman, 1996). It is not surprising since all the RTKs share common downstream components. However, it brings a question that how the specific cell fate in the eye is determined. A study on the specification of cone cells provides a clue for the cell fate specification. The expression of D-Pax2 (the Drosophila homologue of Pax2) is required for cone cell specification. The transcription regulatory region of D-Pax2 is capable of binding to Suppressor of Hairless (Su(H)), Yan, Pointed (Pnt) and Lozenge (Lz). Su(H) is the target of Notch signal, while Yan and Pnt are the target of RTK signalling. Lz encodes a Runt domain containing transcription factor. By examine the expression of D-Pax2 in cone cells in mutant background of Lz, EGFR and Notch, Flores et al. have shown that the specification of cone cell dependants on the presence of Lz in addition to Notch and EGFR signals (Flores et al., 2000). This work gives a good example of how multiple signals work together to specify the cell fate. In this case, EGFR signal is part of the signal network, and the specific cell fate is the results of the integration of various inputs.

Epithelial planar polarity

Signals from the seven transmembrane receptor, Frizzled (Fz) is required to establish the correct epithelial planar polarity (Vinson et al., 1989). In the Fz mutant, the mirror-image symmetry is lost. Genes involved in establishing the EPP in Drosophila eye includes *dishevelled* (*dsh*), *flamingo* (*fmi*), *misshapen* (*msn*), *rhoA*, and *rac* (Fanto et al., 2000; Paricio et al., 1999; Strutt et al., 1997). Genetic evidence has established Dsh as an adaptor protein, which transduces the signal from Fz to the downstream targets. Msn, RhoA and Rac function downstream of Dsh (Mlodzik, 1999; Van Aelst et al., 2002). Fmi is a seven transmembrane cadherin that function genetically downstream of Fz (Usui et al., 1999).

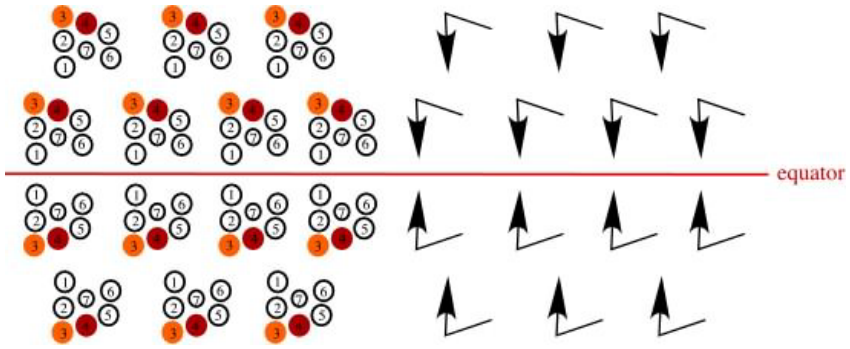

Fig.1-2. The schematic representation of the arrangement of epithelial planar polarity in Drosophila compound eye. In each ommatidium, seven photoreceptor cells (R1 to 7) are arranged in a trapezoid shape, R8 lies beneath the R7. The relative position of R3 and R4 determines the polarity of each ommatidium. Ommatidia are arranged in mirror-symmetry across the dorsoventral midline, the equator (left panel). The polarity of each ommatidium is indicated by arrows (right panel).

1.4.2 FGF signalling during Drosophila embryonic development

Two processes of embryo development require FGF signals exclusively. One is the mesoderm migration, the other is the trachea branching.

Mesoderm migration

Soon after ventral furrow invagination, the cells of the mesoderm primordium that are close to the ectoderm make contact with ectoderm. After the initiation of the contact, mesoderm cells can migrate along the ectoderm dorsally to form single layer of cells (Fig.1-2A). A gene encodes for an FGFR-*heartless* (*htl*) is indispensable for this process. In the *htl* mutant, mesoderm cells invaginate normally but fail to migrate dorsally (Beiman et al., 1996; Gisselbrecht et al., 1996). The FGF ligand for Htl is currently unknown. In the homozygous *dof* (downstream of FGF) mutant embryos, mesoderm cell migration is also defective (Fig.1-2B) (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Later on, the Htl signal together with other inductive signals such as Decapentaplegic (Dpp) signal are required for the differentiation of certain cardiac and somatic muscle cells (Michelson et al., 1998).

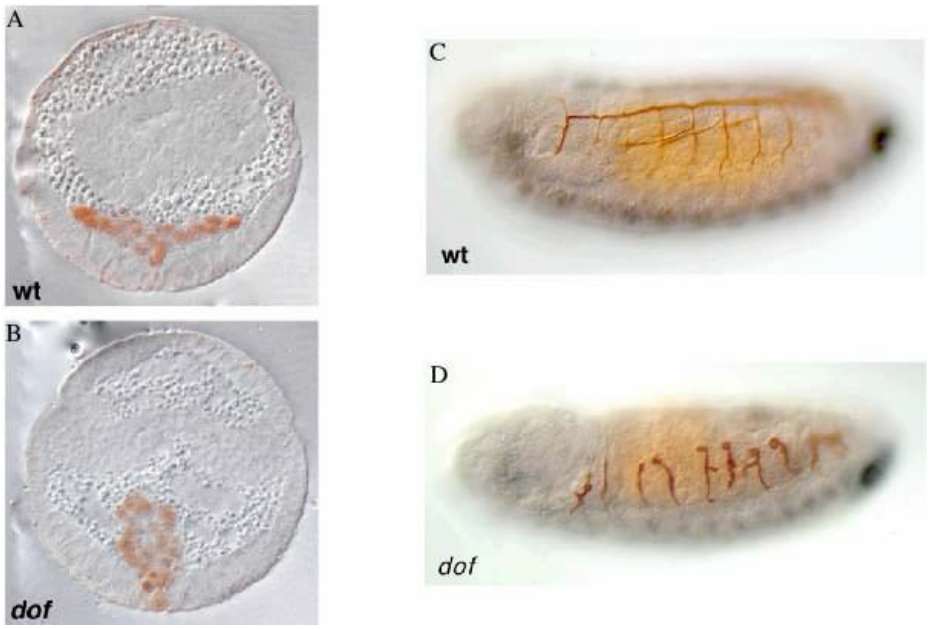

Fig.1-3. The phenotype of homozygous *dof* mutant embryos. Comparing to wild type embryo in similar stage (A&C), in *dof* mutant, mesoderm cells cannot spread normally (B), and tracheal pits do not branch (D). Mesoderm cells are visualized by anti-twi antibody in brown (A&B). Trachea branches are visualized by 2A12 in brown as well (C&D). The mesoderm phenotype of *dof* is similar to *htl* mutant, while the tracheal phenotype is similar to *btl* or *bnl* mutant. Courtesy of R. Wilson (Vincent et al., 1998).

Tracheal branching

The larval tracheal system is a net of interconnected hollow tubules with openings to the environment that provides oxygen to the different tissues by passive diffusion. The development of the trachea starts at the end of the germband extension during embryo development. The placodes of approximately 40 cells per hemisegment differentiate within the surface ectoderm. These cells invaginate while undergo their final round of cell division, forming tracheal pits. Tracheal pits then initiate first, second and third branches to form an interconnected network of hollow tubules (Fig.1-2C). Several signal pathways have been shown to be important for the development of trachea, such as FGF signal, Dpp signal, Notch signal and EGF signal. FGF signal is important for the primary and secondary branching as well as some fine third branching. Branchless (Bnl, a FGF homologue), secreted by mesoderm cells, gives instructive information to the tracheal cells where to extend (Sutherland et al., 1996). The receptor for Bnl is Breathless (Btl) (Klambt et al., 1992). In the *btl* or *bnl* or *dof* mutants, no branching occurrs during the trachea development (Fig.1-2D) (Imam et al., 1999; Klambt et al., 1992; Sutherland et al., 1996), (Michelson et al., 1998; Vincent et al., 1998).

The transduction of FGF signalling

How the two FGF signals in mesoderm and trachea are transduced is largely unknown. The activated forms of either FGFRs, which are the FGFR chimera composed of the dimerization domain of λ repressor and the transmembrane and cytoplasmic domains of FGFRs (Lee et al., 1996; Michelson et al., 1998), can not rescue the *dof* mutant phenotype. Together with its cytoplasmic location, these results imply that Dof functions downstream of FGF. Overexpression of constitutive active form of Ras85D in mesoderm or trachea can rescue the *dof* mutant phenotype partially which place Ras85D genetically downstream or parallel to Dof. In addition, activated Rl (MAPK) have been found at the tip of migrating mesoderm and trachea cells (Gabay et al., 1997). The staining specific for activated Rl disappears in *htl* or *dof* homozygous mutant embryos (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Sprouty, which is a common negative regulator of RTK signals, is required for proper FGF signal during tracheal branching (Hacohen et al., 1998). However, how exactly the signals are transduced in mesoderm and trachea is not clear. As an attempt to understand FGF signals in morphogenesis, I first tried to address how the FGF signal is transduced in general, that is what the other components in the FGF signals are. A general approach to address this question genetically is to perform a screen searching for mutants of genes that affect FGF signals.

1.5 Approaches

A number of screens have been done to search for new components involved in Ras85D-MAPK pathways using Drosophila compound eye as a model system (Huang et al., 2000; Karim et al., 1996; Rebay et al., 2000; Therrien et al., 2000). The major advantages of this system are that Drosophila eye is not required for survival, and is highly sensitive to signal changes and subjective to pattern disruption. Either a gain-of-function (GOF) or a loss-of-function (LOF) screen have been employed in the screens that have been published. It has been estimated that over 2/3 of all Drosophila genes show no obvious LOF phenotypes when mutated, perhaps due to functional redundancy (Miklos et al., 1996). However, over- or mis-expression of these genes could provide information of their functions. Therefore Rorth et al. have generated a collection of flies carrying enhancer P-element (EP-element) that can be used for a GOF screen (Rorth, 1996). Flies carrying the EP-element are able to overexpress the gene downstream of the EP insertions when Gal4 is present.

1.6 Aim of the thesis

The aim of this thesis is to identify molecules involved in the FGF signalling pathway.

2 Results

2.1 The gain-of-function screen

2.1.1 The 'GMR>λ-btl, dof' eye phenotype

Flies expressing the constitutively active form of Btl or Htl (λ-Btl or λ-Htl) respectively together with Dof by GMR-Gal4 had a rough eye phenotype (Robert Wilson, Fig.2-1D). However, expression of *λ-btl* or *λ-htl* or *dof* with GMR-Gal4 had no effect on the morphology of the fly eyes in comparison to that of *gmr-Gal4* flies (Fig.2-1, compare B&C to A). This observation implies that the major downstream targets of the FGF signal are probably present during the Drosophila eye development and therefore offers us a possibility to utilize the Drosophila compound eye to screen for components that are involved in the FGF signal. A forward gain-of-function (GOF) screen was hence designed to identify critical components involved in the FGF signalling cascade. This screen is based on the hypothesis that when the dose of a critical downstream component of the FGF signalling cascade is changed, the strength of the ectopic FGF signal will be affected and thus the 'GMR>λ-btl, dof' eye phenotype can be modified.

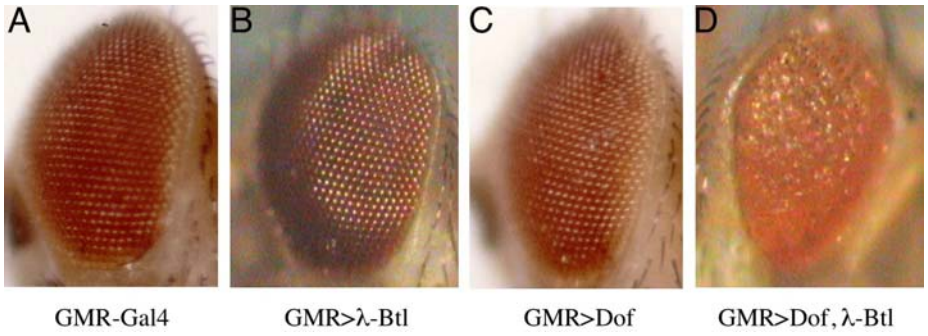

Fig.2-1. Misexpression of Dof and λ-btl by GMR-Gal4 produces a rough eye phenotype. Expression of GMR-Gal4 alone (A), λ-Btl by GMR-Gal4 (B), dof by GMR-Gal4 (C) do not show any rough eye phenotype, while expression of both dof and λ-Btl by GMR-Gal4 (D) produces a rough eye phenotype. The other FGFR homologue in Drosophila, htl has similar effect to btl. Flies were raised at 22°C. Courtesy of R. Wilson.

For the convenience of the screen, transgenes of *gmr-Gal4*, *UAS-λ-btl*, and *UAS-dof* were recombined onto one chromosome. The flies carrying all three transgenes on one

chromosome were denominated as 'GMR>λ-btl, dof'. Flies with one copy of the chromosome show a rough eye phenotype, two copies cause lethality.

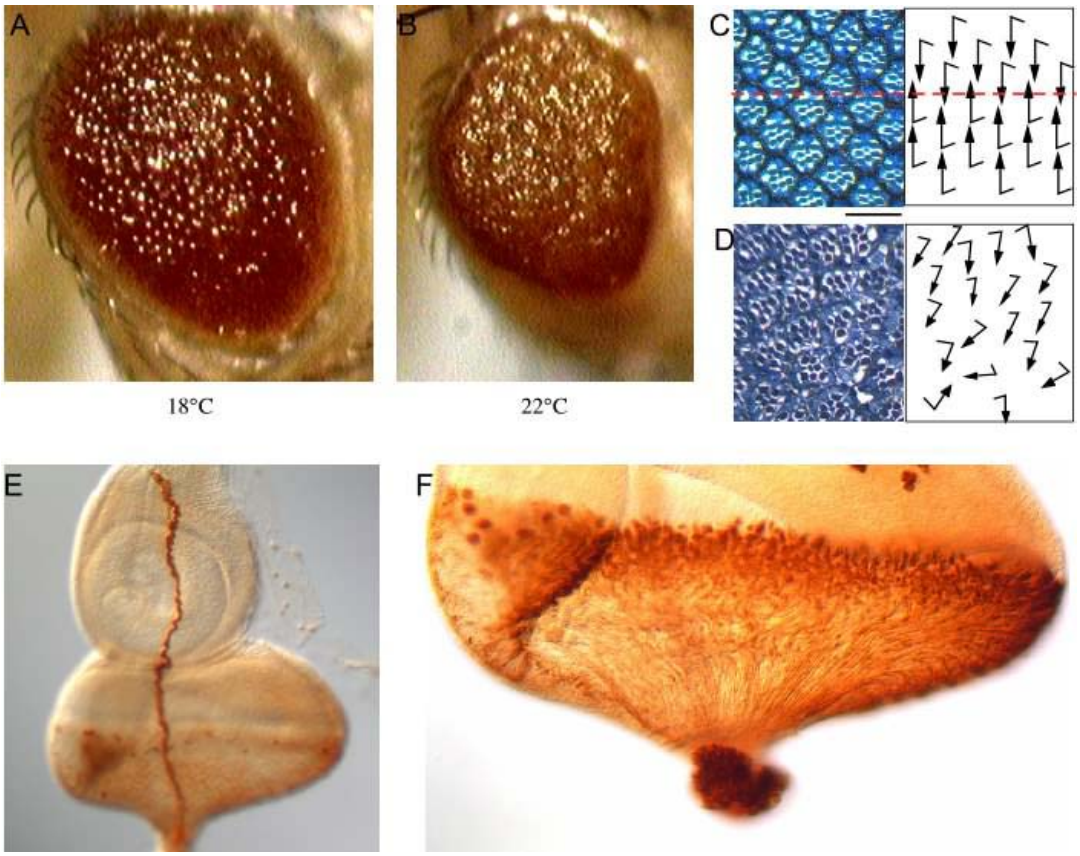

Fig. 2-2. The 'GMR>λ-btl, dof' eye phenotype and the expression pattern of the *gmr-Gal4*. The eyes of 'GMR>λ-btl, dof' flies are rougher and smaller at 22°C (B) than at 18°C (A). Sections through the eye of a 'GMR>λ-btl, dof' fly raised at 18°C show that the orientation of the photoreceptors are irregular (D) in comparison to wild type (C), which suggests that 'GMR>λ-btl, dof' eye has an epithelial planar polarity (EPP) defect at 18°C. In addition, there are loss of photoreceptor cells, shortening of rhabdomeres as well as EPP defects in the eyes of 'GMR>λ-btl, dof' flies raised at higher temperature (Curtiss J., personal communication). Rhabdomeres were stained with toluidine blue. The red dash in C marks the equator. Scale Bar for C & D is 25µm. The *gmr-Gal4* is expressed in Bolwig's nerve (E) and all the developing photoreceptor cells in the eye discs of 3rd instar larvae (F). GMR-Gal4 was visualized by overexpression of lacZ and anti-beta-Gal staining. The schematic drawing for the orientation of photoreceptors followed the example of Fanto et al. 2000.

Before starting the screen, I examined the 'GMR>λ-btl, dof' eye phenotype in more detail. The eye phenotype is mild at 18°C. The eyes become smaller and rougher as the

temperature increases (Fig.2-2, A&B), which is probably due to the increased activity of Gal4 transcription factor. The change of eye size at higher temperature is likely to be due to the loss of photoreceptor cells. The epithelial planar polarity (EPP) in Drosophila compound eye is established in the mirror-symmetric arrangement of ommatidia relative to the dorsoventral midline, the equator (Fig.2-2C). Sections through the eye of the 'GMR>λ-btl, dof' fly raised at 18°C show that the photoreceptor cells did not orient properly compare to wild type (Fig.2-2D). This suggests that the ectopic FGF signal has an EPP defect. In addition, the eyes of the 'GMR>λ-btl, dof' flies raised at higher temperature show defects in loss of photoreceptor cells, and shortening of rhabdomeres (Curtiss J., personal communication). In order to find out in which cells the ectopic FGF signal is induced, the flies carrying the *gmr-Gal4* that were used to express *dof* and *λ-btl*, were crossed to flies with a *UAS-lacZ* transgene. GMR-Gal4 can drive expression of *lacZ* in all the differentiated photoreceptor cells and in Bolwig's nerve in eye disks of the third instar larvae (Fig.2-2, E&F). Therefore the ectopic FGF signals in the 'GMR>λ-btl, dof' fly are induced in all the differentiated photoreceptor cells and Bolwig's nerve.

2.1.2 Tests of the potential candidates

To test some potential candidates and also to determine if the eye phenotype of 'GMR>λ-btl, dof' could be modified in a dose sensitive manner, I carried out a loss-of-function test for those candidates. Several classes of known signalling molecules were tested for modification of the 'GMR>λ-btl, dof' eye phenotype when half of their gene products were mutated. Firstly genes that are involved in Ras85D-MAPK pathway were examined. I have chosen to test *ras85D, raf* (the Drosophila homologue of *MEKK*), *dsor1* (the Drosophila homologue of *MEK*), *rl* (*rolled*, the Drosophila homologue of *MAPK*), *csw (corkscrew)*, *dos (daughter of sevenless)*, *drk (downstream of receptor tyrosine kinase), ksr (kinase suppressor of Ras), gap1 (GTPase activating protein), sos (son of sevenless)*, and *14-3-3ζ*. The adaptor protein, Dock (Dreadlock), has been found to interact genetically with Ras85D (Schnorr et al., 2001), and therefore the *dock* mutant was tested. Since 'GMR>λ-btl, dof' flies show defects in planar polarity, the mutants of genes that mediate planar polarity signals were included in the experiment. These are *dsh (disheveled), fmi (flamingo),* and *fz (frizzled)*. RhoA has also been shown to have a function in EPP signalling. In addition, overexpression of RhoA by Btl-Gal4 shows defects in the formation of dorsal trunk of the trachea (Lee et al., 2002). Given that RhoA is an important effector for cytoskeletal rearrangement, mutants for *rhoA* and one of its activators *rhoGEF2* (Barrett et al., 1997) were also tested. Genes, like *Notch* and *cyclinA*, which have distinct functions and are unlikely to be directly regulated by the FGF signalling pathway, were tested as controls.

Flies carrying mutations in the candidate genes were crossed to the 'GMR>λ-btl, dof' stock. The progeny were raised at 18°C. Eyes of those progeny containing one copy of the mutant gene and one copy of the 'GMR>λ-btl, dof' chromosome were compared to their

siblings with only one copy of the 'GMR>λ-btl, dof' chromosome. The results of this experiment are summarized in table 2-1.

Mutants of *raf, rhoA, rhoGEF* and *fmi* enhanced the 'GMR>λ-btl, dof' eye phenotype. Other mutants such as *rl*, *ras85D, csw, dos, drk, Dsor1, ksr, gap1, sos*, *14-3-3ζ, dock, dsh*, and *fz* did not show any visible modification of the 'GMR>λ-btl, dof' eye phenotype. Mutants of *Notch* and *cyclinA* did not affect the roughness of the eyes of 'GMR>λ-btl, dof' flies.

The fact that among the MAPK signalling cassette only the *raf* mutant enhanced 'GMR>λ-btl, dof' eye phenotype is surprising. It may be explained by the fact that Raf is on the top of the hierarchy of the enzymatic pathway and therefore more sensitive to the dose change in the test. The significance of the other modifiers is difficult to explain based on a single test. The results suggest that it is possible to identify components involved in the FGF signalling cascade when the amount of the molecule in the Drosophila eye is changed in the presence of 'GMR>λ-btl, dof' chromosome. Neither *Notch* nor *cyclinA* mutants show modification of 'GMR>λ-btl, dof' eye phenotype suggesting that the test has some specificity.

Both Cdc42 and Rac1 are known to be downstream targets of RTK signals and to regulate signals leading to cytoskeleton rearrangement, vesicle transport and cell differentiation (Katso et al., 2001; Schlessinger, 2000). During eye development, signalling by Rac1 is also required in the establishment of epithelial planar polarity (Fanto et al. 2000). To test if they also interact with the ectopic FGF signal in fly eyes, I crossed 'GMR>λ-btl, dof' flies to transgenic flies carrying *UAS-cdc42*$^{L89.4}$ or *UAS-rac*^{N17} respectively. *Cdc42*$^{L89.4}$ and *rac*^{N17} are dominant negative alleles (Benlali et al., 2000; Lou, 2001). Neither of the co-overexpression modified the 'GMR>λ-btl, dof' eye phenotype at 18°C, which may imply that Cdc42 and Rac1 are not critical components for this ectopic FGF signal.

The results of this test indicated that it was possible to find components that are important for the ectopic FGF signalling in a dose sensitive screen. Given that other RTK signal pathways are used extensively during the Drosophila eye development and most of the downstream components are shared by different RTK signals, only candidates that are common for RTK signals during eye development could be found in a loss-of-function screen. Many genes are expressed ectopically in a gain-of-function (GOF) screen, which offers a possibility to find components that are more specific for FGF signals in a GOF screen. Therefore, a GOF screen was carried out to search for genes that are important for the FGF signalling pathway.

Table 2-1. The results of the tests of the potential candidates

Genes tested:	Genotype of the flies examined:	Allele class	Eye phenotype of the progeny compared to that of 'GMR>λ-btl, dof'
ras85D	*sev ^{d2} ; Ras1 ^{e2f} / TM3*	hypomorph	~
	Ras^{C40B} FRT82B/TM3^{ftz}	amorph	~
raf	*raf ^{EA75} / FM7*	LOF	Rougher
	raf ^{C110} / FM7	hypomorph	Rougher
dsor1	*y w dsor1 ^{LH110} FRT / FM7a*	amorph	~
rl	*Df(2R)rl ^{10a} / CyO*	–	~
	rl ^{EMS698} / SM1	–	~
csw	*y csw$^{Esev1A–eOP}$ sev^{d2} / FM7*	antimorph	~
dos	*w^{-}; dos^{P115}FRT 2A / TM3*	LOF	~
drk	*w; drk $^{24/1}$ / CyO*	–	~
	drk ^{e0A}	antimorph	~
ksr	*ksr ^{s721} / TM3,Sb e ry sev-Ras^{V12}*	LOF	~
gap1	*Gap1$^{1-16}$ / TM3 ry*	–	~
sos	*sev ^{d2};sos ^{e4G} / CyO*	–	~
14-3-3ζ	*14-3-3ζ^{P07103} cn / CyO; ry*	hypomorph	~
rhoA	*FRT rhoA ^{R2} / CyO*	–	Rougher
rhoGEF2	*FRT DrhoGEF / CyO*	–	Rougher
dock	*dock ^{P04723} cn ^{1} / CyO; ry^{506}*	amorph	~
	y^{1} w^{67} c^{23}; dock ^{P13421} / CyO	amorph	~
dsh	*dsh^{1}*	hypomorph	~
fmi	*y w; fmi ^{E59} / CyO (y^{+})*	LOF	Rougher
fz	*In(3LR)fz / TM1, Me ri sbd^{1}*	amorph	~
cyclinA	*If / CyO; cycA^{C8} / TM3 ^{UbxLacZ}*	null	~
Notch	*Df(1) N ^{81K} / FM6 ; UAS FLP / TM2*	null	~

~: no obvious difference; –: not determined; LOF: loss of function

2.1.3 The gain-of-function screen

A gain-of-function screen was performed at 18°C to identify new components of the FGF signal (Fig.2-3A). 'GMR>λ-btl, dof' flies were crossed to the EP lines individually. The eyes of the progeny containing an EP insertion and the 'GMR>λ-btl, dof' chromosome were compared to their siblings carrying only the 'GMR>λ-btl, dof' chromosome. Those EP lines showing a modified 'GMR>λ-btl, dof' eye phenotype in the F1 generation were selected as potential candidates. When the screen was completed, in total, 153 candidates emerged. Among these candidates, there were 81 enhancers and 72 suppressors.

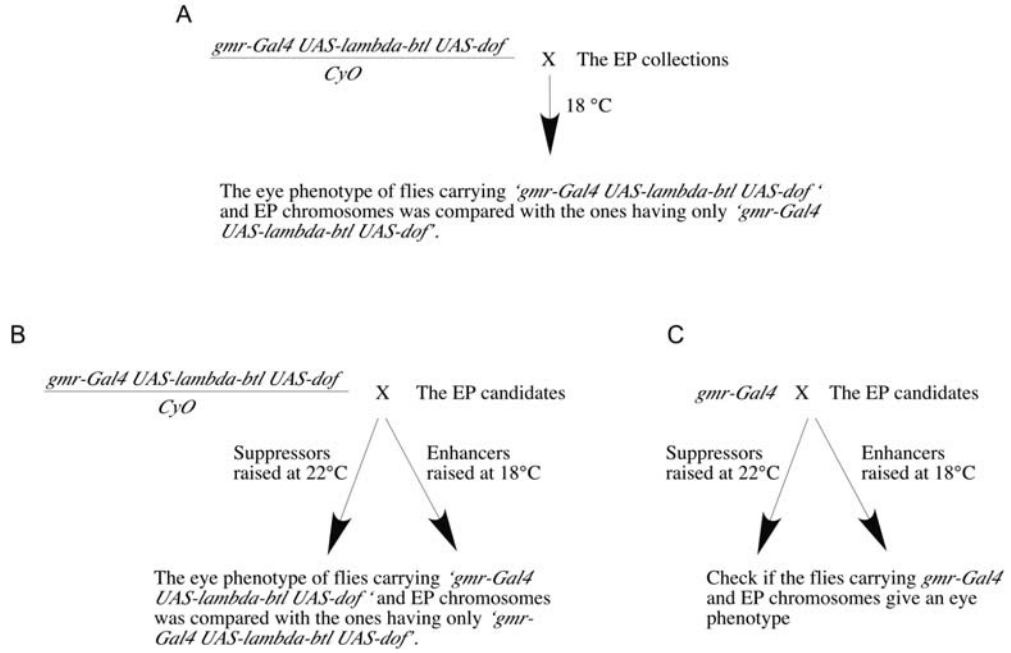

Fig.2-3 Schematic representation of the crosses for the screen. A, illustrates the crosses of the screen. 'GMR>λ-btl, dof' flies were crossed to the individual EP lines. The offspring were scored for the modification of 'GMR>λ -btl, dof' eye phenotype. B, illustrates the retest of the candidates. The retest is similar to the screen with one modification. The offspring of the suppressors were raised at 22°C while the enhancers were raised at 18°C. C, illustrates the test for EP candidates whether they give rough eye phenotype with GMR-Gal4 alone. The offspring were raised in a similar way to 'b'. 'GMR>λ-btl, dof': *gmr-Gal4 UAS-lambda-btl UAS-dof / CyO.*

The 'GMR>λ-btl, dof' eye phenotype is temperature sensitive (Fig.2-2, A&B). It is mild at 18°C but can be enhanced dramatically by raising the temperature by 0.5 to 1 degree. However the eye phenotype does not visibly change when flies are raised between 19-

22°C. This observation implies that the conditions giving rise to 'GMR>λ-btl, dof' eye phenotype at 18°C are finely balanced, and a slight change in the conditions can dramatically affect the eye phenotype. Therefore it is possible that some of the enhancers were picked up by chance due to the temperature fluctuation during the period of eye development. It is difficult to determine a potential suppression of the 'GMR>λ-btl, dof' at 18°C due to its mild phenotype. To confirm the effect of a suppressor, I retested their effect on the 'GMR>λ-btl, dof' phenotype at 22°C. To confirm the potential enhancement, they were retested at 18°C (Fig.2-3B). This retest and the test described below were carried out simultaneously and the results of the two tests were combined. Only those candidates that passed both tests were considered further.

It is possible that some EP lines produce a rough eye phenotype with *gmr-Gal4* alone that act independent of the ectopic FGF signal, in which case the enhancement phenotype observed in the screen may be the additive effect and have nothing to do with the FGF signal. To exclude this class of modifiers, the candidates were crossed to *gmr-Gal4* to test if the progeny with *gmr-Gal4* and EP insertions cause a rough eye phenotype (Fig.2-3C). These crosses were carried out in such a manner that the candidates for enhancers were crossed and raised at 18°C, and those for suppressors were at 22°C in accordance to the retest. Those enhancer candidates giving a rough eye phenotype with *gmr-Gal4* alone were excluded. However, a suppressor giving a rough eye phenotype with *gmr-Gal4* alone could still be considered to be a specific modifier. All the confirmed suppressors had passed the test despite four of them giving mild rough eye phenotype with *gmr-Gal4* alone. These four EP lines are EP1413, EP0355, EP1455 and EP0622.

In the end, there were 26 enhancers and 24 suppressors that passed both the retest and the test for its overexpression phenotype by GMR-Gal4 alone. These 50 candidates are summarized in table 2-2 according to their cytological localization. The candidates are described in the next chapter.

Table 2-2 The list of the candidates found in the screen

EP lines	Cytological region	Phenotype in the screen	Phenotype with GMR-Gal4	Genes possibly affected	Additional remarks	Viability
1200	3F2	S	N	*CG2829* (kinase)	GOF	viable
1408		S	N			viable
1413		S	Sli. rough			viable
1340	7A1	S	N	*fz4*, RE54930, LD39940		viable
1342	7B6	E	N	*ches-1-like (checkpoint suppressor homologue-1-like)*		viable
1453		E	N			viable
1207	9F5	S	N	*CG1679*	No flanking seq. info.	viable
1503	10E2	E	N	*CG2446* (containing DNA-glycosylase domain)	can be overexpressed	viable
1335	12A6-10	S	N	*CG11172* (*NFAT*, nuclear factor of activated T-cells homology)	GOF	viable
1390		S	N			viable
1508		S	N			viable
1353		S	N			viable
0355	14B16-17	S	Sli. rough	*dsp1* (*dorsal switch protein 1*)	can be overexpressed	viable
1455	18D3	S	Sli. rough	*CG14217* (kinase)	can be overexpressed	viable
1323	19A2	S	N	*amnesiac*	No flanking seq. info.	viable
1216	1B14	E	N	*skpA*		viable
	13C7-8			no information available	No flanking seq. info.	
2582	22A2	slight S	N	*robo2 (roundabout 2, or leak)*	can be overexpressed	viable
1211	23B1-2	E	N	*NTPase*	No flanking seq. info.	viable
0719	23C2	E	N	*CG3542* (formin binding protein)	LOF	no
2204	25C1	S	N	*msp300 (muscle specific protein 300)*		viable
2510	28D2	E	N	*CG7231*		viable
2171	34D4	S	N	*CG8954*	can be overexpressed	viable
2571	42E5	S	N	*tetraspanin 42Ef*		viable
0622	48A2	S	Sli. rough	*tou (toutatis)*	GOF	viable
0988	54C3-7	slight S	N	*mesr4 (misexpression suppressor of ras 4)*	GOF	viable
2258	57C6-7	S	N	*CG4266* (containing RNA binding domain)		no
2516	57F3	E	N	*CG10433*		no
2034		E	N			viable
1222	57F5	strong E	N	*CG10321* (transcription factor)		viable
2319	57F6	E	N	*CG10082* (kinase)		viable
0436		E	N			viable
2310		E	N			no
0712		strong E	N			viable
2440		E	N			no
0541	57F8	E	N	*tim10, or CG30290*		viable
2494		E	N			viable
2421		E	N			no
2444	57F9	E	N	*hmgD (high mobility group protein D)*		viable
0467		E	N			viable
1135	64A12	strong S	N	*ago (archipelago)*	GOF	viable
0595	66C	S	Darker*	*CG6765* (transcription factor)		viable
3659	66F4	E	N	*boule*	GOF	no
3348	69E2-4	E	N	*CG10967* or *CG11006*		viable
3443	78A2-4	S	Darker*	*pap (poils aux pattes)*	GOF	viable
3468	78C1-2	E	N	*eip78C (Ecdysome-induced protein 78C)*	No flanking seq. info.	viable
3028	82A5	E	N	*CG1090 (Na^+/Ca^+ exchanger)*	No flanking seq. info.	no
3634	90F7	E	N	*dlc90F (dynein light chain 90F)*		no
3575	94A4	E	N	*sar1*	GOF	no
0863	97D2	S	N	*CG6386* (kinase)	GOF	no
3280	100B2	S	N	*dco (discs overgrown)*	GOF	no

Legend: S, suppressor; E, enhancer; N, no modification; Sli. Rough, slightly rough; can be overexpressed, the EP can drive expression the gene with a proper gal4 driver; GOF, gain-of-function phenotype; LOF, loss-of-function phenotype; No flanking seq. info., no flanking sequence information is available from internet; * , the eye colour becomes darker; no, not viable.

2.2 Candidates

Most of the candidates are homozygous viable. The exceptions are EP0719, EP2258, EP2516, EP2310, EP2444, EP2421, EP3659, EP3028, EP3634, EP3575, EP0863 and EP3280. As part of the Berkeley genome project, all the EP lines have been cytologically mapped by chromosome *in situ* (The Flybase Consortium, 2002). Except EP1207, EP1323, EP1216, EP1211, EP3468 and EP3028, the sequence information of the adjacent genomic region (termed flanking sequence) into which the remaining EP candidates were inserted is also available (The Flybase Consortium, 2002). 16 of the candidates are inserted on the X chromosome, 23 on the second and 11 on the third. Among these, EP0719, EP3575 and EP0863 were studied more extensively.

In this chapter, I discuss the candidates according to their cytological regions. Except EP0719, EP3575 and EP0863, which appear in a separate chapter, I will firstly describe the insertion sites of each candidate and the genes that are possibly affected. Secondly, I will address the question of whether the effects are caused by gain-of-function or loss-of-function of the genes. Thirdly, I will discuss the possible functions of the protein products of the genes. In many cases, several EP-element insertions are close by, for which figures illustrate the insertion sites and the genes nearby are provided. In these figures, I use gray arrows to indicate the transcription/translation directions of the genes or the directions of the UAS promoters in the EP-elements; hollow arrows to indicate the insertion sites of the suppressors; black arrows to indicate the insertion sites of the enhancers; while the EP lines that do not modify the 'GMR>λ-btl, dof' eye phenotype but are close to the insertions having effects are indicated by black arrow heads. As an approach to gain an insight into the possible function of the gene, I analyzed the expression patterns of some of the genes whose protein products show similarity to known components that are likely to be involved in signal transduction. Since in most of the cases, this analysis was the only data concerning the individual candidates, they are presented here.

2.2.1 The description of the candidates

EP1200, EP1408, and EP1413-suppressors

These three EP-elements are spread over a region of 10Kb (Fig.2-4A). There are two predicted genes located nearby, *CG12462* and *CG2829*. The orientation of these three EP insertions makes it impossible that the gene *CG12462* was overexpressed in the screen, although EP1408 is located upstream of this gene. In the presence of Twi-Gal4, EP1413 can drive expression of *CG2829* in the mesoderm of the embryos (Fig.2-4B) although the other two cannot. EP1413 is inserted the furthest away from the gene *CG2829*. Given that different Gal4 driver lines could have different ability to drive the expression of genes downstream of the UAS promoter, it is possible that all these three EP insertions overexpressed the gene *CG2829* in the screen, which leads to the suppression of the 'GMR>λ-btl, dof' eye phenotype.

CG2829 is predicted to be a serine/threonine protein kinase. It is highly homologous to the human form of protein kinase U-α (PKUα). The human homologue of PKUα can be co-immuno-precipitated with 14-3-3ζ. It colocalizes with the cytoplasmic intermediate filament system of cultured fibroblasts in the G1 phase of the cell cycle. It localizes in the perinuclear area in the S phase cells and in the nucleus in late G2 cells (Zhang et al., 1999).

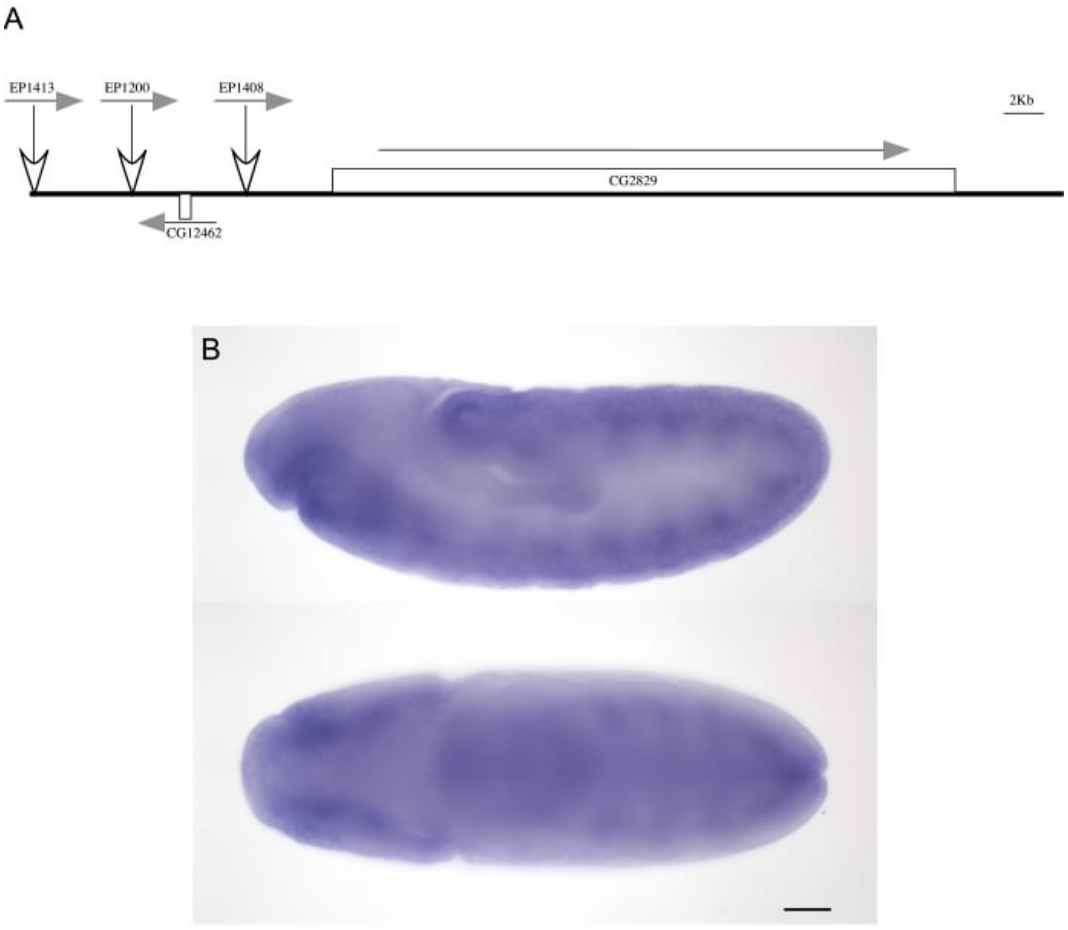

Fig.2-4. EP1413, EP1200 & EP1408 suppress the 'GMR>UAS-λ-btl, dof' eye phenotype probably by overexpression of the gene *CG2829*. A, three EP-elements are inserted upstream of *CG2829*. Two genes are close by, *CG2829* and *CG12462*, which are indicated by two boxes. B, When EP1413 was crossed to Twi-Gal4, *CG2829* was ectopically expressed in the mesoderm. Top panel, lateral view and lower panel, ventral view of the same embryo. Scale Bar: 50μm

In order to analyze the expression pattern of the gene, *in situ* hybridization was performed using an anti-sense RNA probe. The results show that the gene *CG2829* is ubiquitously expressed during the early stage of development, which is probably due to maternal contribution (Fig.2-5A). The transcripts seem to be degraded gradually when embryos develop (Fig.2-5B&C). The signal is very weak at later stage of the embryonic development (Fig.2-5D).

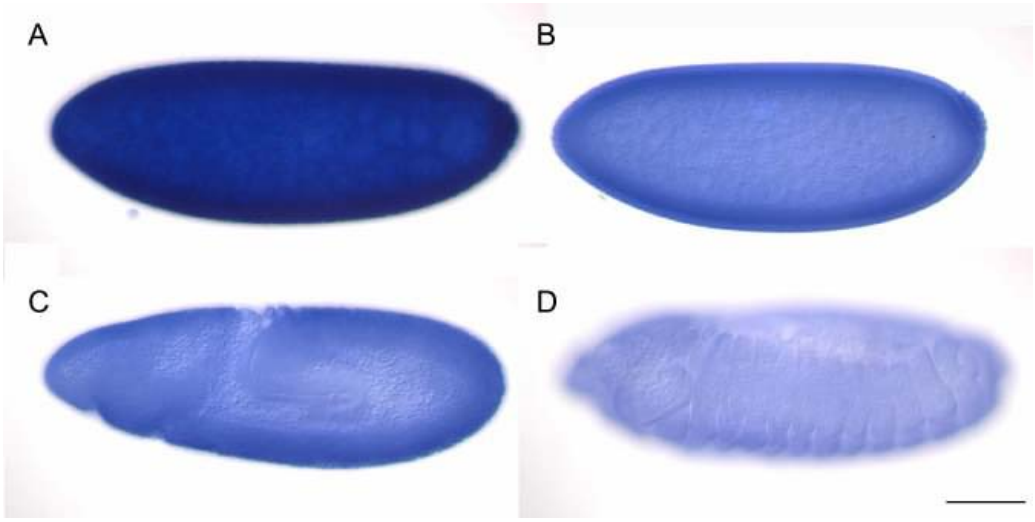

Fig.2-5. The expression pattern of *CG2829*. *CG2829* is expressed ubiquitously during early blastoderm (A), cellularization (B) and germband extension (C). The *in situ* signal starts to fate by stage 14 (D). Scale Bar: 100μm.

EP1340-suppressor

EP1340 is inserted into the first intron of a gene predicted from an isolated cDNA clone, LD39940, in an orientation that could not lead to the overexpression of the gene. At about 4Kb downstream, there is a gene predicted from another isolated cDNA clone, RE54930, which could be possibly overexpressed by EP1340. However, by the time I completed my screen, the nearest predicted downstream gene was *fz4* (*frizzled 4*), which is located about 85Kb downstream of the EP insertion. The direction of the insertion makes it possible to overexpress *fz4* in the presence of Gal4. To determine if the suppression phenotype caused by EP1340 is due to overexpression of *fz4*, I generated transgenic flies carrying *fz4* cDNA with UAS promoter, and tested its ability to modify the 'GMR>λ-btl, dof' eye phenotype. Although there is no visible phenotype caused by overexpression of *fz4* alone by GMR-Gal4, the presence of *fz4* transgene enhances the 'GMR>λ-btl, dof' eye phenotype, which implies that the phenotype caused by EP1340 is unlikely due to the overexpression of *fz4*. The sequence of the predicted protein product of LD39940 shows no similarity to any

known proteins. The cDNA clone, RE54930, has not been completely sequenced. Therefore EP1340 was not studied further.

EP1342 and EP1453-enhancers

EP1342 is inserted in the first intron of the gene *ches-1-like* (*checkpoint suppressor homologue-1-like*), within the 5' untranslated region (Fig.2-6). EP1453 is located 6Kb upstream of the gene. Both of them could lead to overexpression of the gene in the screen. There is no other predicted gene nearby that might be more likely to be affected by EP1453. *CHES-1-like* encodes a protein containing a fork head domain. It is probably a transcription factor. Incubation of S2 cells with *ches-1-like* RNAi results in increasing numbers of cell death (Ramet et al., 2002).

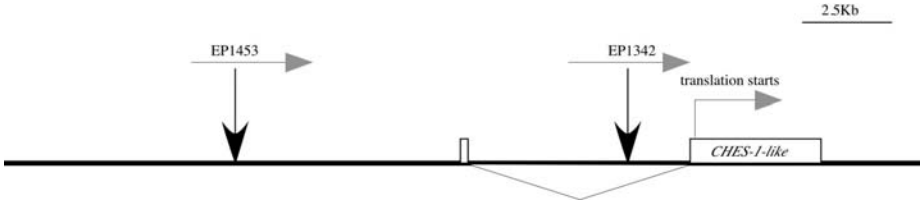

Fig.2-6. EP1453, EP1342 and *CHES-1-like* are located close by. EP1453 is inserted 6Kb upstream of the gene, while EP1342 is inserted into the first intron of the gene. Both have the potentials of overexpression of the gene in the screen.

EP1207-suppressor

There is no flanking sequence information available to map the insertion sites of EP1207. However, it appears to be inserted proximal to a predicted gene *CG1679*, which encodes an IGF-II mRNA-binding protein (The Flybase Consortium, 2002).

EP1503-enhancer

EP1503 is inserted into the first intron of the predicted gene *CG2446*, within the 5' untranslated region. The gene has been overexpressed by this EP insertion with Sca-Gal4, and results in potential shaft-to-socket transformations in extra sensory organs (Abdelilah-Seyfried et al., 2000). Therefore the enhancement phenotype observed in the screen is possibly due to the overexpression of *CG2446*. This gene encodes a protein containing a DNA-glycosylase domain.

EP1335, EP1390, EP1508 & EP1353-suppressors

The first three EP-elements are inserted into the first intron of the gene *CG11172* (*NFAT*) which consists of five exons (Fig.2-7). The fourth is inserted into the first exon. All of them are located in the 5' untranslated region. Two more EP-elements, EP1541 and EP1329, are inserted into the first intron near the insertions of the first three EP lines, but in the reverse orientation. These two EP-elements fail to modify the 'GMR>λ-btl, dof' eye

phenotype. Therefore, the suppression phenotype is likely to be caused by overexpression of *CG11172*. This gene has also been found in the GOF screen for components that can modulate the Ras85D pathway in the Drosophila compound eye (Huang et al., 2000). Its protein product is closely related to the mammalian nuclear factor of activated T-cells (NFAT) family of transcription factors.

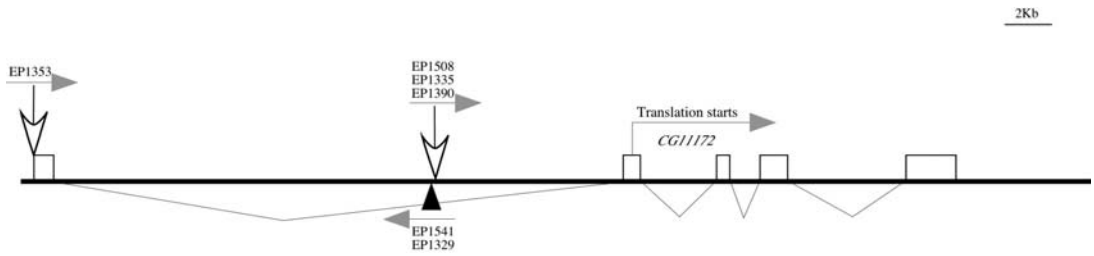

Fig.2-7. The region where *CG11172* is located. The predicted gene consists of five exons, which are indicated by boxes. There are five EP-elements inserted in a small region spanning about one hundred bases. The sixth is inserted into the first exon.

EP0355-suppressor

EP0355 is inserted 37bp upstream of the gene *dsp1* (*dorsal switch protein 1*). The gene has been overexpressed by the EP insertion with Elav-Gal4 or Ap-Gal4 (Kraut et al., 2001; Pena-Rangel et al., 2002). Therefore it is possible that the suppression phenotype observed in the screen was the result of overexpression of *dsp1*. *Dsp1* encodes a transcription co-repressor, which contains HMG (high mobility group) boxes. It can bind to p50 subunit of the NF-κB heterodimer and the Rel domain of dorsal (Brickman et al., 1999).

EP1455-suppressor

EP1455 is inserted into the first exon, within the 5' untranslated region of a predicted gene *CG14217*. EP1455 can drive expression of *CG14217* with Btl-Gal4 in the trachea (Fig.2-8). Therefore it is possible that the suppression phenotype is caused by overexpression of *CG14217*.

CG14217 is highly homologous to the mammalian protein TAO1 (thousand and one amino acid protein kinase 1) (1e-146 to the rat homologue, flybase). The mammalian TAO1 protein can activate MEK3 specifically and therefore activate P38 in transfected cells (Hutchison et al., 1998). The Drosophila homologue of MEK3 is Licorne (Lic). It has been placed genetically upstream of Gurken, a Drosophila homologue of EGF that is required for proper patterning of oocytes and eggs (Suzanne et al., 1999). The

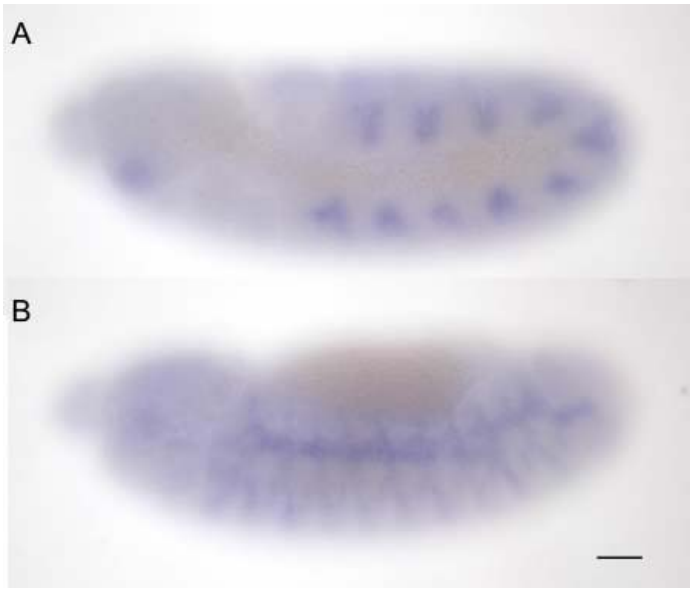

Fig.2-8. When EP1455 was crossed to Btl-Gal4, *CG14217* was ectopically expressed in developing trachea. *CG14217* was overexpressed in the tracheal placodes at stage later 10 to early 11 (A), the expression stays during stage 14 (B). Detected by *in situ* with anti-sense RNA probe made from LD40388. Scale Bar: 50μm.

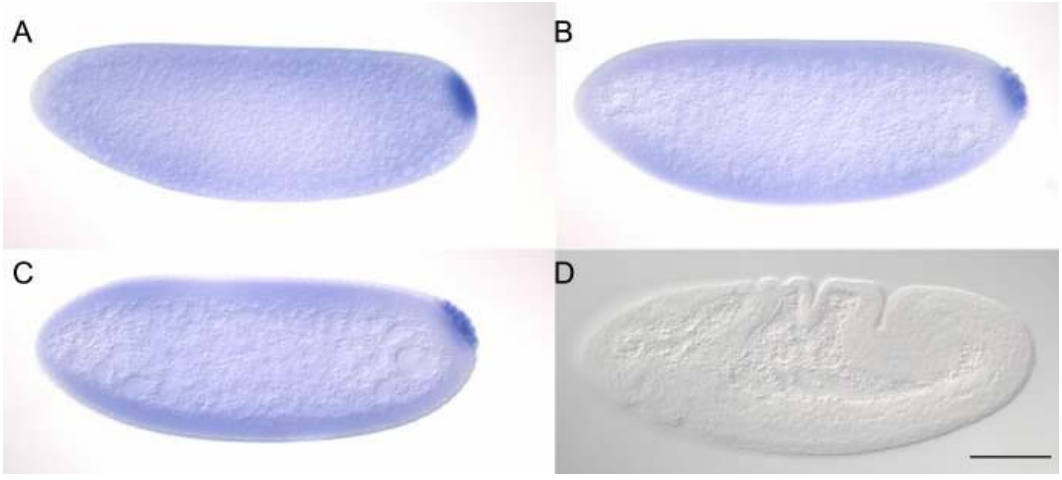

Fig.2-9. Expression pattern of *CG14217*. The expression of *CG14217* is highly concentrated at the posterior position before pole cells are visible (A), it colocalizes with pole cells during later blastoderm (B) although else where there are low ubiquitous expression. The signal seems unchanged during early gastrulation (C), but disappears during germband extension (D). Scale Bar: 100μm.

mammalian homologue of CG14217 can possibly transduce the signal to MEK3, and the Drosophila MEK3 has a role in EGF signalling. Therefore CG14217 is a good candidate downstream of RTK signalling

In order to see if the expression of the gene *CG14217* overlaps with the expression pattern of *dof* or any of the *fgfs*, *in situ* hybridization was performed using the anti-sense RNA probe derived from *CG14217*, which reveals that the transcripts of *CG14217* are most likely deposited maternally (Fig.2-9). Before the formation of pole cells, the transcripts have already concentrated at the posterior pole (Fig.2-9A). After stage 3, the signal is seen mainly at the posterior pole and in pole cells (Fig.2-9, B&C). By the middle of gastrulation, the signal is almost undetectable (Fig.2-9D), and it does not reappear at later stages of embryonic development.

EP1323-suppressor
There is no flanking sequence information available for EP1323. However, it appears to be inserted proximal to *amnesiac* (The Flybase Consortium, 2002). Two other EP insertions, EP0346 and EP1571 that are also inserted proximal to the gene did not modify the 'GMR>λ-btl, dof' eye phenotype. EP0346 and EP1571 were both found in a screen for genes affecting axon path finding, whereas EP1323 was not (Kraut et al., 2001).

EP1216-enhancer
EP1216 contains two insertions, in 1B13 and in 13C7-8. The insertion in 1B13 is 2.1Kb upstream of the gene *skpA* and could potentially lead to overexpression of the gene in the screen. It is also 157bp upstream of *CG13363*, which transcribes in an opposite direction. SkpA is a component of SCF complex involved in protein ubiquitination (Das et al., 2002). *CG13363* encodes a component showing no homology to any known proteins. The other insertion cannot be placed precisely due to lack of flanking sequence information. This EP candidate was not investigated further because it was not clear which of the two insertions, or whether both, give the enhancement phenotype.

EP2582-suppressor
EP2582 is inserted upstream of *robo2* (*roundabout 2*, or *leak*) with the potential to overexpress the gene with Gal4 (Rajagopalan et al., 2000). Overexpression of EP2582 under the control of Elav-Gal4 causes a commissure loss phenotype (Bashaw et al., 2000). It is possible that the suppression phenotype I observed in my screen is due to the overexpression of the *robo2* gene.

Robo2 is one of the three receptors found in Drosophila so far for the secreted midline repellent Slit that are important for the patterning of the embryonic nervous system. Recently, it was shown that Robo2 is required for the outgrowth of the terminal cells of tracheal ganglionic branches in response to Slit signal produced by midline cells, in which Slit acts as an attractive signal for *robo2* expressing cells (Englund et al., 2002).

EP0719-enhancer
The insertion of EP0719 disrupts the gene *CG3542* which leads to the enhancement of the 'GMR>λ-btl, dof' eye phenotype (see chapter 2.4 for details).

EP1211-enhancer
The precise insertion site of EP1211 cannot be mapped due to lack of flanking sequence information. However, it appears to be inserted proximal to *NTPase* (The Flybase Consortium, 2002).

EP2204-suppressor
EP2204 is inserted 4.5Kb downstream of the predicted gene *CG14035*, and approximately 12.5Kb upstream of *msp-300* (*muscle specific protein 300*). The orientation of the insertion suggests that it could lead to the overexpression of *msp-300* in the screen.

In homozygous *msp-300* mutants, the overall shape of each myotube is defective and they do not reach their epidermal attachment sites (Rosenberg-Hasson et al., 1996). Msp-300 is homologous to *C. elegans* ANC-1 (nuclear anchorage defective 1). Both proteins consist of a nuclear envelope localization domain (named KASH domain for Klarsicht/Anc-1/Syne-1 homology) at the C-terminus and an actin-binding domain at the N-terminus. Between the two domains, there is a large central domain that could allow a single molecule to extend over 0.5μm. ANC-1 can possibly anchor the nucleus directly to actin cytoskeleton (Starr et al., 2002). Msp-300 may have similar functions.

EP2510-enhancer
EP2510 is inserted into the 5' untranslated region of the first exon of the predicted gene *CG7231*. The direction of the EP-element insertion will not allow it to drive expression of *CG7231* in the screen. However, EP2510 could lead to the overexpression of the gene *CG7233*, which is located 8Kb downstream. CG7231 does not display any homology to known proteins. CG7233 has weak homology to the ski proto-oncogen (2.2e-57, flybase). The ski proto-oncogen has been shown to influence proliferation, morphological transformation and myogenic differentiation (Ludolph et al., 1995).

EP2171-suppressor
EP2171 is inserted into the first exon of a predicted gene *CG8954*, which has been overexpressed by the EP2171 with Elav-Gal4 (Kraut et al., 2001). Therefore it is possible that overexpression of *CG8954* suppressed the 'GMR>λ-btl, dof' eye phenotype. Larvae expressing *CG8954* by Elav-Gal4 have abnormal synapses although homozygous deletion mutants show no visible phenotype (Kraut et al., 2001), (Ashburner et al., 1999). The gene encodes a protein without any known conserved domains.

EP2571-suppressor
EP2571 is inserted upstream of the gene *tetraspanin 42Ef* (*tsp42Ef*, or *CG12845*) with the potential to overexpress it in the screen. In the region where the EP-element is inserted,

there are 18 *tetraspanins* clustered together. Tetraspanins are four transmembrane proteins that are abundantly expressed in mammals, Drosophila and *C. elegans*. Genetic studies in mammals show that some Tetraspanins have important roles in lipid signals and integrin signals, although most of them function redundantly (Hemler, 2001). The Tetraspanin superfamily has been found to be a component of membrane rafts (Boesze-Battaglia et al., 2002; Todres et al., 2000). There are in total 37 *tetraspanins* found in Drosophila genome. One of them, *late bloomer* is involved in synapse formation (Kopczynski et al., 1996). Fradkin et al. deleted 9 *tetraspanins* from the region where EP2571 lies. This deletion included *tsp42Ef*. They did not detect any visible defects, which suggests that there is significant molecular compensation for most Tetraspanin functions (Fradkin et al., 2002).

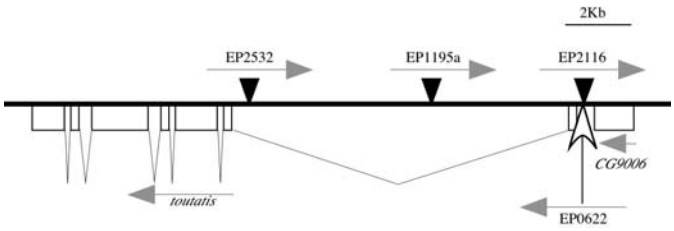

Fig.2-10. The region in which EP0622 is inserted. The exons of the genes in the region are indicated by boxes.

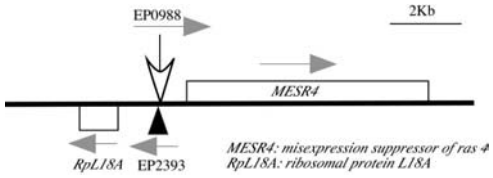

Fig.2-11. The region in which EP0988 is located. Two genes, *MESR4* and *RpL18A,* are next to the EP insertions, which are indicated by boxes. EP0988 is inserted upstream of *MESR4* with the potential to overexpress the gene, while EP2393 cannot.

EP0622-suppressor

EP0622 is inserted about 140bp upstream of *toutatis* (*tou*), in a direction that could lead to overexpression of the gene in the screen (Fig.2-10). Three other EP-elements are inserted in this region. EP2116 lies about 160 upstream of the gene. EP2532 and EP1195a are in the first intron of *tou*. All three are inserted in the opposite orientation to EP0622, and do not modify the 'GMR>λ-btl, dof' eye phenotype. EP0622 in combination with GMR-Gal4 alone leads to a slightly rough eye phenotype when the flies are raised at 22°C.

These facts suggest that the suppression phenotype caused by EP0622 is possibly caused by overexpression of the *tou* gene.

The protein product of *tou* has weak similarity to various homeotic proteins. *Tou* mutant homozygous flies are poorly viable, and most of them have an interrupted wing vein L5 and the wing has a downward-turned appearance (Fauvarque et al., 2001).

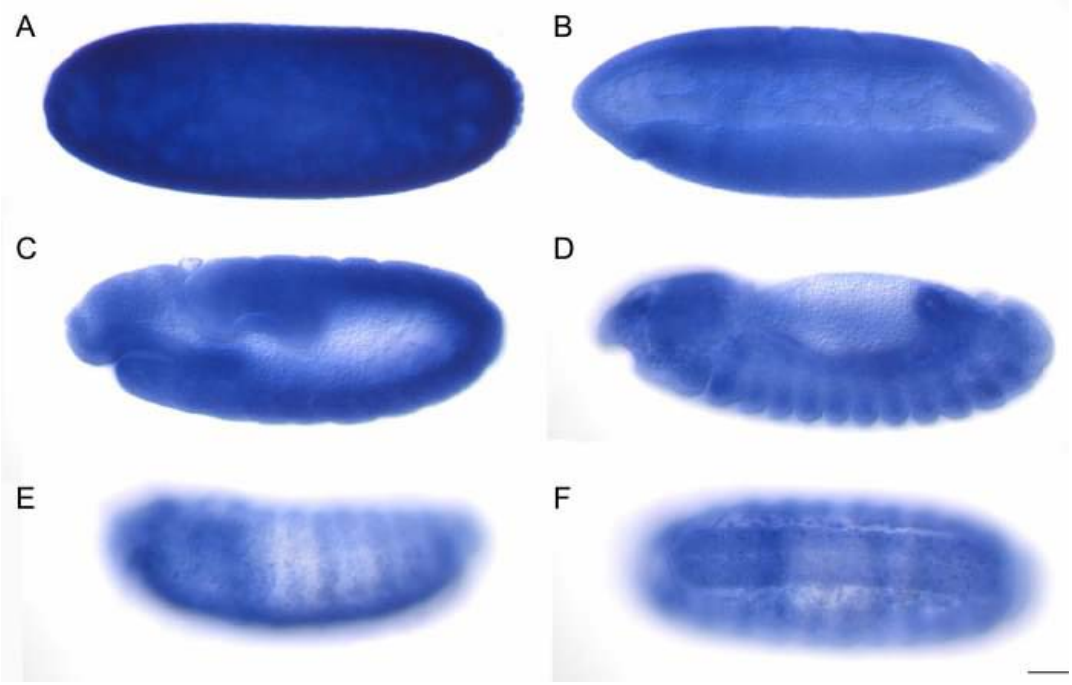

Fig.2-12. The expression pattern of *CG4266*. *CG4266* is expressed ubiquitously during blastoderm (A), the expression stays throughout embryonic development (B, C, D, E). The signal starts fading during stage 16 (E, F), though it seems to be stronger in the central nervous system (F). Scale Bar: 50μm.

EP0988-suppressor

EP0988 is inserted 560bp upstream of *mesr4* (*misexpression suppressor of ras 4*) with potential of overexpression of the gene in the screen (Fig.2-11). EP2393 is inserted 11bp away from EP0988 but in the opposite orientation. EP2393 does not modify the 'GMR>λ-btl, dof' eye phenotype, which implies that the EP0988 may suppress the 'GMR>λ-btl, dof' eye phenotype by overexpression of *mesr4*. MESR4 is a zinc-finger protein found as a suppressor for Ras signalling (Huang et al., 2000).

EP2258-suppressor

EP2258 is inserted into the 5' untranslated region of the predicted gene *CG4266*, and oriented in such a way that the gene could be overexpressed in the screen. CG4266 contains RNA-binding domains.

I examined the expression pattern of *CG4266* to see if it overlaps with that of *dof* or any of the *FGFRs*. *In situ* hybridization by the RNA anti-sense probe suggests that the expression of the gene *CG4266* is ubiquitous (Fig.2-12). At early stage of the embryos, it is probably deposited maternally (Fig.2-12A). The signal stays throughout embryonic development (Fig.2-12, B, C, D&E). The signal starts to fade during stage 16, though seems to be stronger in the central nervous system (Fig.2-12F).

EP2516 and EP2034-enhancers

EP2516 and EP2034 are inserted upstream of the predicted gene *CG10433* (Fig.2-13). The orientation of these two EP-elements suggests that the gene could be overexpressed in the screen. CG10433 has no homology to any characterized proteins. It may be a gene involved in circadian rhythm (Claridge-Chang et al., 2001).

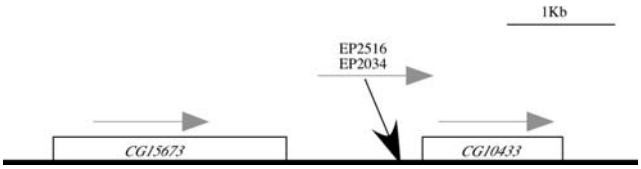

Fig.2-13. The region that EP2516 and EP2034 are inserted. *CG10433* is located downstream of the two EP-elements insertions. All the genes close by are indicated by boxes.

EP1222-enhancer

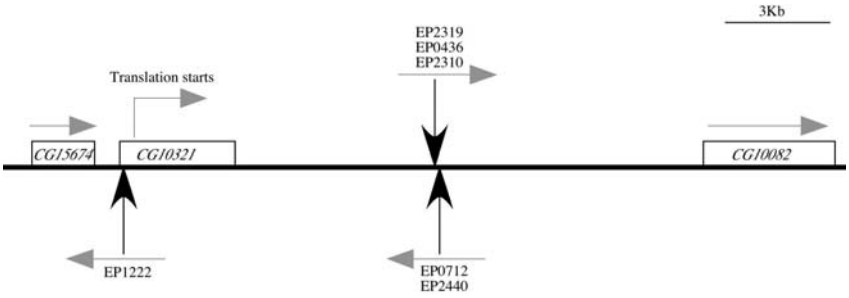

Fig.2-14. The region that EP1222, EP2319, EP0436, EP2310, EP0712 and EP2440 are inserted. All the genes close by are indicated by the boxes.

EP1222 is inserted in the 5' untranslated region of *CG10321* (Fig.2-14). The direction of the insertion does not allow the overexpression of the gene *CG10321* in the screen. There is no other gene that is more likely to be affected. Therefore, it is possible that the enhancement of the eye phenotype is caused by the disruption of the gene expression. *CG10321* encodes a predicted transcription factor, which contains C2H2 and C2HC zinc finger domains.

EP2319, EP0436, EP2310, EP0712 and EP2440-enhancers

EP2319, EP0436, EP2310, EP0712 and EP2440 are inserted in a small region and are no more than 400bp apart from each other (Fig.2-14). The orientation of two of them, EP0712 and EP2440, is different from the other three. They are located about 6Kb downstream of the predicted gene *CG10321*, and about 8Kb upstream of the predicted gene *CG10082*. Since all of the EP elements are inserted nearby, and are far away from the closest two genes, it is likely to be a loss of function phenotype that I found in the screen. CG10082 has weak similarity to human Inositol Hexakisphosphate Kinase 3 (5e-38, NCBI BLAST) (Saiardi et al., 2001).

EP0541, EP2494 and EP2421-enhancers

EP0541, EP2494 and EP2421 are inserted in a region no more than 400bp away from each other, between the *CG30290* and *tim10* genes (Fig.2-15). It is not clear whether the effects of either or both genes enhanced the 'GMR>λ-btl, dof' eye phenotype. *CG30290* is 480bp away from *tim10* and transcribes in the reverse direction in comparison to *tim10*. These three insertions are upstream of both genes. However, the orientation of the insertions only allows EP0541 overexpression of *Tim10* and the EP2494 and EP2421 overexpression of *CG30290*. Therefore these three EP insertions have the potential to affect either or both of the two genes.

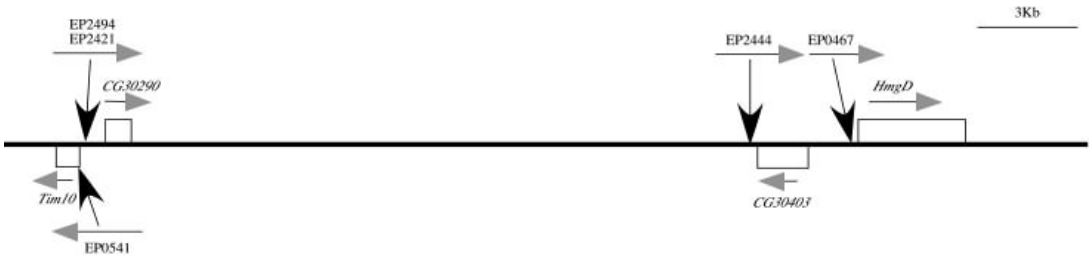

Fig.2-15. The region that EP2494, EP2421, EP0541, EP2444 and EP0467 are inserted. All the genes close by are indicated by boxes.

Tim10 is a component of the mitochondrial inner membrane translocase complex. I examined the expression pattern of *tim10* to see if it overlaps with that of *dof* or any of the *fgfs*. *In situ* hybridization using the RNA anti-sense probe shows that *tim10* is maternally

provided during early stages of development (Fig.2-16A). The maternal transcripts seem to degrade during gastrulation (Fig.2-16, B&C). However, during posterior midgut formation, the signal comes up again in the posterior midgut primordium (Fig.2-16C). During stage 10, the *tim10* transcripts are present in both posterior midgut and anterior midgut primordia (Fig.2-16D), the signals are visible till the end of the midgut formation (Fig.2-16, E&F). The mRNA of both *dof* and *btl* are expressed in the midgut at similar stages although the significance of this expression of *dof* and *htl* is unknown.

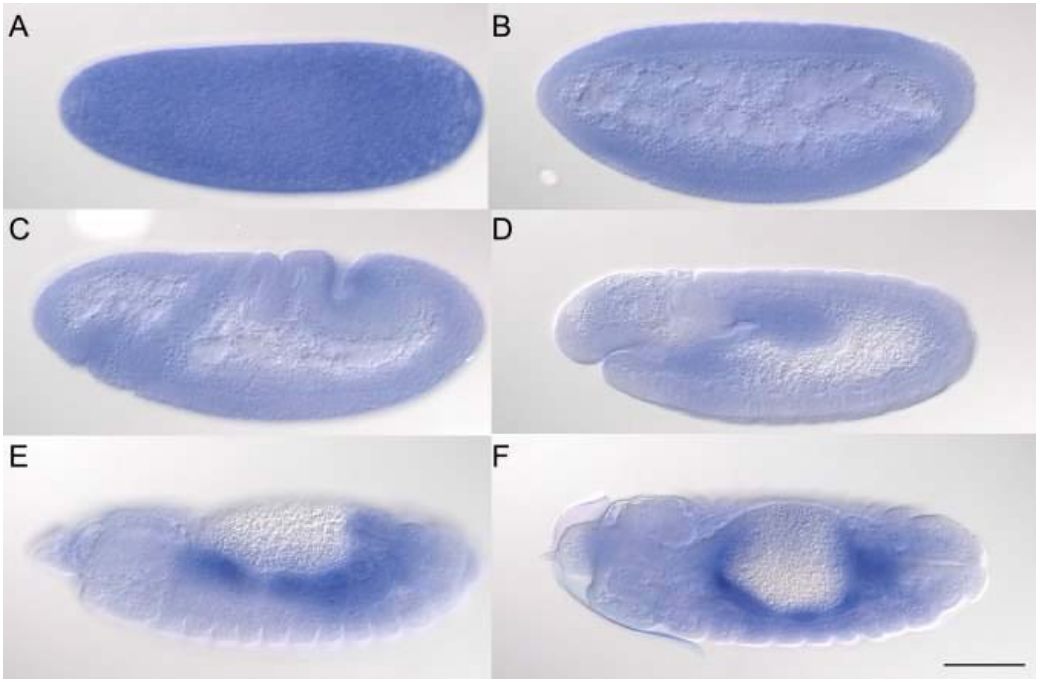

Fig.2-16. The expression pattern of *tim10*. Transcripts are ubiquitously distributed during blastoderm of the embryos (A), during cellularization and gastrulation the staining fades away (B, C). However, it seems there are local concentrated staining in posterior mid-gut primordium (C, arrowhead). At the fully extended germband stage, signal becomes stronger in both posterior and anterior mid-gut primordia (D, arrow heads), and the transcripts accumulate at later stages during the formation of the mid-gut (E, F). Scale Bar: 100μm.

The protein product of *CG30290* does not have obvious functional domains, although data bank search show that it has weak homology to the yeast halotolerance protein Hal3a (3e-44, NCBI BLAST). This protein belongs to a superfamily of Flavoproteins, which can bind to Flavin MonoNucleotide (FMN) molecules. The yeast *Saccharomyces cerevisiae* HAL3 gene is important in regulation of cation efflux. It may also have a function in cell cycle control (de Nadal et al., 1999).

EP2444 and EP0467-enhancers

Both EP2444 and EP0467 have the potential to overexpress the gene *hmgD* (*high mobility group protein D*) in the screen (Fig.2-15). Insertion EP0467 lies 40bp upstream of the gene whilst EP2444 is approximately 4Kb upstream of the gene. Alternatively these EP insertions can affect *CG30403*, which lies between EP2444 and *hmgD*. This gene, transcribed in the reverse direction in comparison to *hmgD*, is 1.7Kb upstream of *hmgD*. EP2444 is 200bp downstream of the gene *CG30403*.

hmgD encodes a component involved in DNA packaging. It can either by itself, or in conjunction with other chromosomal proteins, induce a condensed state of chromatin that is distinct from and less compact than the His1 containing fiber (Ner et al., 2001). Its transcripts were found in the germarium in the adult males and females, ubiquitously present during early development, and then from about stage 9 on, it is highly concentrated in the neuronal cells. This dynamic expression pattern implies that it might have a more specialized rather than a general role (Stroumbakis et al., 1994). CG30403 has no similarity to any known proteins.

EP1135-suppressor

EP1135 is inserted 17bp upstream of the gene *ago* (*archipelago*) with the potential to cause the overexpression of *ago* in the screen. It is located also 457bp upstream of the gene *CG1265* which transcribes in a reverse direction in comparison to *ago* (Fig.2-17). The insertion does not cause lethality.

Two imprecise excisions of EP1135 have been generated (Fig.2-17). Excision #17 uncovers the 2.2Kb region including the predicted first exon and the major part of the first intron of the gene *ago* but does not extend to the protein coding region of the gene. This deletion leads to lethality of homozygous larvae. Excision #1 has not been fully sequenced. The confirmed sequence shows that the excision starts 1.4Kb upstream of *CG1265*, and it takes away at least half of the protein coding region of *ago*. A fragment of more than 700bp of the P-element remained. These two alleles do not complement each other. Given that the EP insertion does not cause lethality while the two excisions do, it is likely that the expression of *ago* is impaired. Both excisions fail to modify the 'GMR>λ-btl, dof' eye phenotype, which suggests that the suppression phenotype I observed is probably due to the overexpression of *ago*.

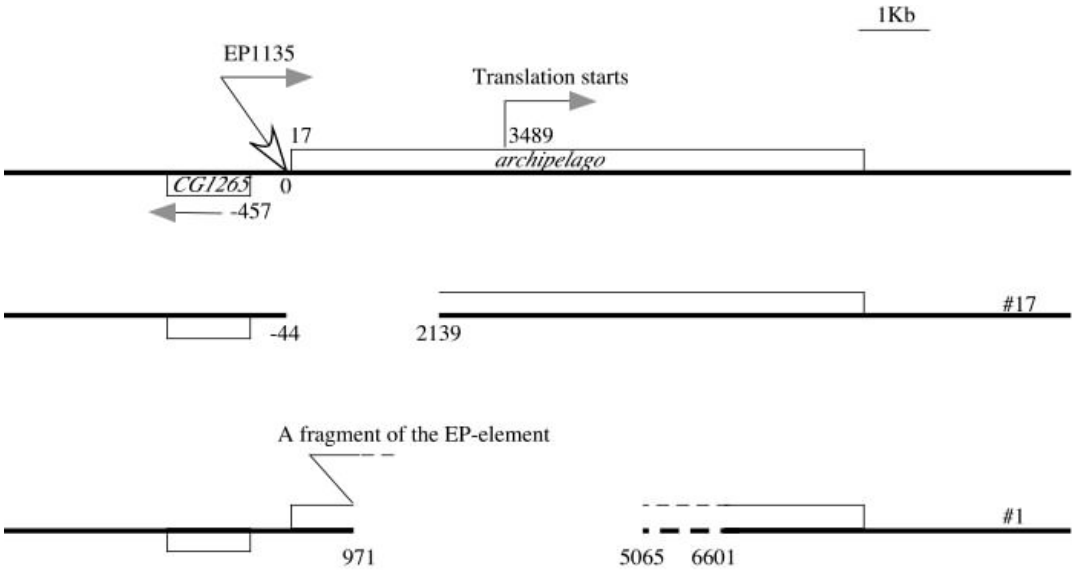

Fig. 2-17. The schematic representation of the region of EP1135 and two imprecise excisions of the EP-element. The boxes indicate the genomic region that two genes *archipelago* and *CG1265* are located. The relative positions of transcription/translation starting sites, and the confirmed breaking points of the excisions are indicated by numbers in comparison to the insertion site of EP1135. The dashes mark the sequences that have not been confirmed and therefore are not certain if they are still present.

Ago is an ubiquitin-protein E3 ligase that targets cyclin E for degradation (Moberg et al., 2001). CG1265 encodes a component having no homology to known proteins. Given that overexpression of *ago* strongly suppresses the eye phenotype, I suspect that ago targets the degradation of the critical components of the FGF signal and therefore suppresses the ectopic signals in the eye. I first examined the expression pattern of ago. Revealed by *in situ* hybridization by anti-sense RNA probe, ago is provided maternally (Fig.2-18, A&B). The signal starts to fade during germband retraction (Fig.2-18C), and almost no signal is detectable at stage 14 (Fig.2-18D). I also crossed EP1135 to Twi-Gal4 and Btl-Gal4 to see if overexpression of *ago* alone will affect the mesoderm or the tracheal development. There were no defects in the mesoderm or the tracheal system. Homozygous mutants for both imprecise excision alleles die during the larval stage (from L1-L3), and no mesodermal or tracheal defects were observed in the mutant embryos.

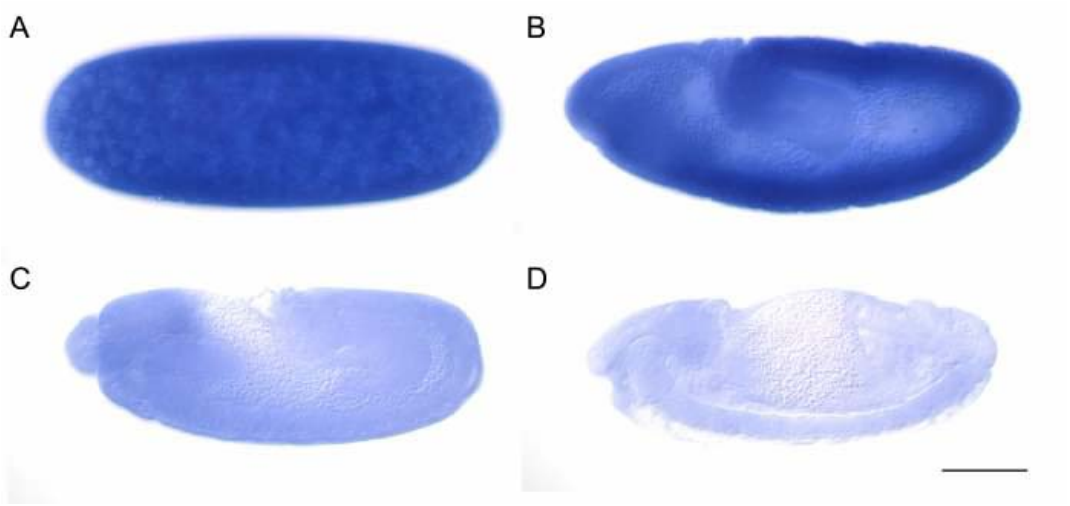

Fig.2-18. The expression pattern of *archipelago*. *Archipelago* is ubiquitously expressed during blastoderm and gastrulation (A &B), the signal starts to fade during germband retraction (C), and is almost invisible at stage 14 (D). Scale Bar: 100μm.

EP0595-suppressor
EP0595 is inserted 17bp upstream of a predicted gene *CG6765* and has the potential to overexpress *CG6765* in the screen. *CG6765* encodes a protein containing a BTB/POZ domain and a C2H2 type Zinc finger.

EP3659-enhancer
EP3659 is inserted in the 5' untranslated region of *boule* and has the potential to overexpress *boule* in the screen. Another EP insertion, EP0548, is inserted 21bp upstream of EP3659 but in a reverse direction and did not modify the 'GMR>λ-btl, dof' eye phenotype. Therefore it is likely that the EP3659 modifies the eye phenotype by overexpression of *boule*.

Boule is an RNA binding protein. It has been found to interact genetically with cdc25 phosphatase. Homozygous *boule* mutants have a defect in entry into meiosis (Maines et al., 1999). Boule is the Drosophila homologue of human DAZ (deleted in azoospermia) which when mutated in humans causes a severe defect in sperm production (Eberhart et al., 1996).

EP3348-enhancer
EP3348 is inserted into the first exon of *CG10967*, but its orientation suggests that it is impossible to lead to overexpression of *CG10967* in the screen. However, it has the potential to overexpress *CG11006*, which is 950bp away. CG10967 is homologous to a protein kinase called UNC-51 [5e-96, (The Flybase Consortium, 2002)]. UNC-51 is a *C.*

elegans serine/threonine kinase that is required for axonal elongation (Ogura et al., 1994). There is no other UNC-51 homologue in Drosophila. CG11006 is a predicted protein having no known homologues.

EP3443-suppressor

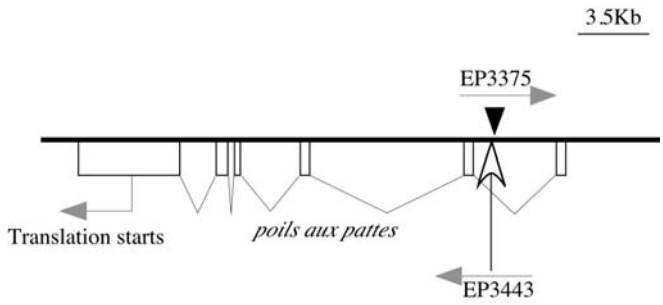

Fig.2-19. The localization of gene *pap* (*poils aux pattes*). *Pap* consists of 6 exons, which are indicated by boxes. EP3443 and EP3375 both inserted into the first intron of the gene pap. EP3443 can potentially cause overexpression of the gene while EP3375 cannot.

EP3443 is inserted upstream of *pap* (*poils aux pattes*) and has the potential to overexpress *pap* in the screen. Another EP insertion, EP3375, which is inserted 90bp upstream of EP3443, did not show any modification in the screen (Fig.2-19). EP3375 lies in the opposite orientation, which makes it more likely that the suppression phenotype found in the screen for EP3443 is caused by overexpression of the gene *pap*.

Pap encodes a transcription regulator. It was first isolated in a screen for genes with effects on specific cell identities sensitive to Ras85D signalling activities (Bourbon et al., 1998). Pap is required for the normal progression of photoreceptor differentiation in the eye disc and for the normal patterning of other imaginal discs (Treisman, 2001).

EP3468-enhancer

EP3468 appears to be inserted proximal to the gene *eip78C* (*ecdysone-induced protein 78C*) (The Flybase Consortium, 2002). There is no information available on flanking sequences. This gene is required for the maximal transcription activity for certain regions of the chromosome during larval development although loss of function experiments show that this gene is not essential for survival (Russell et al., 1996).

EP3028-enhancer

EP3028 appears to be inserted proximal to the predicted gene *CG1090*, which could encode a Na^{+}/Ca^{2+} Exchanger (The Flybase Consortium, 2002). No flanking sequence information is available to precisely map the insertion site.

EP3634-enhancer
EP3634 is inserted in the 5' untranslated region of the gene *dlc90F* (*Dynein light chain 90F*, also called *dtctex-1*). The insertion disrupts the transcription of *dlc90F* (Caggese et al., 2001). However the orientation of the insertion suggests that the gene could be overexpressed in the screen. Loss of expression of the gene leads to sterility in male flies without much effect on viability. This gene is expressed throughout the life cycle of flies with a higher level in female adults, and in 1-3 hour embryos, which suggests a high maternal contribution. The embryonic expression of this gene is ubiquitous. There is less expression of the gene in the L1-L2 larval stages (Caggese et al., 2001).

EP3575-enhancer
The enhancement phenotype found in the screen is caused by overexpression of *sar1* by EP3575 (see chapter 2.4 for details).

EP0863-suppressor
The suppression of the 'GMR>λ-btl, dof' eye phenotype by EP0863 is due to the overexpression of *CG6386* (see chapter 2.4 for details).

EP3280-suppressor
EP3280 is inserted 61bp upstream of *dco* (*double time* or *discs overgrown*). The EP-element is inserted in an orientation that could lead to overexpression of *dco*. One EMS allele and one P-element insertion allele were crossed to 'GMR>λ-btl, dof' flies to test their abilities to modify the eye phenotype. Both of them failed to suppress the eye phenotype as observed for EP3280. Therefore overexpression of gene *dco* suppresses the 'GMR>λ-btl, dof' eye phenotype. *Dco* encodes a homologue of the Casein kinase Iδ/ε subfamily protein. Weak alleles of *dco* affect the circadian rhythm, thus the name *double time*. Mitotic clones of null alleles in imaginal discs show strong effects on cell survival and growth control. Dco protein is a crucial component in the mechanism that links cell survival during proliferation to growth arrest in imaginal discs (Zilian et al., 1999).

2.2.2 Summary of the candidate genes

I have found 50 EP lines in the screen. In total, 38 genes are likely to be affected. Five EP lines have no information on flanking sequence (EP1207, EP1323, EP1211, EP3468 and EP3028) and one line has double insertions (EP1216). The gene possibly affected in one line is not sequenced (EP1340). Therefore the genes possibly affected by these EP lines are not listed here. The remaining 31 genes were classified into the following groups according to the possible functions of their protein products.

1. kinases: *CG2829, CG14217, CG10082, CG10967, CG6386, dco*
2. cytoskeleton related proteins: *CG3542, msp300, dlc90F*
3. protein involved in vesicle transport: *sar1*
4. membrane proteins: *robo2, tsp42Ef*
5. mitochondra proteins: *tim10*

6. transcription factors/regulators: *CHES-1-like, CG2446, CG11172, dsp1, tou, mesr4, CG10321, hmgD, CG6765, pap*
7. RNA binding proteins: *CG4266, boule*
8. component involved in protein degradation: *ago*
9. proteins without known homologues:*CG8954, CG10433, CG30290, CG11006, CG7231*

There are five genes whose protein products cannot be classified into any cellular processes due to lack of known homologues. The remaining genes encode proteins that are involved in a diversity of cellular processes, suggesting that the ectopic FGF signal in the Drosophila eye could be modulated in many ways directly or indirectly.

2.3 Genetic interaction of the candidates with other signalling pathways

One of the major problems for a screen is how to determine *bona fide* candidates, in my case, that is, how I could find out whether the candidates of the screen are involved in FGF signalling. One approach would be to examine the FGF signal in its normal biological environment, and test if the FGF signal could be processed properly in the absence of the candidate genes. This approach would involve studies on the loss of function mutants of the candidates. Considering the number of candidates I have found, I could only selectively investigate some of them. The studies on EP0719, EP3575 and EP0863 that are presented in the next chapter were based on such an approach. In this chapter, I tested the genetic interaction of the candidates with other signalling pathways, and therefore to gain some insights into the functions of the genes and whether they are involved in FGF signalling.

2.3.1 Testing for genetic interactions of the candidates with other signalling pathways

EGF signalling is involved in the eye development. Therefore I first tried to test whether the candidates interact also with the EGF signalling pathway. I crossed *UAS-λ-torpedo* (an active form of EGFR) flies to *gmr-Gal4* flies to make a line constitutively expression of *λ-torpedo* by GMR-Gal4. The progeny that have activated EGFR expressed by GMR-Gal4 ('GMR>λ-top') gave a more severe rough eye phenotype in comparison to 'GMR>λ-btl, dof' at 18°C. I crossed the candidates to the 'GMR>λ-top' flies and raised the progeny at 18°C. 36 of the EP candidates tested did not modify the 'GMR>λ-top' phenotype. Only EP1335, EP1455 and EP2204 suppressed the eye phenotype, while EP1216, EP1211, EP719, EP2510, EP2310, EP1135. EP3659, EP3468, EP3028 and EP3634 enhanced the eye phenotype.

Table 2-3: The summary of the lines that show phenotype in other genetic interactions and GOF experiments

EP lines	modi. of 'GMR>λ -btl, dof' at 18°C	modi. of $Cdc42^{L89.4}$ at 22°C	modi. of Rac^{N19} at 22°C	modi. of 'GMR>λ -PDGFR' at 22°C	modi. of 'GMR>λ -top' at 18°C	'GMR >EP' at 22°C	modi. of KDN / ras^{V12} [ref1]	GOF effec. on ES dev. [ref2]	GOF effec. on DT form. [ref3]	GOF effec. on axon guid. [ref4]
1200	S	N	N	N	N	N				
1408	S	E	E	slight S	N	N		2	E,d	
1413	S	E	E	S	N	darker				
1340	S	N	N	S	N	N				
1342	E	N	N	slight S	N	N				
1453	E	N	N	slight S	N	N				
1207	S	N	E	S	N	N				
1503	E	N	N	E	N	N		3		
1335	S	E	N	S	slight S	N		1		Y
1390	S	E	N	S	N	N				
1508	S	E	N	strong S	N	N		1	E,d	Y
1353	S	E	N	strong S	N	N	E/S		E,a	Y
0355	S	N	E	?E	N	R			E,d	Y
1455	S	E	N	strong S	S	N				
1323	S	N	N	S	N	N				
1216	E	N	N	N	slight E	N		2		
2582	slight S	N	N	S	N	N				Y
1211	E	E	E	E	E	N				
0719	E	N	N	slight E	E	N				
2204	S	N	S	S	S	N				
2510	E	E	E	E	E	N				
2171	S	N	N	S	N	N				
2571	S	N	N	n.d.	n.d.	n.d.				
0622	S	N	E	S	N	N		2	E,d	
0988	slight S	N	N	S	N	N	E/S*			
2258	S	N	N	S	N	N				
2516	E	N	N	N	N	N				
2034	E	N	N	S	N	N				
1222	strong E	n.d.	n.d.	slight S	N	N				
2319	E	N	S	strong S	N	N				
0436	E	S	N	S	N	N				
2310	E	N	N	S	slight E	N				
0712	strong E	N	N	slight S	N	N				
2440	E	N	N	slight S	N	N				
0541	E	N	N	N	N	N				
2494	E	n.d.	n.d.	N	N	N				
2421	E	N	N	N	N	N				
2444	E	N	N	slight E	N	N				
0467	E	N	N	E	N	N				
1135	strong S	N	N	E	slight E	N				
0595	S	n.d.	n.d.	E	N	N		1		
3659	E	E	n.d.	E	E	N				
3348	E	N	N	N	N	N				
3443	S	N	N	S	N	N				
3468	E	E	E	N	E	N				
3028	E	N	N	E	E	N				
3634	E	N	N	E	E	N				
3575	E	N	N	N	N	N				
0863	S	N	N	S	N	N				
3280	S	E	E	S	N	N				

EP lines are grouped according to their effects on the possible common genes in table 2-2. Different groups of EP lines are indicated by alternative shades. *EP0386 was found in the screen. According to molecular information both EP lines possibly affect the same downstream gene. [ref1]: Huang et al. 2000; [ref2]: Abdelilah-seyfried et al. 2000; [ref3]: Pena-Rangel et al. 2002; [ref4]:Kraut et al. 2001. 1:loss of external cells; 2: supernumerary ES (extra sensory) organs or support cells; 3: potential cell fate transformation. DT: Dorsal Thorax; E,d/a Enhancer in class d (effect on multiple structures) / class a (effect on chaetae). Y: yes, have an effect. Dev: Development. Form: formation. Guid: guidance. E: enhancer. S: suppressor. N: no modification. R: rough. n.d. not determined

PDGF signalling is involved in both hemocytes and border cell migration (Duchek et al., 2001; Heino et al., 2001). In order to test whether candidates interact also with the PDGF signalling pathway, I crossed *gmr-Gal4* to *λ-pdgfr* flies. Being raised at 22°C, the 'GMR>λ-pdgfr' flies show a mild rough eye phenotype. I crossed EP candidates to the 'GMR>λ-pdgfr' flies, and raised the progeny at 22°C. Most of the EP candidates tested modified the 'GMR>λ-pdgfr' eye phenotype. Only EP1200, EP1216, EP2516, EP0541, EP2494, EP2421, EP3348, EP3468 and EP3575 had not effect on the rough eye phenotype of 'GMR>λ-pdgfr'. EP1503, EP1211, EP0719, EP2510, EP2444, EP0467, EP1135, EP0595, EP3659, EP3028 and EP3634 enhanced the 'GMR>λ-pdgfr' eye phenotype, while the remaining lines suppressed the phenotype.

Overexpression of *UAS-cdc42$^{L89.4}$* and *UAS-rac^{N17}* in the 'GMR>λ-btl, dof' flies did not modify the 'GMR>λ-btl, dof' eye phenotype. However a weak rough eye phenotype was obtained when either of the transgenes was overexpressed by GMR-Gal4 alone at 22°C, which was a phenotype that did not show up at 18°C. This observation provides a possibility to further evaluate the candidates. To test if there were any genetic interactions between either of these two small GTPases and the candidates, lines overexpressing either Cdc42$^{L89.4}$ or Rac^{N17} by GMR-Gal4 ('GMR>cdc42$^{L89.4}$' or 'GMR>rac^{N17}') were generated. These two lines were then crossed to the candidates and the offspring were raised at 22°C. Results of interactions are summarized in table 2-3. In brief, EP1408, EP1211, EP2510, EP3468 and EP3280 enhance the eye phenotype of both 'GMR>cdc42$^{L89.4}$' and 'GMR>rac^{N17}'. EP1207, EP0355 and EP0622 only enhance the phenotype caused by overexpression of Rac^{N17}, while EP2204 and EP2319 suppress the 'GMR>rac^{N17}' phenotype. EP1335, EP1390, EP1508, EP1353, EP1455 and EP3659 only enhance the phenotype caused by overexpression of Cdc42$^{L89.4}$, while EP0436 suppresses the 'GMR>cdc42$^{L89.4}$' phenotype. All the other lines that were tested did not modify the eye phenotype caused by the overexpression of *UAS-cdc42$^{L89.4}$* and *UAS-rac^{N17}*. Altogether, 12 of the candidate genes interact genetically with Rac and Cdc42. They are *CG2829, CG7231, eip78C, dco, CG1679, dsp1, tou, msp300, CG10082, CG11172, CG14217* and *boule*. The protein products of these candidates are involved in diverse cellular processes.

2.3.2 The results from other gain-of-function screens in comparison to my screen

There are four gain-of-function screens that have been published recently, in which the same collection of EP lines were used as in my experiment (Abdelilah-Seyfried et al., 2000; Huang et al., 2000; Kraut et al., 2001; Pena-Rangel et al., 2002). I compared their candidates to mine.

A misexpression screen had been carried out to identify the genes that modify the rough eye phenotype caused by overexpression of a dominant negative form of Kinase Suppressor of Ras (KDN) in the cells expressing the gene *sev* (Huang et al., 2000). This gain-of-function screen was aimed at finding out critical components of Ras85D-MAPK pathway. There were two overlapping candidates between their findings and mine (table

2-3). One was EP1353, found as a suppressor in my screen affecting *CG11172*, which can suppress the eye phenotype caused by KDN and enhance the eye phenotype caused by overexpression of Ras^{V12}. Another candidate, EP0386, has a similar effect (The Flybase Consortium, 2002). EP0386 is able to overexpress the same gene as EP0988, *mesr4*. EP0988 appears to be a weak suppressor in my screen. Huang et al. did not pick up EP0988. EP0386 is missing from the collection that I have screened, which is the reason that I did not identify it. Both of these overlapping genes encode transcription factors. Since they are found in the screen for downstream components involved in RTK signals, it is possible that they also have functions in the FGF signalling pathway.

Two recent GOF screens searched for genes exclusively involved in neuronal cell differentiation (table 2-3). In the screen conducted by Abdelilah-Seyfried et al., 2000, the effects of the misexpression of EP lines by scaGal4 in external sensory organs were examined. In comparison to my results, Abdelilah-Seyfried et al. have also identified EP1408, EP1503, EP1335, EP1508, EP1216, EP0622 and EP0595. Except for EP1216, the remaining lines possibly affect *CG2829, CG2446, CG11172, tou* and *CG6765* respectively. Rena-Rangel et al., have screened the effects of the misexpression of EP-lines by pnrGal4 on dorsal thorax formation. In comparison to my results, they have also found EP1408, EP1508, EP1353, EP0355 and EP0622. These lines possibly affect *CG2829, CG11172, dsp1* and *tou* respectively. Except *CG2829*, which encodes a kinase, all others are transcription factors or regulators.

Kraut et al. have screened the effects of the misexpression of EP lines by a pan-neuronal Gal4 on motor axon guidance and synaptogenesis (Kraut et al., 2001). They identified genes whose products are required for axon guidance and synaptogenesis. In comparison to my results, they have also identified EP1335, EP1508, EP1353, EP0355 and EP2582. These lines possibly affect *CG11172, dsp1* and *robo2* respectively, two transcription factors or regulators and one membrane receptor.

2.3.3 Summary

8 EP candidates found in the primary screen do not show any interactions in other signalling pathways that I have tested, which are the EGF signalling pathway, the PDGF signalling pathway, and signalling pathways involve Cdc42 or Rac1. These candidates were also not found in other published GOF screens, which are screens for genes that modify KDN signalling pathway, have effects on development of extra sensory cells, dorsal thorax, or axon guidance. These 8 candidates possibly affect functions of 4 genes, which are *CG10967, sar1, tsp42Ef,* and *tim10.*

2.4 Studies on three of the candidates -EP0719, EP3575 and EP0863

As an approach to determine whether a gene is involved in FGF signalling, I chose three candidates to study further in detail, which are EP0719, EP3575 and EP0863. All three lines are homozygous lethal. There are two reasons that I chose these three candidates. Firstly, these EP lines affect genes involved in different cellular processes. Secondly, they are inserted into the exons of the affected genes and are therefore easy to manipulate.

In order to determine the function of the genes during Drosophila development, I mobilized the three EP elements by providing transiently a copy of transposase and searched the progeny for small imprecise excisions by PCR. All the imprecise excisions recovered were sequenced. At least two independent lines of the precise excision events were identified in each case and later on confirmed by sequencing. In all cases, precise excisions revert the lethality, implying that the homozygous lethal phenotype of these three lines is caused by the EP insertions. All of the imprecise excisions were recombined on FRT chromosomes. Experiments for generating germline clones and somatic clones were performed.

2.4.1 EP0719

EP0719 is inserted into the genomic region that codes for the amino acid 637 of the predicted protein CG3542, which contains 806 amino acids (Fig.2-20). It is possible that a truncated form of the gene encoding a protein that lacks the C-terminal 170 amino acids is still present.

CG3542 is homologous to a Formin Binding Protein
The N-terminal 561 amino acids of the predicted protein CG3542 are homologous to the N-terminal 721 amino acids of human FBP11 (Formin Binding Protein 11) with 39% identity and 53% similarity (Fig.2-20). The FBP11 together with other FBPs have been found in a yeast two-hybrid screen searching for interaction partners of Formin (Chan et al., 1996).

FBP11 has been implicated to be a homologue of the yeast protein Prp40. Sequence alignment shows that the N-terminal 630 amino acids of Drosophila FBP are homologous to the Prp40 protein with 23% identity and 43% similarity (Fig.2-20). Prp40 was found as a suppressor in a genetic screen for proteins that interact with *S. cerevisiae* U1 small nuclear RNA. It has been shown that Prp40 can bind U1 RNA *in vitro* and is important for the splicing of the RNA (Kao et al., 1996). Based on the protein sequence similarity, it is possible that CG3542 is involved in RNA splicing (Mount et al., 2000).

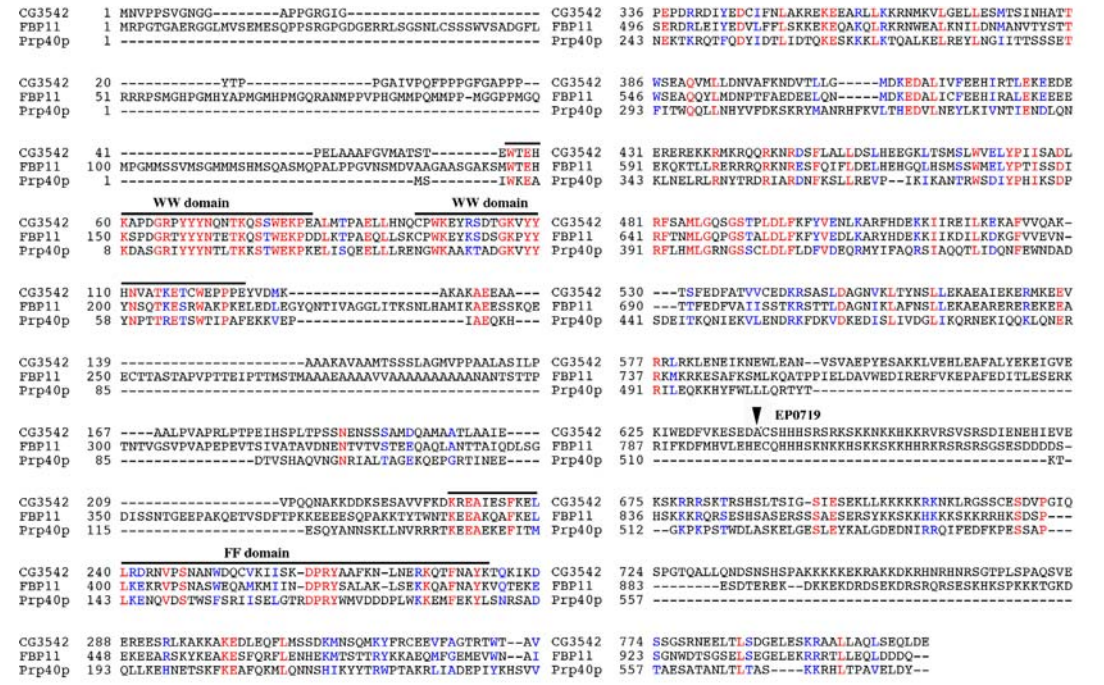

Fig.2-20. Sequence comparison of CG3542 with FBP11 and Prp40p. WW domains and FF domain are highly conserved among the three proteins. EP0719 is inserted into the coding region of the amino acid 637.

The N-terminal region of CG3542 contains two WW domains, and one FF domain (Fig.2-20). The FF domains are protein-protein interaction motifs capable of binding multiple phosphorylated proteins (Carty et al., 2000). WW domains are conserved protein motifs of 38-40 amino acids found in a broad spectrum of proteins. They mediate protein-protein interactions by binding to proline-rich domains (Bedford et al., 2000). The WW domains of the mammalian homologue of CG3542, FBP11, can bind to formin, which contains proline-rich domains. Formin is required for limb development in vertebrates (Trumpp et al., 1992) in which it is localized mainly in the nucleus. However its homologues in Drosophila and *S. pombe* are exclusively cytoplasmic and important for a number of actin-dependant processes, including polarized cell growth and cytokinesis (Frazier et al., 1997). The yeast homologue of Formin, Bni1, has been shown to interact with Profilin. It is a downstream target of Cdc42. A recent paper has shown that in yeast, when Formin is stimulated by Profilin, it can mediate actin nucleation *in vitro* and possibly therefore organize the actin network (Sagot et al., 2002). There are two Drosophila homologues of Formin identified so far. They are Diaphanous and Cappuccino (Afshar et al., 2000; Castrillon et al., 1994; Emmons et al., 1995). Both are required for cytokinesis. In addition, Cappuccino has also been indicated to have a role in regulating microtubule assembly. It is possible that proteins potentially interact with Formin could have a role in cytoskeleton regulation.

The analysis of functions of CG3542 during Drosophila development

CG3542 or *fbp* may be expressed at a low level ubiquitously during the embryonic development. No specific expression pattern can be seen in RNA *in situ* (Fig.2-21). It seems that there is maternal contribution of the transcripts during early stages (Fig.2-21A), and the mRNA signal gradually fades during embryonic development (Fig.2-21, B, C&D). The EP insertion is homozygous lethal at very late embryonic development or the L1 larval stage, and no defect is detected in either the mesoderm or the trachea.

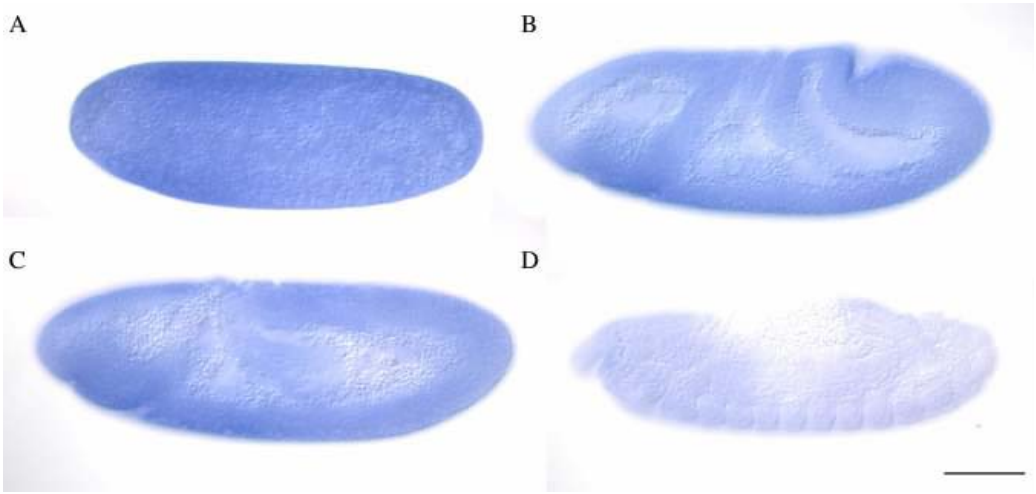

Fig.2-21. The expression pattern of *CG3542*. *CG3542* is likely to be ubiquitously expressed in the blastoderm embryos (A). The transcripts levels appear to decrease as the embryos develop (B, C). The staining is much weaker at stage 14 (D). Scale Bar: 100μm

In order to determine the function of the protein during Drosophila development, I generated imprecise excisions of EP0719. In total, 6 distinct excisions were identified by PCR (Fig.2-22). These excisions were sequenced. Three imprecise excisions, #3.3, #9.4 and #35.1, have large deletions that extend in both directions from the original insertion, but are all within the protein coding region. The deletions in these three excisions lead to frame shifts of the remaining 3' codon sequence, which induce premature stop codons. Therefore, #3.3 and #9.4 may produce truncated proteins missing more than half of the C-terminal amino acids, while #35.1 may produce a protein with C-terminal 375 amino acids deleted. The excisions #9.4, #15.5 and #18.2 extend only towards the 3' end of the EP insertions. Two of these, #15.5 and #18.2 are very small internal deletions with the 3' untranslated region remaining intact. The deletion #15.5 took away the coding region for 104 amino acids between Nr.640 and Nr.745. However, this excision left an insertion of a fragment of 8bp (see appendix 1 for details), which leads to the introduction of a stop codon 120bp away from the break point. The deletion #18.2 leads to frame shift and

therefore introduces a stop codon 18bp away from the break point. Both excisions may produce a truncated protein with C-terminal 166 amino acids missing. The deletion in excision #9.4 includes the C-terminal protein coding region and 3' untranslated region of the gene, but does not go beyond the 3' untranslated region of a cDNA sequence derived from this gene. The homozygous mutant phenotype of all these excisions is lethal at a similar stage to the homozygous EP insertion. Both precise and imprecise excisions were crossed to 'GMR>λ-btl, dof' flies to examine their ability of modifying the eye phenotype. All the imprecise excisions enhance the eye phenotype while the precise excisions had no effect. These results confirm that the modification of the 'GMR>λ-btl, dof' eye phenotype by EP0719 is caused by disruption of the Drosophila homologue of FBP.

The *in situ* data suggests that it is possible that maternally deposited RNA or protein could support embryonic development. In order to find out if maternal product is required, I generated germline clones for the imprecise excisions. It proved impossible to obtain embryos from germline clones for excisions #3.3, #9.4, #35.1 and #9.3. The ovaries of these lines developed till stage 4, but not beyond this point. In the *OvoD* mutant, egg development also stops at this stage. This result suggests that the protein is probably required for early germ cell development. However, embryos from germline clones of excisions of #15.5 and #18.2 were generated. The appearance of the eggs is normal. Half of the eggs are expected to be homozygous excision mutants, while the remaining half should contain one copy of wild type genes inherited from the heterozygous father. Half of the fertilized eggs developed normally till adulthood, suggesting that the paternally provided wild type gene can rescue the germline mutant phenotype. The remaining fertilized eggs developed till the end of the embryonic stage or early larval stage and died. These embryos do not show any defects in the mesoderm or the trachea. These results imply that the excisions #3.3, #9.4, #35.1 and #9.3 probably represent null alleles, and #15.5 and # 18.2 are likely to be hypomorphic alleles. The fact that #15.5 and #18.2 germline clones can survive till the later embryonic stages of development suggests that the protein with C-terminal 166 amino acids removed can still fulfill its function in oogenesis.

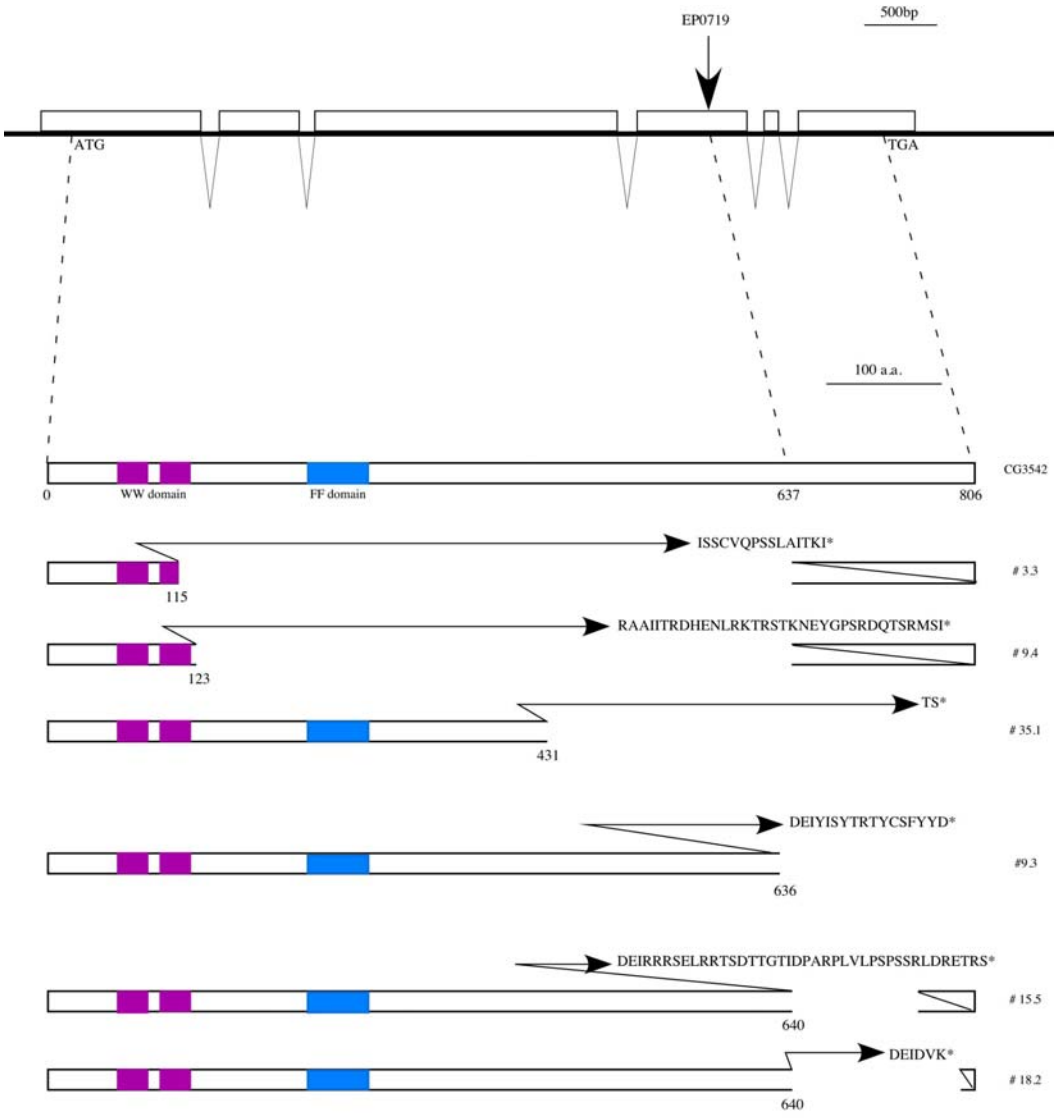

Fig.2-22. Imprecise excision of EP0719 generated six independent deletions. The insertion of EP0719 is close to the C-terminal protein coding region of *CG3542*. The protein contains three recognizable domains, two WW domains and one FF domain, indicated by purple and blue boxes respectively. The imprecise excisions of EP-element generated six deletions. Five, #3.3, #9.4, #35.1, #15.5 and #18.2, are internal deletions that the excisions do not go beyond protein coding region. Among these, #35.1 deleted part of the stop codon, and the remaining excisions cause frame shift which are indicated by a line cross the region. #9.3 is a deletion also including the 3' untranslated region of the gene. However the deletion does not go beyond the 3' end untranslated region of a cDNA sequence derived from this gene. The possible amino acids being translated after the breaking point of individual excisions are shown. See appendix for DNA sequence details.

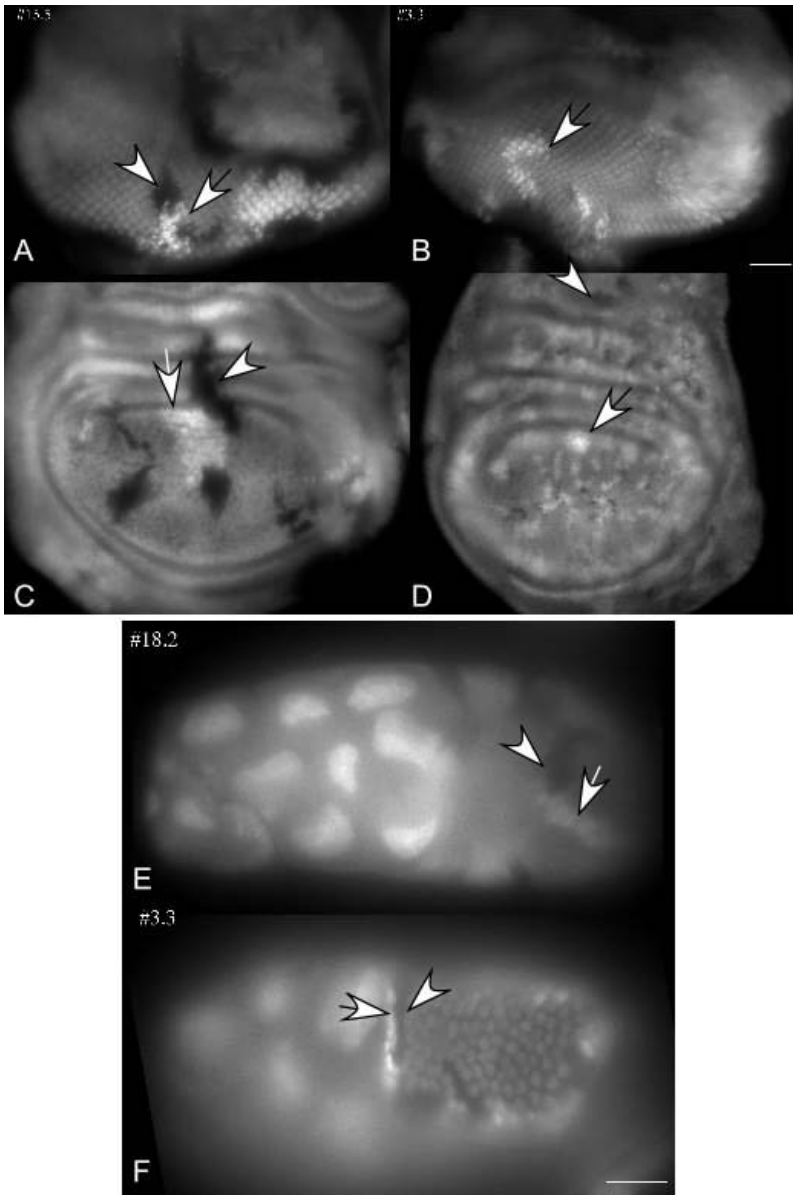

Fig.2-23. Somatic clones of mutants that affect protein coding region of *CG3542*. The cells lack of GFP marker are the mutant clones of imprecise excisions of #15.5 (A&C), #18.2 (E), #3.3 (B, D&F). The examples of mutant clones were indicated by arrow heads, while the examples of wild type twin clones containing two copies of GFP transgenes and therefore brighter than the non-clonal cells were indicated by arrows. It was almost impossible to recover clones homozygous for mutant #3.3 in photoreceptor cells (B) although with #15.5, mutant clones were generated (A). However, clones for mutant #3.3 were recovered in some cell types in wing disc, although the size is rather small (D), clones for mutant #15.5 were also generated in wing disc (C). Clones for mutants of either #3.3 or #15.5 were recovered for somatic cells in egg chamber (E & F). Scale Bar: 50μm

In order to examine whether the function of CG3542 is essential for cell survival, clones in imaginal discs and somatic cells in the egg chamber were generated. The mutant clones were marked by lack of nuclear GFP (Fig.2-23). In the wing and eye imaginal discs, large mutant clones of excision #15.5 were generated. The size of the mutant clones is comparable to their wild type twin clones, which contain two copies of GFP transgene and therefore have a stronger GFP signal (Fig.2-23, A&C). For excision #3.3, there is almost no or only very small mutant clones in photoreceptor cells in comparison to their twin clones (Fig.2-23B). It is possible to generate small clones for excision #3.3 in the wing discs (Fig.2-23D). In the ovaries, homozygous clones of both excisions #18.2 and #3.3 in follicle epithelial cells were generated (Fig.2-23, E&F). The size of the clones was comparable to the wild type twin clones. Therefore, it is likely that the gene is not generally required for cell survival.

2.4.2 EP3575

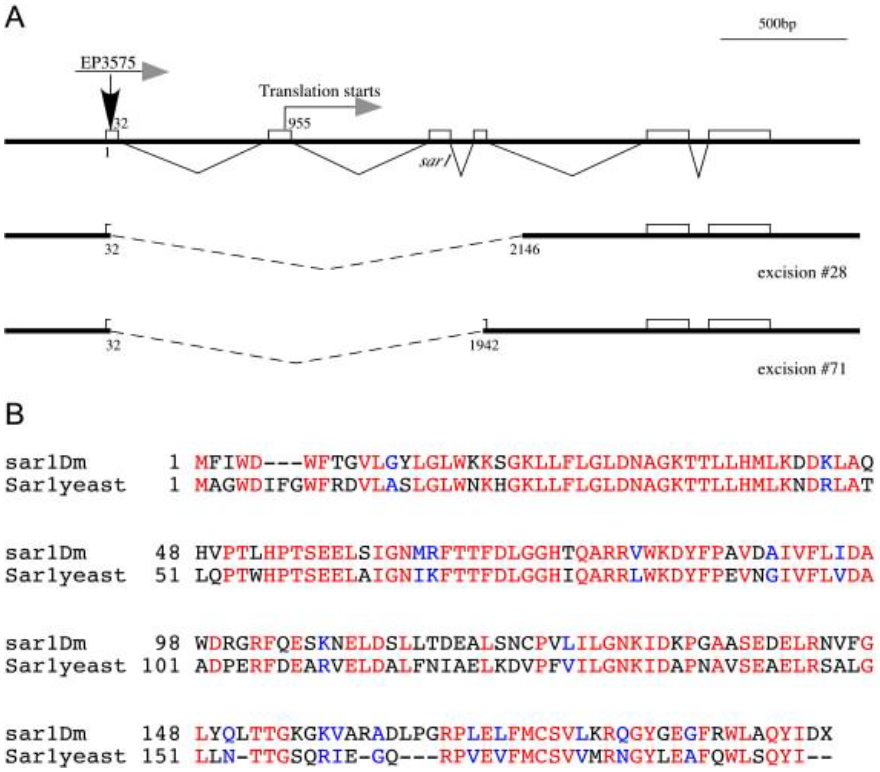

Fig.2-24. EP3575 is inserted into the first exon of Drosophila *sar1* (A). The gene consists of 6 exons, which are indicated by boxes. Sar1 is highly conserved from yeast to Drosophila (B). The EP-element was mobilized and two imprecise excisions have been recovered (A). The imprecise excision #28 uncovers the region between first exon to fourth intron, while #71 uncovers a slightly smaller region. Both excisions are likely to generate null alleles.

EP3575 is inserted into the first exon of a gene whose product is highly homologous to Sar1. The EP insertion is located within the 5' untranslated region of the Drosophila *sar1*, and the orientation of the insertion allows overexpression of the *sar1* gene (Fig.2-21A). The insertion causes lethality in later homozygous embryos or early larvae. However neither the mesoderm nor the trachea show any defects in homozygous embryos.

Sar1 is a small GTPase that together with Arfs form a subfamily of Ras small GTPases that mostly regulate vesicle budding (Takai et al., 2001). The function of Sar1 has been mainly studied in the yeast S. cerevisiae. It is associated with the ER and is involved in the formation of COPII-coated transport vesicles from the ER (Barlowe et al., 1994; Matsuoka et al., 1998). Sar1 is highly conserved from yeast to Drosophila (Fig.2-21B). There is only one *sar1* gene in yeast and two in mammals.

In Drosophila, only one Sar1 homologue exists. *Sar1* transcripts are likely to be expressed at a low level and ubiquitously during most embryonic developmental stages (Fig.2-25, A, B&C). However the signal seems to decrease before germband retraction (Fig.2-25D) and does not become stronger again till the end of the embryonic development. The early ubiquitous staining in the embryos suggests that the *sar1* mRNA may be deposited maternally.

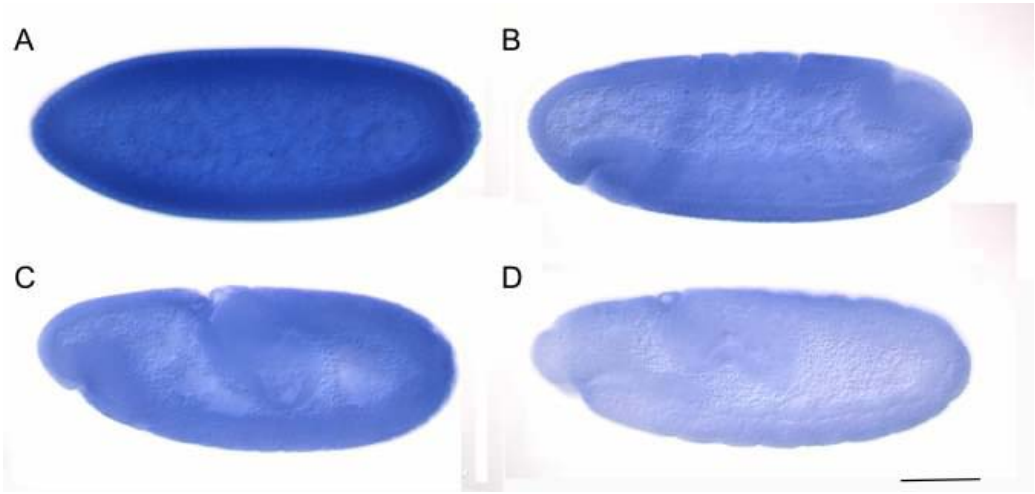

Fig.2-25. The expression pattern of *sar1*. *Sar1* is expressed ubiquitously during embryo development. From blastoderm (A), gastrulation (B), to fully extended germband (C), the level of *sar1* transcripts gradually decreases. The signal goes down dramatically before the germband retraction (D) and remains at a low level. Scale Bar: 100μm.

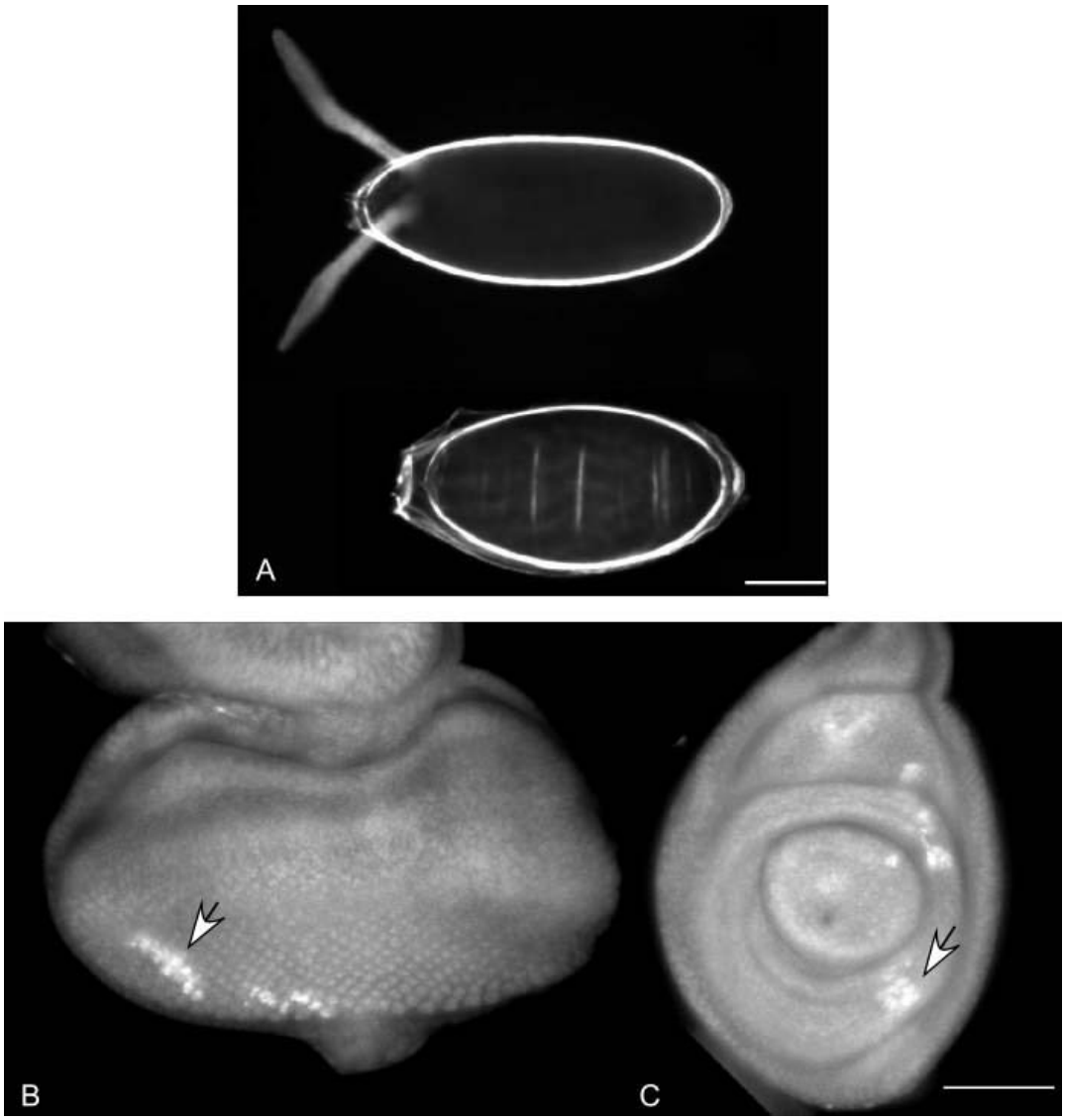

Fig.2-26. Clones of *sar1* mutants. Both deletions that affect *sar1* give an identical phenotype. Only a few of eggs were produced when germline clones of the *sar1* mutants were generated. The eggs were smaller (A, button) in comparison to wild type egg (A, top). The *sar1* germline clones lack of dorsal appendages. In the experiment to generate *sar1* mutant clones in the imaginal discs, mutant clones are marked by lack of GFP, while their wild type twin clones containing two copies of GFP transgenes are brighter than the other non-clonal cells. There is no *sar1* mutant clones recovered in either eye imaginal discs (B) or leg imaginal discs (C), although the twin clones are clearly visible (arrows in B&C). Scale Bar for A: 100μm, for B&C: 80μm.

In order to investigate further the function of the gene and its possible relation to the FGF signal pathway, I generated two imprecise excisions, excision #28 and #71 (Fig.2-24A). The *sar1* gene is composed of 6 exons and encodes a small protein of 193 amino acids. The imprecise excision #28 deleted part of the 1st exon and the 2nd to 4th exons of *sar1*. The deletion removes 2114bp of DNA including the coding sequence of the first 76 amino acids. This deletion probably leaves a splicing problem for the remaining transcript, and the C-terminal part of the protein is unlikely to be transcribed. Excision #71 deleted part of 1st exon, 2nd to 3rd exons completely, and most of exon 4. This deletion uncovers a region of 1910bp, and removes the coding sequence of 72 amino acids and a truncated protein is also unlikely to be translated due to the lack of a recognizable sequence coding for Met in the remaining protein sequence. Both excisions are homozygous lethal. When these excisions were crossed to the 'GMR>λ-btl, dof' flies, they were not able to enhance the eye phenotype. Therefore, the enhancement of the 'GMR>λ-btl, dof' eye phenotype found in the screen was due to overexpression of gene sar1.

Embryos that were homozygous for either of these deletions do not show any defects in the mesoderm or the trachea. However, the *in situ* hybridization data suggests that maternally supplied product may be sufficient for embryonic development. To investigate the function of the gene further, both deletions were recombined to FRT chromosomes and germline clones were generated. Females induced to produce germline clones laid a few eggs, which were very abnormal. These eggs do not have dorsal appendages (Fig.2-26A). When the ovaries were dissected, few egg chambers had developed beyond stage 4 which is the point at which the *OvoD* mutant affects the egg development. Thus the function of the *sar1* gene is required for the early development of the germline cells. The eggs that were laid may survive at the early stages of egg development due to the residual product generated before the mitotic recombination.

In order to find out whether *sar1* is required for cell survival, I tried to generate mutant clones of *sar1* in somatic tissues. *Sar1* mutant clones are not visible in eye discs and leg discs while the wild type twin clones are present (Fig.2-23, B&C), indicating that *sar1* is required for the cell survival.

2.4.3 EP0863

EP0863 is inserted in the second exon, within the 5' untranslated region of the predicted gene *CG6386* (Fig.2-27). The orientation of the insertion permits overexpression of the gene in the presence of Gal4. This insertion causes lethality in later embryos and the L1 larvae stages in homozygous mutants. However, the mutant embryos do not show any defects in the mesoderm or the trachea.

The gene encodes a predicted kinase of 599 amino acids. It is highly homologous to human and mouse Vaccinia-Related Kinase 1 (VRK1). It shows approximately 40% identity and 60% similarity to human VRK1 (Fig.2-24). The most similar region of the three proteins is the kinase domain. The human VRK1 has been shown to be a functional kinase, which phosphorylates murine p53 on threonine 18. The human VRK1 is likely to be an upstream regulator of p53 that may belong to a new signalling pathway (Lopez-Borges et al., 2000). In the C-terminus of the Drosophila gene, there are several nuclear localization signals.

To analyze the expression of CG6386, I performed *in situ* hybridization using the anti-sense RNA probe. The results suggest that the transcripts of the gene are provided maternally. The mRNA is degraded gradually during embryonic development (Fig.2-29).

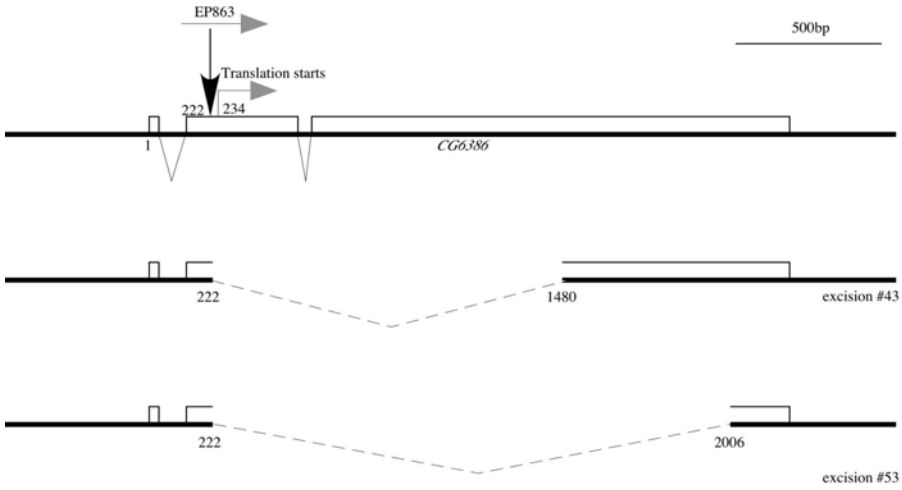

Fig.2-27. A schematic representation of the genomic region around EP863 and the deletions generated as results of imprecise excision of this P-element. EP863 is inserted into the second exon of *CG6386*. Imprecise excision of the EP insertion produced two internal deletions, which are likely to be null alleles of the gene. The sequence information can be found in appendix.

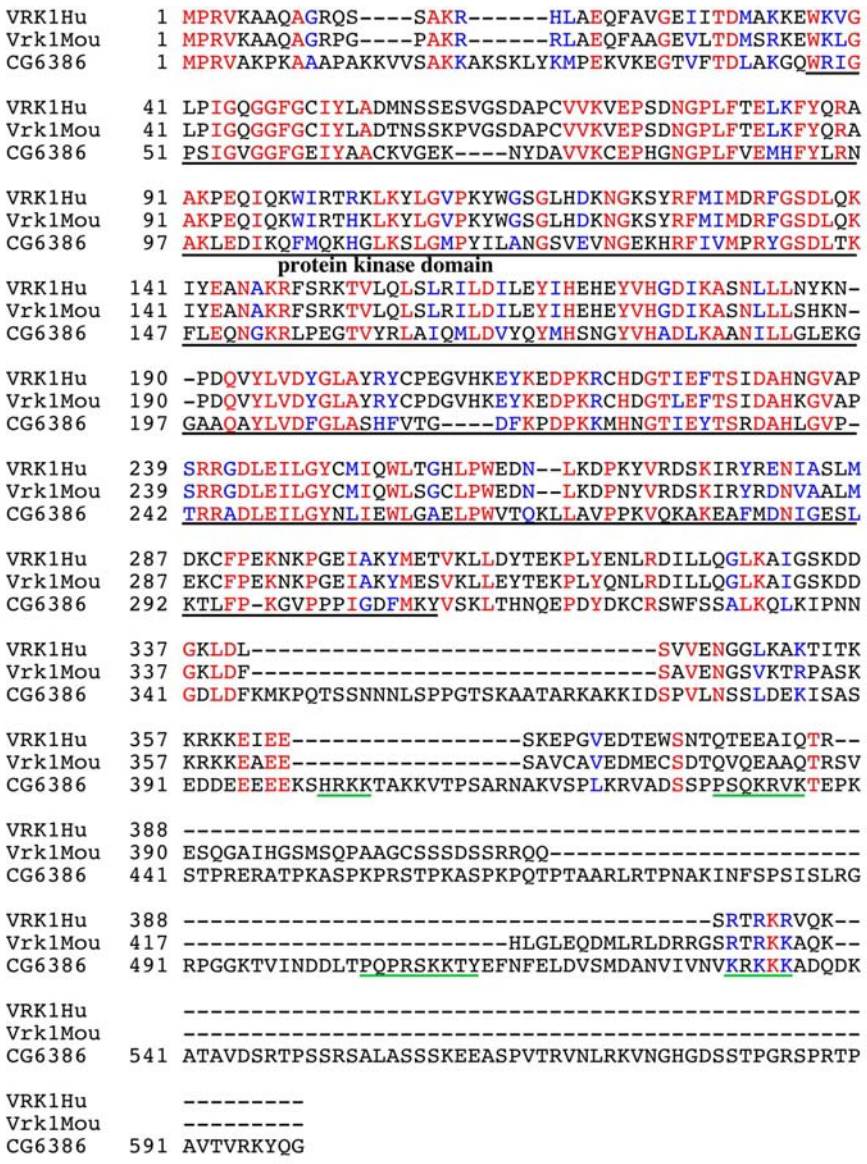

Fig.2-28. Sequence comparison of the predicted protein encoded by *CG6386* with human and mouse VRK1. The protein kinase domain are highly conserved between all three proteins. The green lines mark the potential nuclear localization sequences that match the consensus sequences.

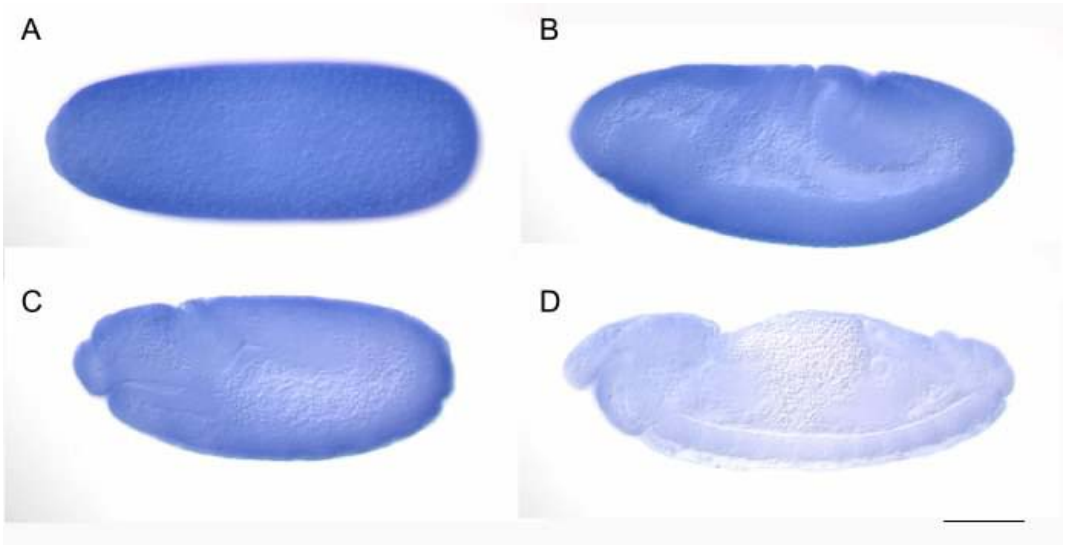

Fig.2-29. The expression pattern of *CG6386*. The gene is expressed ubiquitously during blastoderm (A), gastrulation (B), and the fully extended germband stage (C), it becomes less abundant during dorsal closure (D). Scale Bar: 100μm.

To study the function of the gene during development, I generated imprecise excisions of the EP-element. Two imprecise excisions, #43 and #53, were identified (Fig.2-27). #43 deleted 1258bp and #53 deleted 1784bp of the genomic DNA starting from the EP insertion site. In both cases, a large part of protein coding region is missing. Both excisions lead to frame shift and are likely to represent null alleles. The excisions cause lethality in homozygous embryos or larvae. Both imprecise and precise excisions fail to modify the 'GMR>λ-btl, dof' eye phenotype, implying that the suppression phenotype of EP863 is due to overexpression of the gene.

No defects in the mesoderm and the trachea were detected in these mutant embryos. In an experiment to generate germline clones for both excisions, females induced to produce germline clones laid few eggs, which were abnormal. These eggs did not display a consistent phenotype. When the ovaries were dissected, no egg chamber developed beyond stage 4. Therefore, it is likely that the gene product is required for the early egg development. To test whether the gene is required for cell survival, I generated mutant clones in eye imaginal discs. I could generate mutant clones in photoreceptor cells having similar size to their wild type twin clones (Fig.2-26), indicating that the gene is not generally required for cell survival.

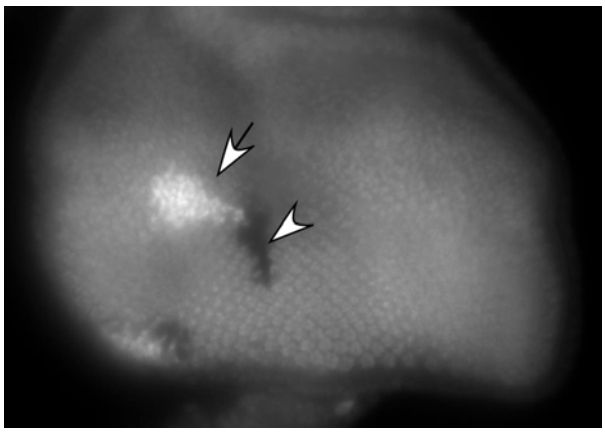

Fig.2-30. Mutant clones of allele #53 of *CG6386* were generated in eye imaginal discs. Excision #43 gives an identical phenotype. The mutant clones are marked by lack of GFP. An example is indicated by an arrow head. The wild type twin clones containing two copies of GFP transgene are brighter than the non-clonal cells. An example is indicated by an arrow. The size of the mutant clone is comparable to its twin clone, implying the gene is not essential for cell survival.

2.4.4 Summary

The three candidate genes that I have investigated in more detail all appear to be important for early egg development, which present difficulties to study whether they are involved in FGF signalling or not during embryonic development. There is no known function for the FGF signal during early egg development, which suggests that these three candidates function not only in FGF signalling although they may still have a role at later stages during embryonic or larval development.

2.5 Studies on the mutants of *X-144*

The mutation *X-144* was first mapped to 7A, close to *fz4* (*frizzled 4*), the gene downstream of EP1340. EP1340 was picked up in my screen as a suppressor of the 'GMR>λ-btl, dof' eye phenotype. Therefore I started to work on this mutant.

X-144 was identified in a screen conducted in Dr. Nuesslein-Volhard's lab. The cuticle prepared from the homozygous embryos of *X-144* shows a tail-up phenotype, which implied that germband retraction was not complete (Dr. B. Moussian, personal communication). After examining the cuticle preparation, Dr. S. Roth thought there may either be gastrulation or dorsal-ventral patterning defects.

There are two alleles that fall into the same complementation group. One is homozygous viable but female sterile, and the other is homozygous lethal. The eggs laid by the

homozygous mother of the viable allele, or by the trans-heterozygous mother of the two alleles, or by the germline clone of the lethal allele give similar embryonic phenotype.

2.5.1 Mapping of the mutant

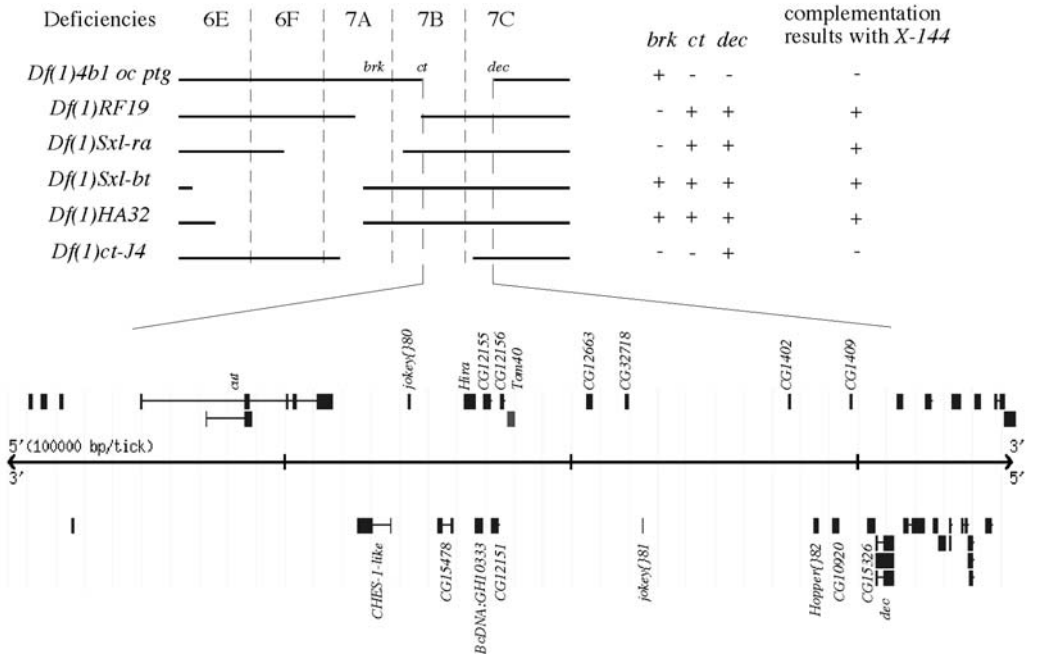

Fig.2-31 The summary of the complementation tests. The regions uncovered by the six deficiencies that were used in the complementation tests are indicated by the gaps in the horizontal lines. The molecular markers for the deficiencies, the test results and the candidate genes in the region are listed. Abb: *brk*: *brinker*; *ct*: *cut*; *dec*: *defective chorion 1*.

To precisely map the location of the gene, I crossed the viable mutant to different deficiencies in the region. They are *Df(1)4b1 oc ptg, Df(1)RF19, Df(1)Sxl-ra, Df(1)Sxl-bt, Df(1)HA32, Df(1)ct-J4*. Among them, *Df(1)RF19* and *Df(1)Sxl-ra* uncover the gene *brk (brinker)* but do not uncover the genes *ct (cut)* and *dec (defective chorion 1)*, *Df(1)Sxl-bt* and *Df(1)HA32* uncover the region from 6E to 7A, outside the region that contains *ct*, *dec* and *brk*. *Df(1)4b1 oc ptg* uncovers the region that includes *ct* and *dec*, but not *brk*, while *Df(1)ct-J4* uncovers the region that includes *brk* and *ct*, but not *dec*. I crossed males of the viable *X-144* allele to the deficiencies, selected in the F1 generation females having both *X-144* and deficiency chromosomes, and examined whether these females were sterile. The results are summarized in figure 2-31. *X-144* does not complement *Df(1)4b1 oc ptg* and *Df(1)ct-J4*, but complements all the other deficiencies. These results narrowed down

the candidates that could be affected in the mutant to 15 annotated genes (The Flybase Consortium, 2002).

2.5.2 The mutant phenotype

In order to investigate whether the mutant has a gastrulation phenotype, anti-twist antibody staining was performed to visualize the mesoderm. The staining shows that embryos have their germband twisted and folded to various degrees (Fig.2-32B, C&D) in comparison to wild type (Fig.2-32A). Statistical analysis indicates that about 67% (14/21) of the embryos homozygous of viable mutant show the phenotype in fig.2-32B or C, in which the germband is extended to certain degree. The remaining 33% (7/21) show the extreme phenotype of fig.2-32D, in which germband almost does not extend at all. In the case for the germline clones of lethal allele, all the embryos at germband extension stage show the phenotype between fig.2-32B and C.

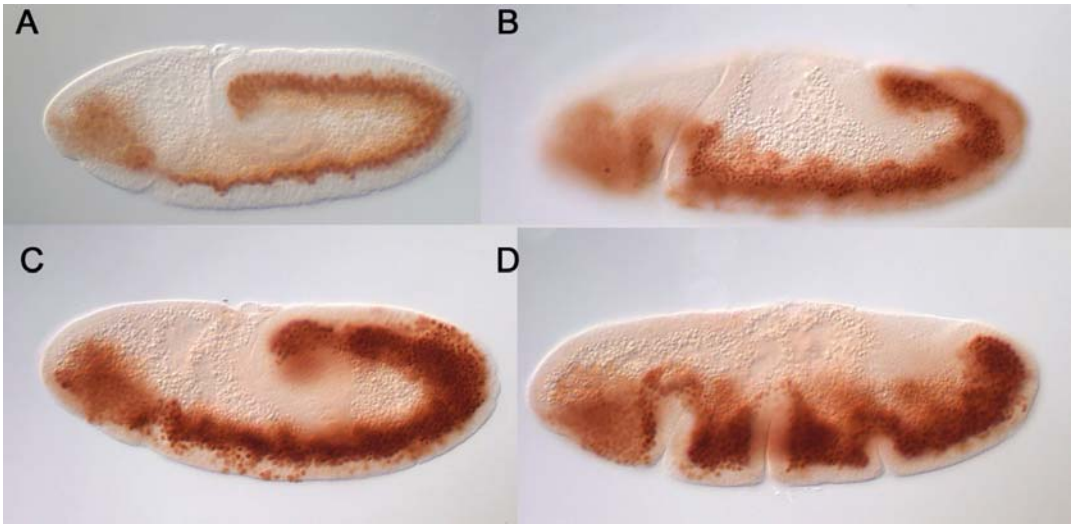

Fig.2-32. Anti-Twist antibody staining of wild type (A), and *X-144* mutant (B, C, D) embryos. *X-144* mutant embryos display various degrees of twists and folds during germband extension. Statistical analysis shows that about 67% (14/21) embryos display phenotype between B and C, while the remaining 33% (7/21) embryos display the extreme phenotype of D. However, all the embryos from the germline clones of the lethal allele display phenotype between B and C.

In order to find out whether there is defect during germband extension, I did time-lapse live recording for wild type embryos and homozygous mutant embryos from both alleles. Both alleles show similar phenotype except in viable alleles, germband extension was not observed in some embryos after the cellularization was completed. The records show that the germband extension in mutant embryos is slower than in the wild type embryos (Fig.2-

33. right panel shows an example of a mutant embryo, compare to the left wild type panel).

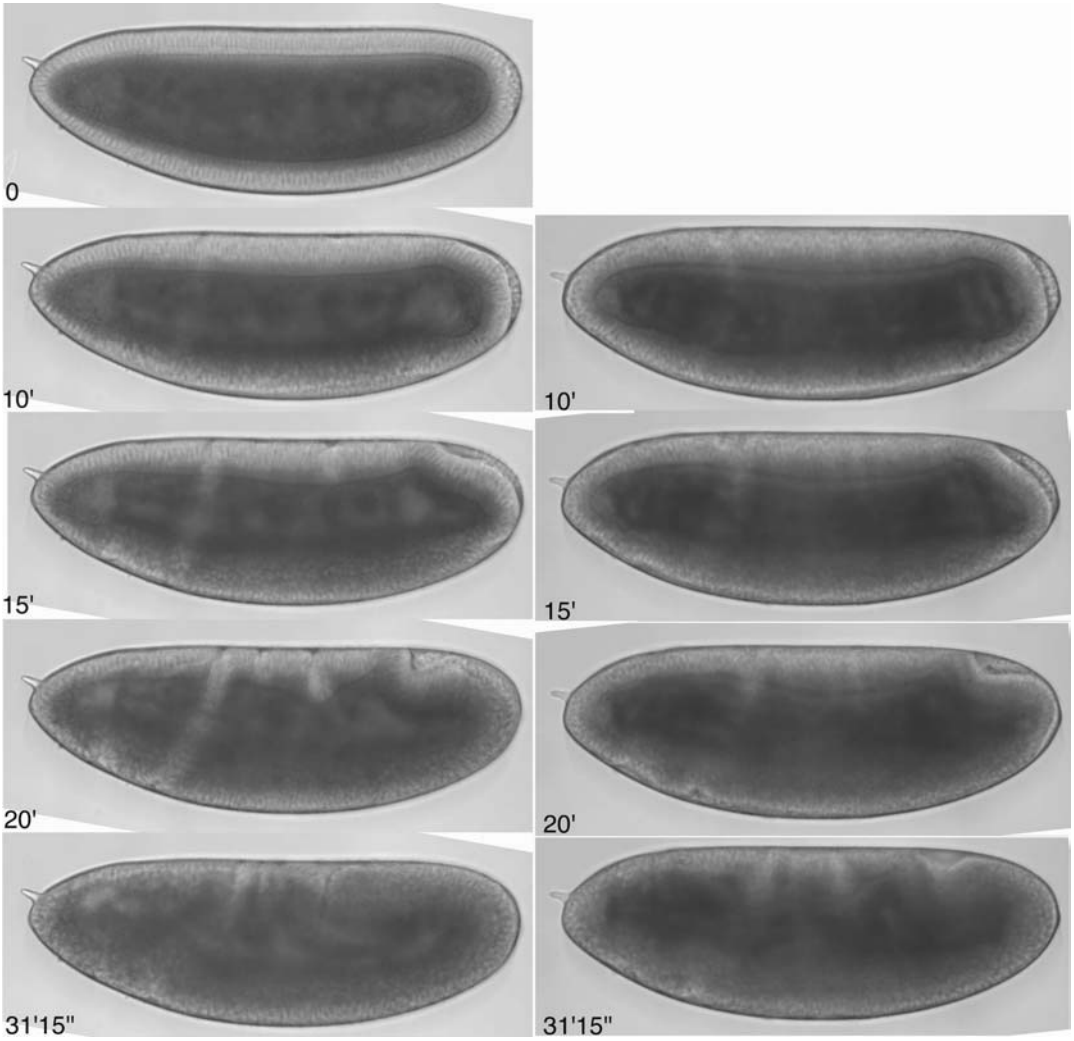

Fig.2-33. The germband extension of *X-144* embryos is slower than the wild type embryos. Left panel shows series of pictures taken from a time-lapse living recording during early gastrulation of a wild type embryo. Right panel shows series of pictures taken from a recording during the same period of gastrulation of an *X-144* embryo. If one considers the end of cellularization as time point zero, after 10 min. germband extension in the mutant starts to show a delay. The delay becomes more obvious when gastrulation progresses.

In order to find out whether there is defect during germband extension, I did time-lapse live recording for wild type embryos and homozygous mutant embryos from both alleles. Both alleles show similar phenotype except in viable alleles, germband extension was not observed in some embryos after the cellularization was completed. The records show that the germband extension in mutant embryos is slower than in the wild type embryos (Fig.2-33. right panel shows an example of a mutant embryo, compare to the left wild type panel).

One possibility that could account for this phenotype would be defects in early embryonic patterning. The anti-activated MAPK staining is a good readout of the Torso signal which is responsible for the terminal cell fates, and the DEGFR signal which is important for early dorsal-ventral patterning (Gabay et al., 1997). The anti-activated MAPK staining in the germline clones of lethal allele show that the embryonic patterning appears to be normal, which suggests that the early patterning is unlikely to be affected (Fig.2-34, B, D, F compare to A, C, E). There is some MAPK activity at the anterior end of the mutant embryos (Fig.2-34D&F) that does not exist in the wild type embryos (Fig.2-34C&E). The development of amnioserosal cells is the indication of a proper dorsal fate. From stage 11 to 14, *Krüppel (kr)* is expressed in amnioserosa cells (Lamka et al., 1999). The anti-*kr* antibody staining of the stage 12 embryos shows that the amnioserosal cell fate determination is normal in the germline clones of lethal alleles (Fig.2-34H compare to G). Even in the extreme case observed in the viable mutant embryos that germband does not extend at all, one could observe a differentiated single layer of amnioserosa cells (Fig.2-34I, arrowhead). Therefore, there is no obvious patterning defect in the mutant embryos.

As presented already, the mutants display defects at the onset of germband extension, which does not seem to be the result of early patterning failure. Therefore, I examined younger embryos in detail. First, I investigated embryos at the end of cellularization by phalloidin staining. The staining revealed that at the end of cellularization, the shapes of the cells formed in the mutant embryos is irregular (Fig.2-35B compare to A). This is the case for both alleles.

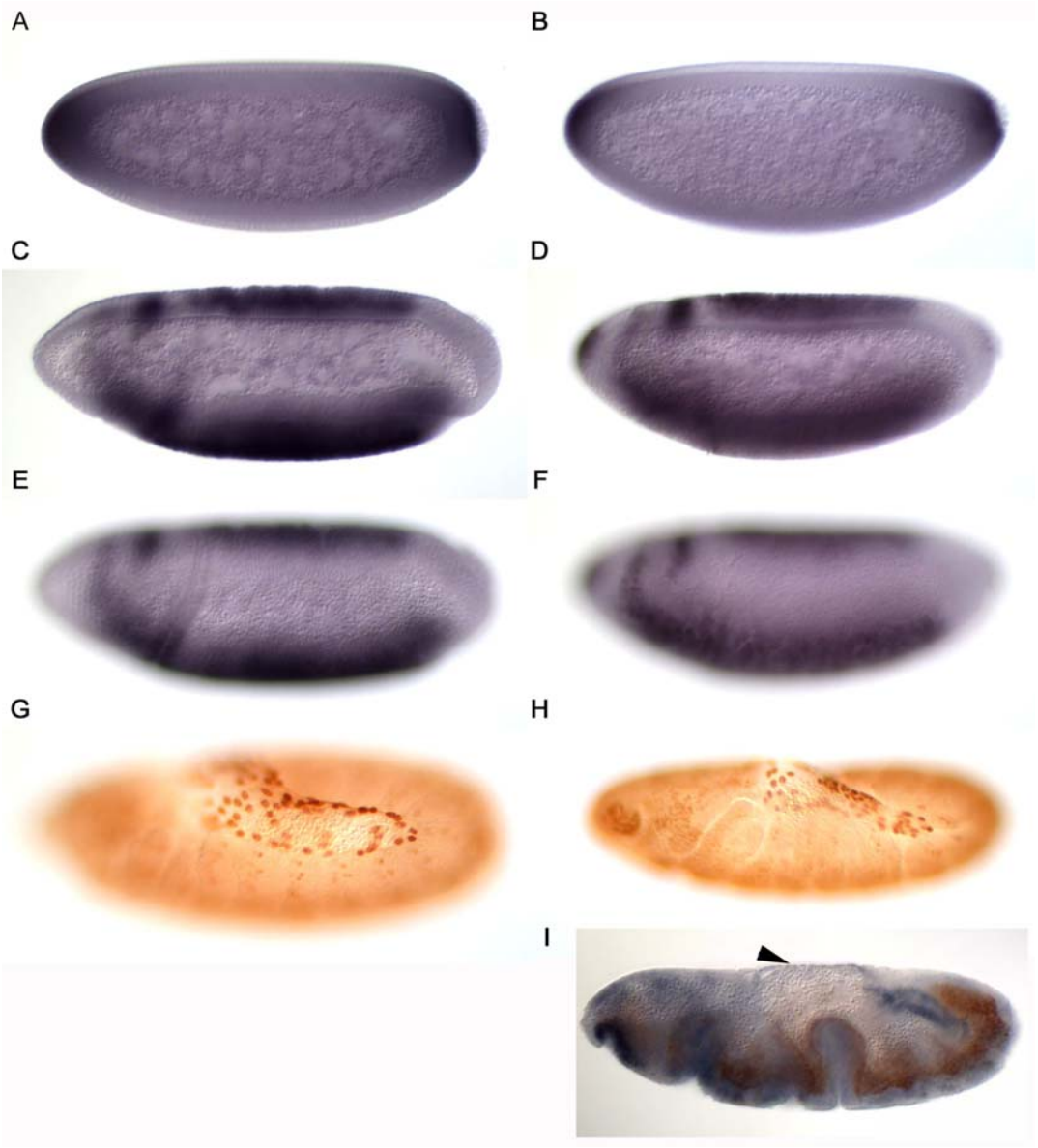

Fig.2-34. The dorsal-ventral patterning appears to be normal in mutant embryos. A-F are anti-activated MAPK staining of wild type embryos (A, C, E) and *X-144* germline clone lethal allele (B, D, F). A and B are blastoderm embryos, while C, E and D, F are in early gastrulation stage. C and E, D and F are pictures from different focal plans of same embryos. G (wild type) and H (germline clone lethal allele) are anti-*krüppel* staining of stage 12 embryos. I shows the extreme case of the viable mutant embryo double stained with anti-Twist (brown) and anti-Krüppel (blue) antibody. The arrowhead in I indicates a layer of amnioserosa cells.

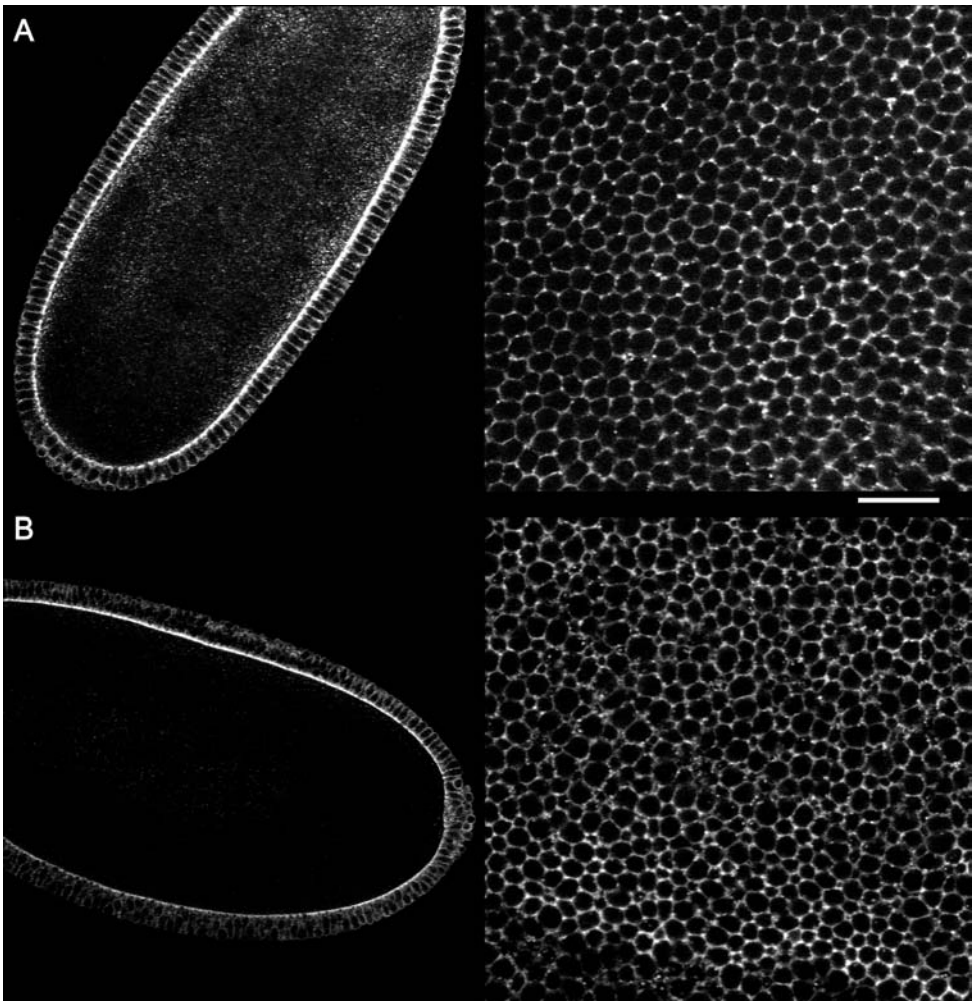

Fig.2-35 The actin staining of embryos at the end of cellularization. A, wild type; B, *X-144* mutant. Scale Bar for left panel is 50μm, for right panel is 20μm.

To gain a detailed look at the cellularization process, I stained embryos of homozygous viable allele with anti-phosphotyrosine, and propidium iodide to visualize the nuclei. In wild type embryos, a single layer of nuclei are formed and maintained during cellularization (Fig.2-36). Detailed examination of the mutant embryos shows that at the beginning of cellularization, some nuclei are not directly under the cell cortex (Fig.2-37A); as cellularization progresses, the multi-layers of nuclei become obvious, and some nuclei fall into the center of the embryos (Fig.2-37B, C, D&E). Multi-layers of cells are formed by the end of cellularization (Fig.2-37F).

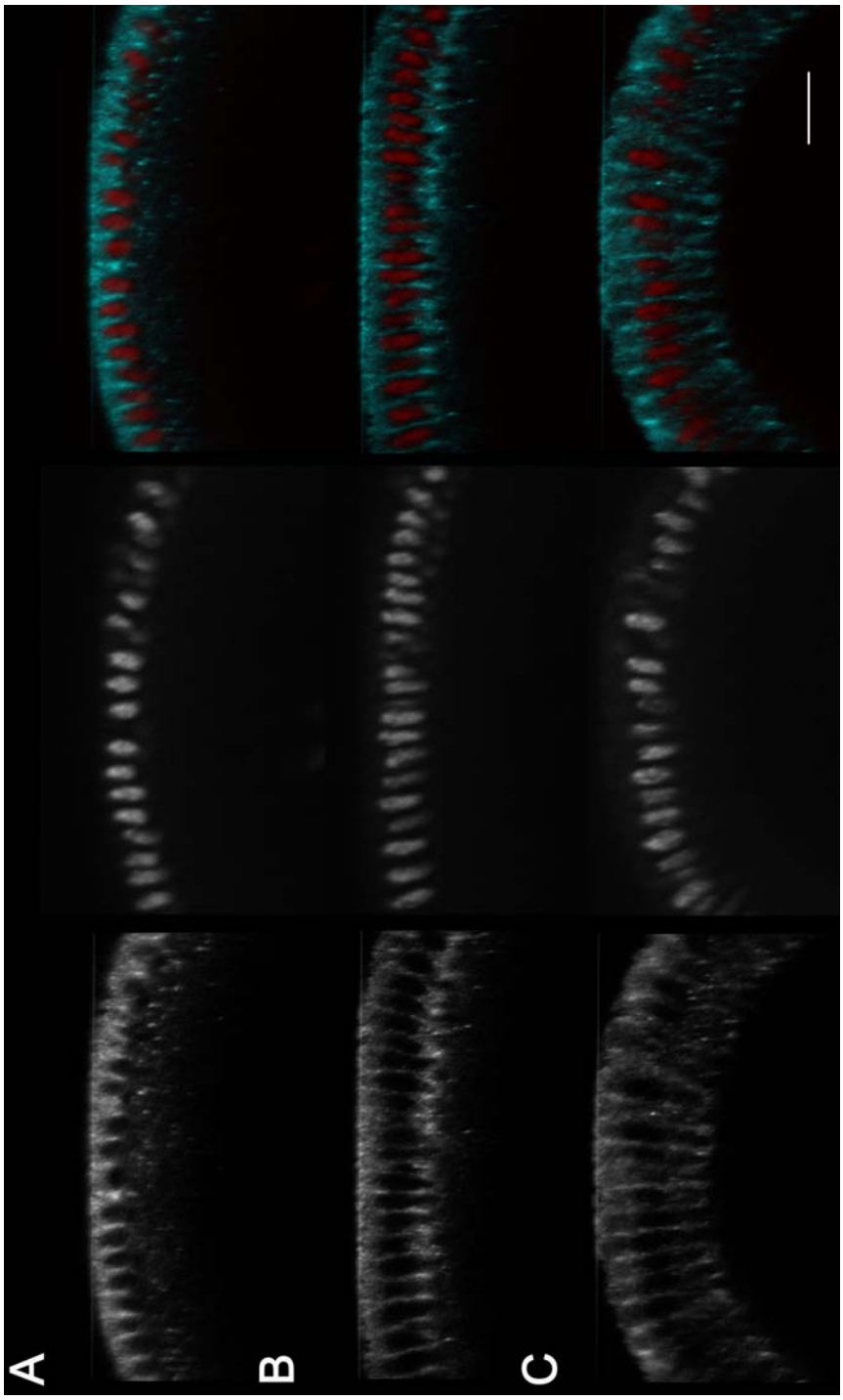

Fig.2-36. The progression of the cellularization of wild type embryos. From Left to right, phosphotyrosine staining, nuclear staining by propidium iodide, and merge of the two stainings. Scale Bar, 20μm.

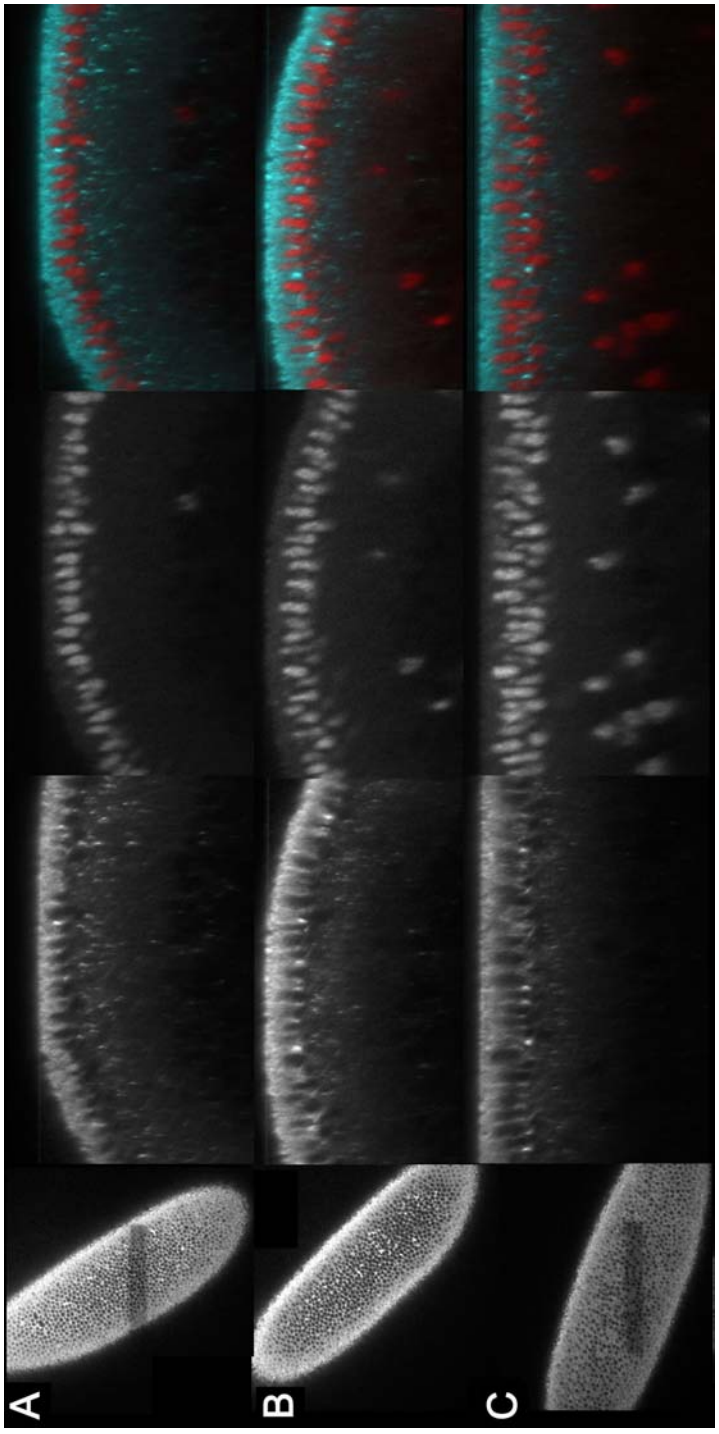

Fig 2-37

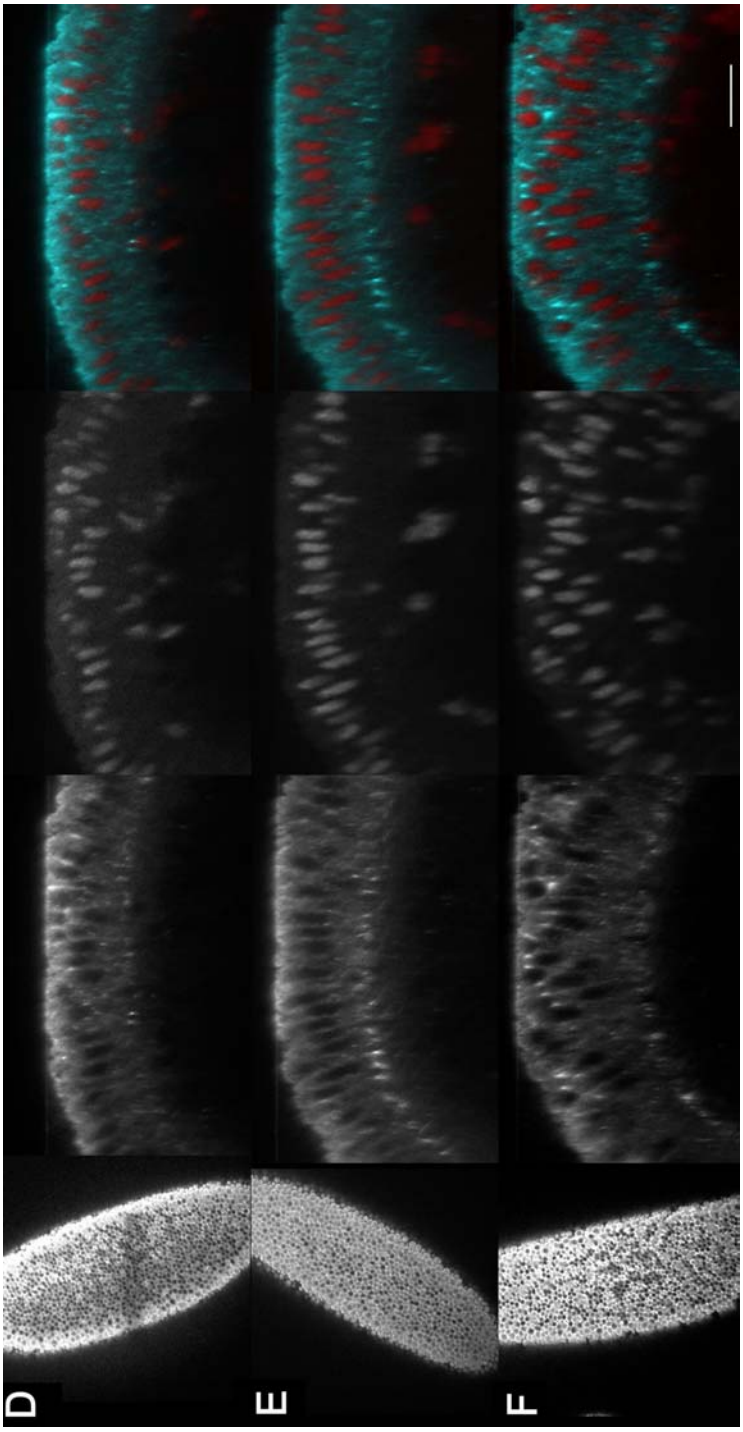

Fig.2-37 The cellularization defects of *X-144* mutant embryos. From left to right, whole mount embryos; phosphotyrosine staining; propidium iodide staining for nuclei, and merge of second and third columns. Scale bar for second, third and fourth columns, 20μm

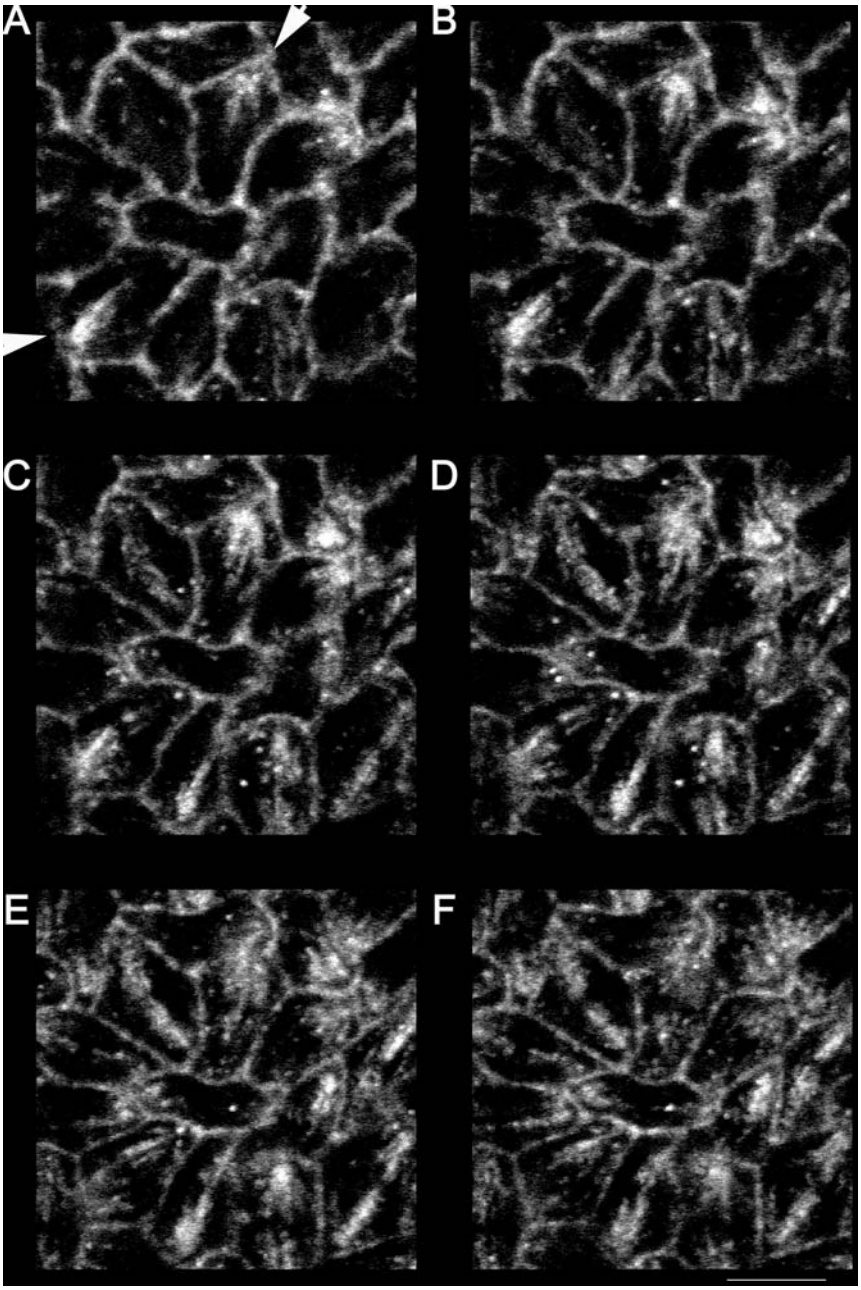

Fig.2-38. Series of continuous sections through an anaphase *X-144* mutant embryo. Different spindles from different cells or within same cells are in different focal plane. Embryos were stained by anti-phosphotyrosine and anti-tubulin antibody. Two sections next to each other have 244nm distance between them. Two cells with defective spindle anchors are indicated by arrows in A. The invisible spindles from these two cells appear gradually in the sequential sections from B-F. Scale Bar: 2μm

It seems that at the beginning of cellularization, there have already been problems in the proper localization of nuclei. Therefore, I examined the cell division during blastoderm stage. Anti-tubulin and anti-phosphotyrosine double staining of the mutant embryos in anaphase of cell cycle 13 shows some of the spindles are falling off from the cell cortex (Fig.2-38, arrows), which is the case for both alleles. This may be the reason why some of the nuclei are no longer directly under the cell cortex at the beginning of cellularization.

3. Discussion

In a previous study, Dossenbach *et al* swapped the intracellular domains of Htl and Btl with Torso and EGFR, and assayed the function of these chimerical proteins by rescuing the FGFRs and/or dof mutant phenotype in mesodermal spreading and tracheal branching in *Drosophila melanogaster*. They concluded that RTKs generate a generic signal that is interpreted in responding cells according to their developmental history (Dossenbach et al., 2001). However, this cannot explain why various RTKs are evolutionarily involved in the first place and why different RTKs are required in distinct *in vivo* situations. As they also indicated in their discussion, the results of the rescue by chimerical proteins are not as good as by the endogenous receptors. Therefore, there are possibly some fine tunings of distinct RTK signals *in vivo*. In this thesis, I presented a forward genetic screen to identify components that are involved in one of the RTK signals - the FGF signal, and thereby provide an in sight into how the FGF signal is regulated *in vivo*.

The screen searched for genes that could modify the phenotype caused by an ectopic FGF signal in the eye in a gain-of-function (GOF) situation. This ectopic FGF signal is able to induce a rough eye phenotype, which implies that the ectopic signal transduced in the eye is likely to utilize the existing downstream components common to all RTK signal pathways. Two examples are the overlapping candidates of my screen and the screen done by Huang *et al*. It is generally believed that the activation of various RTKs transduce the signals to common downstream events. In many cases, it has been shown that RTK signals are functionally replaceable. For instance, in PC12 cells, a long lasting activation of MAPK by NGF induces neuronal differentiation, while EGF induces a much shorter MAPK activation leads to proliferation. Experimental manipulations that lengthen the response to EGF signalling can also induce neuronal differentiation (Marshall, 1995). Activated EGF signal could replace the requirement of Sev signal for R7 differentiation (Freeman, 1996). The ectopic FGF signal in the eye could in principle mimic other signals. Therefore, many of the candidates found in this screen could be common modulators of various RTK signals.

However, in the GOF screen, many genes were ectopically expressed in the Drosophila eye, which offers me a chance to identify some candidates that may be more specific for the FGF signal. This is the advantage that a loss-of-function screen does not have. The disadvantage is that in a GOF screen, it is also possible to identify genes that interfere with downstream pathways while not having a role in the endogenous FGF signal because they are not present at the time for the signal transduction. Therefore the best way to find out if the gene is involved in FGF signalling *in vivo* would be to study the loss of function phenotype. However, if the gene is also involved in other signalling cascades, the possible function of the gene in the FGF signal may not be discovered easily in this assay. In this case, the candidates that can be demonstrated to be involved in the FGF signal are either not required before the FGF signal is required during the Drosophila embryonic

development, or only function in the FGF signal cascade. There is so far no example of downstream components that are demonstrated to be involved only in the FGF signal in vertebrates. In Drosophila, there is only Dof that has not been found to be required in other RTK signals to date. There is no Dof homologue found in mammals till now. The only genes that have weak similarity to Dof, BCAP (B cell antigen receptor) and BANK (B-cell scaffold protein with ankyrin repeats), are involved in activating B-cells during immune response in mammals (Okada et al., 2000; Yamazaki et al., 2002; Yokoyama et al., 2002). Therefore, it is possible that Dof is exclusively required in the FGF signal in Drosophila, which leads to the question-why there are any other components required to ensure the specificity of the FGF signal in addition. Although I have only analyzed three candidates in detail, the results implied that most, if not all of, the candidates are not only involved in the FGF signal in my screen.

3.1 The screen is likely to have some specificity for FGF signalling

There is no easy way to determine if this screen is specific for the FGF signal. In the tests of potential candidate genes, the results that null mutants of cyclinA and notch had no effects on 'GMR>λ-btl, dof' eye phenotype imply that the screen has some specificity. RTK signals, especially signals from EGF and sev, are indispensable during the Drosophila eye development. Therefore, one outcome of such a screen could be that the most of the candidates are common for RTK signals. The mutations in two critical downstream components of EGFR/Sev signals in the Drosophila eye, Sos and Ras85D, can weaken the threshold Sev signalling and disrupt the otherwise normal R7 determination (Simon et al., 1991). However, neither *sos* nor *ras85D* mutants could modify the 'GMR>λ-btl, dof' eye phenotype. Only mutants for *raf*, a known downstream component of EGFR/Sev signalling, affected the ectopic FGF signal. Therefore, it is likely that the critical components for the endogenous EGFR/Sev signals are different from that of the ectopic FGF signal.

One approach to estimate the specificity of the screen is to compare the results with other screens. Abdelilah-Seyfried et al. screened for genes involved in extra sensory organ differentiation, while Pena-Rangel et al. screened for genes involved in dorsal thorax formation. Potentially, both screens could find genes that interfere with neuronal cell differentiation. 7/105 of the candidates in the screen performed by Abdelilah-Seyfried et al. and 5/190 of the candidates in the screen performed by Pena-Rangel et al. overlap with mine. There are 36 overlapping genes between these two screens, while only 4 genes in total found overlap with the ones found in the screen described here. The small amount of overlap with these screens suggests that the ectopic FGF signal in the Drosophila eye did not simply disrupt the differentiation programme of the photoreceptor cells.

In the screen performed by Huang et al., 13 lines that represent 12 genes were found, which specifically interact with Ras85D pathway during photoreceptor cell differentiation.

Two of these genes were also identified in my screen. One is the gene *CG11172* that has been identified in almost all GOF screens published. Another gene that overlaps between the screens is *mesr4* that was not found by other groups. This gene encodes a zinc-finger protein, which is possibly involved in FGF induced MAPK signalling pathway.

3.2 Many candidates are possibly involved in various receptor tyrosine kinase signals

Many of the candidates also interact genetically with the ectopic PDGF and/or EGF signals in the Drosophila eye. 13 of the 50 EP candidates interact with the EGF signal, among which, only one candidate, namely EP1135 which is a suppressor for the ectopic FGF signal, functions as an enhancer in the ectopic EGF signalling pathway. The other overlapping modifiers either suppress or enhance both signalling pathways. Consistent with what I have argued before, the ectopic FGF signal does not seem to share many downstream components with the EGF signal.

Only 9 of the candidates do not interact with the PDGF signal. The endogenous PDGF signal is not known to have a function during Drosophila eye development. The results may imply that the ectopic FGF signalling pathway share many common downstream events with the PDGF signalling pathway in the Drosophila eye, and therefore could be modified by similar candidates. However, 12 candidates show contradictory effects with respect to the ectopic PDGF signal and the ectopic FGF signal. Among them, EP1342, EP1453, EP2034, EP1222, EP2319, EP0436, EP2310, EP0712 and EP2440 are enhancers for the ectopic FGF signal but suppressors for the ectopic PDGF signal. These 9 candidates represent 4 different genes, namely *ches-1-like*, *CG10443, CG10321,* and *CG10082*. CG10443 has no known homologues, CG10082 is a kinase and the remaining two are transcription factors and/or regulators. The remaining three candidates, EP0355, EP1135, and EP0595 are suppressors for the ectopic FGF signal but enhancers for the ectopic PDGF signal. They represent *dsp1, ago*, and *CG6765*, two of which, *dsp1* and *CG6765* encode transcription factors and/or regulators, while Ago is an E3 ligase for protein degradation. The 12 candidates that show contradictory effects together with the 9 candidates that do not interact with the PDGF signal, in total these 21 candidates represent 13 annotated genes, which also distinguishes the ectopic FGF signal from the ectopic PDGF signal.

To summarize, different sets of candidates, although some are overlapping, found in the screen could modulate the ectopic FGF, EGF or PDGF signals in the eye, which implies that at least these three RTK signals are regulated differently.

3.3 Various cellular processes may regulate FGF signalling

A signal transduced from a membrane receptor such as FGFR could in principle be regulated at various levels. The secretion of the receptors is tightly regulated. On the membrane, there are membrane rafts that could regulate both the efficiency and specificity of the signal transduction. In most of the cases, if not all, the signal transduction requires kinases. In most levels of signal transduction, cytoskeletal proteins are involved.

Consistent with the expectation, genes involved in many cellular processes were identified in the screen. The candidates range from kinases, cytoskeleton related proteins, proteins involved in vesicle transport, membrane proteins, mitochondrial protein and proteins interact with DNA or RNA.

Two membrane proteins were found in the screen, Tsp42Ef and Robo2. Tsp42Ef has been found in the membrane rafts, which may have a role in regulating the FGF signal in the membrane. Robo2 is involved in the axon guidance. It is possible that the suppression phenotype caused by Robo2 is due to some antagonistic signals generated by overexpression of Robo2. Recent findings show that both Robo and Robo2 are expressed in different tracheal branches and mediate repellent and attractant signals for the branch growth (Englund et al., 2002). A novel Dbl family RhoGEF, GEF64C, that functions upstream of RhoA, was found to interact genetically with Robo signalling in CNS (Bashaw et al., 2001). As I will discuss later, RhoA is likely to be an important component in FGF signalling. The genetic interaction in the eye may imply that the two signalling pathways work coordinately in tracheal branching. Gene products like Dlc90F and Sar1 are probably involved in cytoskeletal organization and/or protein/RNA trafficking, and therefore they may have a role in transporting receptors or other signal molecules.

Both *btl* and *dof* are also expressed transiently in the midgut anlage during the midgut formation although the significance of this expression pattern is unknown. The transient expression pattern of *tim10* in the midgut is similar to that of *btl* or *dof*, which implies that it may have a role in the FGF signal. It is not clear how Tim10 could influence FGF signalling. One possibility is that the ectopic FGF signal has a role in apoptosis. Mitochondria are involved in apoptosis. Mammalian Raf-1 has been found to localize to mitochondria, which is important for inhibiting Bad-dependant apoptosis. Interestingly, this function of Raf is ERK independent (Neshat et al., 2000). The mutant of *raf* enhances the 'GMR>λ-btl, dof' eye phenotype, while mutants for *dsor1* and *rl* have no effects. Therefore one explanation could be that the function of Raf in mitochondria is involved in the FGF signal.

3.4 The involvement of the Ras85D-MAPK pathway in FGF signal transduction

When the constitutively active form of Ras85D is expressed by Btl-Gal4 in the embryonic tracheal system, the *dof* mutant phenotype can be partially rescued. Rl is activated in the leading cells during mesoderm migration, and this activation is abolished in *dof* mutant embryos (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). These results imply that the FGF signal is transduced by Ras85D to the classical MAPK pathway, that is to Raf and then to Dsorl and Rl. However, the Ras85D mutant did not modify the 'GMR>λ-btl, dof' eye phenotype. One possible explanation could be that the Ras signal is just one of the downstream pathways and the reduction of the Ras signal could be compensated by other signalling pathways downstream of the FGF signal. However, when the dose of Raf is reduced by half, 'GMR>λ-btl, dof' eye phenotype was enhanced. If only Ras85D activates Raf, it is illogical that the eye phenotype is more sensitive to the dose change of Raf than Ras85D. It has been shown *in vivo* that Drosophila Raf can be activated in the *ras85D*, *sos* or *grb2* mutant backgrounds to transduce the Torso signal (Hou et al., 1995), which implies that it is possible to activate Raf without Ras85D. Therefore, it is possible that Raf is one of the critical downstream components of the FGF signal but Ras85D may not. In the Ras85D rescue experiment, activated Ras85D probably activates Raf, which leads to the activation of Rl, as a consequence partially rescuing the *dof* mutant phenotype. However, like Ras85D, neither *dsor1* nor *rl* mutants enhance the 'GMR>λ-btl, dof' eye phenotype. To see whether this is a phenomenon in general in these types of screens, I have compared the results of my LOF test with other LOF screens.

Table.3-1 Summary of genetic interactions of different components in Ras85D-MAPK pathway

Mutants for:	[1]Modification of sev-ras^{V12} phenotype	[2]Modification of sev-yan^{act} phenotype	[3]Modification of sev-KDN phenotype	Modification of 'GMR>λ-btl, dof' eye phenotype
drk				
sos		E	E	
ksr	S		E	
14-3-3ζ			E	
Ras85D			E	
raf	S			E
dsor1	S		E	
rolled	S	E	E	

1: Karim et al. 2: Rebay et al. 3: Therrien et al. E: enhancement, S: suppression

Three saturating EMS screens for components of the Ras85D-MAPK pathway have found known genes in the cascade (Table3-1) as well as previously unknown components of the pathway (Karim et al., 1996; Rebay et al., 2000; Therrien et al., 2000). In the screen for modifiers of the Sev-ras1^{V12}, Karim et al. found that mutants for *ksr, raf, dsor1*, and *rl* suppress the eye phenotype, while mutants for *drk, sos, 14-3-3ζ*, and *ras85D* had no effect. Yan is a transcription repressor downstream of MAPK. In the screen performed by Rebay et al. for modifiers of *sev-yan^{act}* phenotype, mutants for *sos* and *rl* were found to

enhance the eye phenotype. Kinase Suppressor of Ras (KSR) is a scaffold protein required for the proper signal transduction from Ras to Raf (Roy et al., 2002). In the screen done by Rubin et al. for modifiers of the *sev-KND* (dominant negative form of KSR) phenotype, only mutants for *drk* and *raf* had no effect on the phenotype. Mutants for other genes such as *sos, ksr*, *14-3-3ζ, ras85D, dsor1*, and *rl* enhanced the sev-KND signal. Ras, Yan and Ksr are involved in the Ras-MAPK pathway. Therefore, these three screens were designed for critical components or regulators of the Ras-MAPK pathway. In my test, only mutants for Raf among all the genes mentioned here give an enhancement phenotype, which implied that the additional FGF signal in the Drosophila eye development did not interact extensively with the endogenous Ras85D-MAPK pathway. Ras85D may not be the critical component for the transduction of the FGF signal.

The results with the three components of the MAPK signalling cassette, namely Raf, *Dsor*1 and Rl suggested that, depending on the assay system, not all of the three genes have been identified in all the screens. Therefore, it is not an exception that only *raf* mutants among the components of the MAPK signalling cassette affecting the 'GMR>λ-btl, dof' phenotype. Given that Raf is on the top of the MAPK signal hierarchy, it is possible that 'GMR>λ-btl, dof' eye phenotype is more sensitive to the dose changes of *raf* rather than the two other downstream genes. It is likely that MAPK pathway is critical for the FGF signalling pathway, but Ras85D may not be as important.

3.5 The effects of epithelial planar polarity signal

Introducing an ectopic FGF signal into the Drosophila eye by GMR-Gal4 results in a planar polarity defect, which suggests that the FGF signal may be interfering with the planar polarity signal. The mutants of two known planar polarity genes, *fmi* and *rhoA* enhance the 'GMR>λ-btl, dof' eye phenotype. Fmi is a seven-pass transmembrane cadherin that was first found to regulate the planar cell polarity (PCP) under the control of Fz (Usui et al., 1999). In addition, mutants of *fmi* have been identified to affect embryonic dendrite growth, which is independent of Fz (Gao et al., 2000). Therefore, Fmi may have a function in signals other than the PCP signal. Besides its role in planar cell polarity during Drosophila eye development (Strutt et al., 1997), RhoA is required in many other processes, such as cell shape changes during dorsal closure (Lu et al., 1999). It has been shown that overexpression of UAS-RhoA^{N17} by Nos-Gal4 affects mesoderm formation (Barrett et al., 1997), and by Btl-Gal4 shows defects in the formation of the dorsal trunk of the trachea (Lee et al., 2002). As listed above, both Fmi and RhoA have a role in cell migration or cell shape change and therefore, they might have potential roles during the FGF signal induced cell migration.

The mutant for one of the RhoA activators, RhoGEF2 also enhanced the ectopic FGF signal in the eye. This result supports the idea that RhoA plays a role in the FGF signalling pathway. However, because the germline clones of RhoGEF2 mutants show a defect in

ventral furrow invagination (Barrett et al., 1997), it is impossible to assess the function of RhoGEF2 during mesoderm spreading. Therefore, whether this particular RhoGEF has a role in FGF signalling *in vivo* remains to be tested. There are 8 annotated RhoGEFs in the Drosophila genome. It is possible that other RhoGEFs activate RhoA in the FGF signal if RhoA is indeed involved. Recently, it was found that one of the RhoGEFs, Pbl (pebble), is important for mesoderm cell migration (Mueller, H.-A. J., personal communication).

3.6 Detailed analysis of some of the candidate genes

Analysis of a cytoskeleton related protein-CG3542

From sequence homology, gene CG3542 encodes a protein that is likely to be involved in RNA splicing (Mount et al., 2000). This is the only gene so far found in the Drosophila genome that is predicted to be a homologue of the yeast gene Prp40 and the mammalian gene FBP11. However, there is no similarity among the C-termini of the three proteins. In addition to the conserved FF domain and WW domains, there are 3 more recognizable FF domains in Prp40, which are not conserved in CG3542. One important amino acid of the Prp40 protein is Ser240, which is located in the second FF domain of Prp40. This amino acid is not conserved between the human and Drosophila proteins. When this Ser is changed to Phe, it can suppress the splicing defect of sar1 gene in the U1 RNA mutant (U1-4U) in yeast. It is likely that yeast Prp40 is involved directly in the splicing of sar1 gene (Kao et al., 1996). However, the function of CG3542 is unlikely to relate to sar1 based on two observations. One is that it is impossible to generate *sar1* mutant clones in wing and leg disks while small clones in null alleles of CG3542 can be generated in wings. Secondly, the disruption of the gene *CG3542* enhances the 'GMR>λ-btl, dof' eye phenotype while the overexpression of *sar1* leads to the enhancement of the eye phenotype. My results suggest that CG3542 is important for the survival of only some cell types, while Prp40 in yeast is an essential gene for survival (Kao et al., 1996). Therefore, CG3542 may not be the Drosophila homologue of Prp40.

The germline clones of the homozygous embryos of C-terminal truncated protein mutant can develop till the end of embryonic development, implying that the predicted protein has multiple functions during Drosophila development. The germline clones of the null mutants do not yield any egg, therefore it is difficult to assess the possible involvement of the component in the FGF signalling pathway during embryonic development.

Analysis of a protein involved in the secretion pathway-Sar1

It was impossible to generate *sar1* null clones in wing and leg disks, which suggests that *sar1* may be essential for cell survival. However, I could generate *sar1* germline clones. It is possible that the *OvoD* transgene is not fully penetrant, and therefore the eggs that were laid might not be germline clones. However, it is unlikely to be the case for the following reasons. First, the eggs that I obtained lacking dorsal appendage were not rare cases. Most of the eggs laid have this phenotype. Secondly, according to literature, although the eggs

occasionally laid by *OvoD* transgenic mother have defects on dorsal appendage, there is no report about having eggs without any sign of dorsal appendage (Chou et al., 1993). Thirdly, both *sar1* and *CG6386* are located on the third chromosome left arm. Germline clones were generated for the deletions of both genes at the same time with the same *OvoD* stock. None of the eggs laid in the experiment with *CG6386* mutant had a similar phenotype. Therefore, the germline clones of the *sar1* mutants are likely to be the ones that maintained residual amounts of mRNA/protein generated before the mitotic recombination, which sustained further development of the germline cells. If this is the case, the phenotype I observed is potentially interesting. The generation of dorsal appendage is the indication of dorsoventral patterning of the follicle cells. This patterning event is the consequence of localized Gurken/EGFR signalling (Roth et al., 1999). The lack of the dorsal appendage may indicate the failure of the Gurken/EGFR signal during oocyte development. Therefore lack of dorsal appendage in *sar1* germline clones may imply that *sar1* is required specifically for the EGF signal during oogenesis in addition to its other functions. Sar1 is one of the 4 candidate proteins that do not interact with the PDGF and EGF signalling pathway during the Drosophila eye development. These may imply that either the ectopic EGF and PDGF signals are not sensitive to the change of Sar1 amounts in the Drosophila eye, or that Sar1 is not required for both signals in the eye. However, it is still possible that Sar1 has a function in the FGF signalling pathway.

Analysis of a predicted kinase-CG6386

CG6386 contains a highly conserved serine/threonine kinase domain. It is homologous to the mammalian vaccinia-related kinase. This mammalian kinase is able to bind to and phosphorylate p53 in an *in vitro* assay (Lopez-Borges et al., 2000). P53 is involved in cell differentiation, and apoptosis. It is possible that p53 also has a role in the FGF signal, which is regulated by CG6386. Drosophila homologue of p53 was identified recently (Brodsky et al., 2000; Jin et al., 2000b; Ollmann et al., 2000). P53 is highly expressed in the nurse cells and oocyte of the stage 10 egg chamber. It is also expressed in blastoderm embryos which is probably due to maternal contribution, in mesoderm and posterior mid gut at stage 10 embryos, and primordial germ cells within the two gonads at stage 16 (Ollmann et al., 2000). This expression pattern implies that p53 may have a role during oogenesis and embryonic development. In my results, eggs of the *CG6386* mutant germline clones could not be generated. There is also high maternal contribution of the *CG6386* mRNA in the early embryos. These observations suggest a possible relation between p53 and CG6386. However, more experiments are required to find out if CG6386 interacts with the p53 pathway and if this interaction has a function in the FGF signal.

Potential approaches to characterize the possible roles of CG3542, Sar1 and CG6386 in FGF signalling

All these three genes appear to be required during oogenesis. Therefore it is not clear whether the three genes that I have studied in more detail are involved in the FGF signal or not. Further experiments are required to address this question. One method would be to generate clones in trachea and examine the behavior of mutant cells during larval development, and compare these to the dof mutant cells. Indirectly, I could also generate

antibodies against the protein products and examine the subcellular localization of the protein, or generate tagged version of the genes and study their localization in an overexpression situation in flies or cultured cells. In the case of Sar1, one would be able to study if Sar1 colocalizes with FGFR or Dof, or co-immuno-precipitates with FGFR or dof. In the case of CG6386, co-immuno-precipitation or yeast two-hybrid could be performed to test if CG6386 interacts with p53. Kinase assay could be used to test if CG6386 could phosphorylate Drosophila p53. If p53 is found to be the substrate of the kinase, the activation or inhibition of p53 could be an indicative readout as to whether the FGF signal activates the CG6386.

Characterisation of the mutant *X-144* that is close to EP1340

The mutant *X-144* was first mapped to the region downstream of the EP1340 insertion. Line EP1340 was a suppressor found in the screen. Therefore I started to work on two aspects on the mutant, one to find out the gene whose mutation is responsible for the phenotype and the other the characterization of the phenotype. So far I only managed to map the mutation to a region containing 15 candidates. The phenotype of the mutant includes spindle anchor defects at the end of cell cycle 13, cellularization defects, and later on gastrulation defects. The first two phenotypes can be explained by same reason that is there are defects of microtubule anchoring of nuclei. In blastoderm embryos where myosin VI was prevented from binding to actin by injecting anti-myosin VI antibody, spindle anchor defects during syncytial division was observed (Mermall et al., 1995). This myosin is proposed to have a function in particle transportation for membrane furrow formation during blastoderm stage. Among the 15 candidates within the region, there is a ser/thr phosphatase, which could potentially inactivate myosin and is a good candidate for the gene. It is hard to say whether there is no other earlier defect at the moment. I have generated lines of transgenic flies carrying histone-GFP or tao-GFP in *X-144* mutant background, and hope that I could find out if there is any earlier defect by following the behavior of nuclei/microtubule during early stages of embryonic development.

3.7 Conclusions

The work presented in this thesis was aimed at identifying molecules involved in the FGF signal based on the modification of a rough eye phenotype caused by an ectopic FGF signal. The candidates identified in the screen are involved in many cellular processes, which implies that the screen was successful in finding molecules that could modulate the FGF (or RTK) signalling at different levels. However, it is difficult to determine if the modulators are specific for the FGF signal that is if they function endogenously in the FGF signalling pathway or not.

4. Materials and methods

4.1 Reagents

Tween20, tRNA, RNase and amino acids were purchased from Sigma. Acelylated Bovine Serum Albumin (BSA), 1KB ladder, restriction enzymes and their buffers were supplied by New England Biolabs. Herring sperm DNA (hsDNA), Nitroblue tetrazolium (NBT), 5'-bromo-4-chloro-indoxylphosphate (BCIP), unlabeled nucleotides, Hexanucleotide Mix, DIG-DNA labeling mix, anti-DIG antibody, Proteinase K and Expand Enzyme Mix were from Roche Diagnostics GmbH. Heparin was bought from Serva. Unless otherwise mentioned, all the other chemicals were from Merck, Sigma or Roth.

4.2 Fly genetics

Drosophila stocks
2300 EP collections are from Szeged Drosophila stock center (http://gen.bio.u-szeged.hu/servlet/jate.genetics.servlet.EPlines) with 165 lines missing. The missing lines are EP3034, EP3040, EP3053, EP3059, EP3076, EP3082, EP3106, EP3134, EP3122, EP3137, EP3166, EP3178, EP3193, EP3195, EP3198, EP3203, EP3204, EP3206, EP3220, EP3248, EP3249, EP3265, EP3279, EP3281, EP3294, EP3336, EP3358, EP3425, EP3509, EP3569, EP3601, EP3605, EP3627, EP3637, EP3643, EP3651, EP3661, EP3665, EP3712, EP3713, EP3717, EP3725, EP2004, EP2028, EP2089, EP2090, EP2100, EP2119, EP2120, EP2157, EP2158, EP2159, EP2166, EP2191, EP2276, EP2340, EP2357, EP2380, EP2401, EP2435, EP2476, EP2482, EP2483, EP2493, EP2497, EP2500, EP2507, EP2508, EP2527, EP2533, EP2539, EP2541, EP2542, EP2546, EP2550. EP2551, EP2558, EP2560, EP2566, EP2575, EP2576, EP2588, EP2596, EP2619, EP2629, EP2642, EP2646, EP0365, EP0376, EP0377, EP0378, EP0381, EP0387, EP0396, EP0408, EP0410, EP0417, EP0446, EP0457, EP0508, EP0513, EP0520, EP0531, EP0536, EP0538, EP0556, EP0593, EP0609, EP0611, EP0618, EP0620, EP0667, EP0674, EP0675, EP0691, EP0693, EP0703, EP0756, EP0760, EP0764, EP0766, EP0767, EP0768, EP0770, EP0772, EP0773, EP0779, EP0783, EP0784, EP0787, EP0790, EP0804, EP0805, EP0809, EP0844, EP0847, EP0875, EP0883, EP0885, EP0888, Ep0920, EP0927, EP0934, EP0937, EP0966, EP0968, EP0969, EP1001, EP1036, EP1076, EP1082, EP1105, EP1119, EP1121, EP1132, EP1144, EP1146, EP1155, EP1161, EP1171, EP1173, EP1196, EP1215, EP1226, EP1227.

Other stocks used in the studies:

Fly stocks	**Source**
P[w^{+} GMR-Gal4] P[w^{+} UAS-λ-btl] P[w^{+} UAS-dof] / CyO	R. Wilson
w; P[w^{+} twiGal4] on second chromosome	
P[w^{+} GMR-Gal4 #12] drk ^{e0A}	F. Sprenger
If / CyO; C8 / TM3 ^{UbxLacZ}	
bco / CyO; P[w^{+} UAS-λ-torpedo]	S. Roth
Df(1)ct-J4 In(1)dl^{49} f/C(1)DX w cv; Dp(1;3)sn^{13A}	
Df(1)HA32/FM7	
y w Df(1)Sxl-ra sn^{3}/y^{+} ct^{+} sxl^{+}	
Df(1)RF19/FM7, y^{31d} sc^{8} w^{a} sn^{X2} v^{Of} g^{4} dn^{1} B^{1}	
y w Dsor1 ^{LH110} FRT / FM7a	Bloomington stock center
14-3-3ζ^{P07103} cn / CyO; ry	
w^{-}; dos^{P115}FRT 2A / TM3	
dock ^{P04723} cn ^{1} / CyO; ry^{506}	
y^{1} w^{67} c^{23}; dock ^{P13421} / CyO	
w^{1118}; P[ry^{+} neoFRT]82B P[w^{+}]87E	
w^{1118}; P[w^{+}]36F P[ry^{+} neoFRT]40A	
P[ry^{+} hsFLP]12, y^{1} w; noc^{Sco}/CyO*	
P[w^{+} ovoD$^{1-18}$]2La P[w^{+} ovoD$^{1-18}$]2Lb P[ry^{+} neoFRT]40A / Dp(?;2)bw^{D},S^{1} wg^{Sp-1} Ms(2)M^{1} bw^{D} / CyO	
w; P[ry^{+} neoFRT]82B P[w^{+} ovoD$^{1-18}$]3R/st^{1} betaTub85D^{D} ss^{1} e^{s}/TM3, Sb^{1}*	
w^{1118}	
w^{-}; al^{1} dp^{ov1} b^{1} pr^{1} Bl^{1} c^{1} px^{1} sp^{1}/SM1	
w; TM3, Sb^{1} Ser^{1}/TM6B, Tb^{1}*	
w^{-}; P[w^{+} UAS-cdc42^{L89}]	
w^{-}; P[w^{+} UAS-Rac^{N17}]	
Df(1)Sxl-bt, y^{1}/Binsinscy	
w ovoD^{1} v24 P[w^{+mW} FRT^{whs}]101/C(1)DX*	
y^{1} f^{1}/Y;P[ry$^{+t7.2=hs}$FLP]38	
Df(1)ct4b^{1} oc^{1} ptg^{1}; Dp(1)ct^{J8}/Ubx;C(1)DX, y^{1}*	
y^{1} w; CyO, H[w^{+} Δ2-3] / Bc1 Egfr^{E1}*	
y w; P[w^{+} btlGal4] on second chromosome	M. Affolter.
Df(1) N ^{81K} / FM6 ; UAS FLP / TM2	T. Klein
P[ry^{+} hsFLP]; 2XP[w^{+} GFP] P[ry^{+} neoFRT]40A / SM6a-TM6b	
P[Ubi-GFP] P[w^{+} FRT]101	
w^{-}; P[ry^{+} neoFRT]82B P[w^{+} GFP^{3-13-7}]	
sev ^{d2} ; Ras1 ^{e2f} / TM3	M. Mlodzik
raf ^{EA75} / FM7	
raf ^{C110} / FM7	
Df(2R)rl ^{10a} / CyO	
rl ^{EMS698} / SM1	
y csw$^{EsevIA-eOP}$ sev^{d2} / FM7	
w; drk $^{24/1}$ / CyO	
ksr ^{s72I} / TM3,Sb e ry sev-Ras^{V12}	
Gap1$^{1-16}$ / TM3 ry	
sev ^{d2};sos ^{e4G} / CyO	
dsh^{1}	
y w; fmi ^{E59} / CyO (y^{+})	
In(3LR)fz / TM1	
Me ri sbd^{1}	
FRT RhoA ^{R2} / CyO	M. Narasimha
FRT DRhoGEF / CyO	K. Barrett
X-144^{1}/FM7 (viable)	C. Nuesslein-volhard
X-144^{2}/FM7 (lethal)	

Genetic crosses

To make imprecise excisions, EP0719, EP3575 and EP0863 were crossed to *y[1] w*; CyO, H [w[+] Δ2-3] /Bc1 Egfr[E1]*. In F1 generation, the flies with EP insertion and Δ2-3 (on CyO balancer) were crossed to proper balancer (*w[-]; al[1] dp[ov1] b[1] pr[1] Bl[1] c[1] px[1] sp[1]/SM1* for EP0719 and *w*; TM3, Sb[1] Ser[1]/TM6B, Tb[1]* for EP3575 and EP0863) flies. The offspring with white eyes were selected for detecting imprecise excisions by PCR. ΔEP0719 were crossed to *w[1118]; P[w[+]]36F P[ry[+] neoFRT]40A*, offspring were selected by G418 resistance and at the same time for loss of w^+ eye phenotype, which were then tested against original EP0719 for recombination of *ΔEP0719 P[ry[+] neoFRT]40A / CyO*. ΔEP3575 or ΔEP0863 were crossed to *P[ry[+] neoFRT]82B P[w[+]]87E*. The offspring were selected by G418 resistance and loss of w^+ eye phenotype, and were then tested against original EP lines for recombination of *P[ry[+] neoFRT]82B ΔEP3575 / TM3*, or *P[ry[+] neoFRT]82B ΔEP0863 / TM3*.

To recombine *X-144[2]* to FRT chromosome, *X-144[2]/FM7* were crossed to *P[Ubi-GFP] P[w[+] FRT]101,* female offspring with *X-144[2] /P[Ubi-GFP] P[w[+] FRT]101* genotype were selected and crossed to *FM6/Y* male. The offspring were selected for GFP negative during larval stage. Among the grow-ups, FM6 and w^+ female adults were selected and crossed to *FM6/Y* male again individually. Among the progenies, lines without male other than *FM6/Y* are the ones with genotype *X-144[2] P[w[+] FRT]101/FM7.*

To generate germline clones for CG3542, female *P[ry[+] hsFLP]12, y[1] w*; noc[Sco]/CyO* were crossed to *P[w[+] ovoD[1-18]]2La P[w[+] ovoD[1-18]]2Lb P[ry[+] neoFRT]40A / CyO* males. The male offspring *P[ry[+] hsFLP]12, y[1] w* / Y; P[w[+] ovoD[1-18]]2La P[w[+] ovoD[1-18]]2Lb P[ry[+] neoFRT]40A / CyO* were collected and crossed to *ΔEP0719 P[ry[+] neoFRT]40A / CyO* females, progenies were heat shocked at 38°C for 1 hour at 3[rd] instar larval stage. The female adults *P[ry[+] hsFLP]12, y[1] w* / +; ΔEP0719 [ry[+] neoFRT]40A / P[w[+] ovoD[1-18]]2La P[w[+] ovoD[1-18]]2Lb P[ry[+] neoFRT]40A* were collected and crossed to *ΔEP0719 P{ry[+] neoFRT}40A / CyO*. Their eggs were collected and analyzed. For sar1 and CG6386, female *P[ry[+] hsFLP]12, y[1] w*; noc[Sco]*/CyO were crossed to *w*; P[ry[+] neoFRT]82B P[w[+] ovoD[1-18]]3R/TM3,* Sb[1] males, male offspring *P[ry[+] hsFLP]12, y[1] w*/Y; P[ry[+] neoFRT]82B P[w[+] ovoD[1-18]]3R/+* were collected and crossed to female *P[ry[+] neoFRT]82B ΔEP3575 / TM3*, or *P[ry[+] neoFRT]82B ΔEP0863 / TM3*. The progenies were heat shocked at 38°C for 1 hour at 3[rd] instar larval stage. Female adults with *P[ry[+] hsFLP]12, y[1] w*/+; P[ry[+] neoFRT]82B P[w[+] ovoD[1-18]]3R/P[ry[+] neoFRT]82B ΔEP3575, or P[ry[+] hsFLP]12, y[1] w*/+; P[ry[+] neoFRT]82B P[w[+] ovoD[1-18]]3R/P[ry[+] neoFRT]82B ΔEP0863* were collected and crossed to male *P[ry[+] neoFRT]82B ΔEP3575 / TM3*, or *P[ry[+] neoFRT]82B ΔEP0863 / TM3*. Their eggs were collected and analyzed.

To generate germline clones for *X-144[2]*, *X-144[2] P[w[+] FRT]101/FM7* were crossed to *w* ovoD1 v24 P[w[+mW] FRT[whs]]101/C(1)DX, y1 f1/Y;P[ry[+t7.2=hs]FLP]38*, the progenies were heatshocked at 3[rd] instar larvae stage. Adults were selected for genotype *X-144[2] P[w[+]*

FRT]101/ w ovoD1 v24 P[w^{+mW} FRT^{whs}]101; P[ry$^{+t7.2=hs}$FLP]38/+* and crossed to *X-144^{1}/Y*. The eggs laid by them were analysed.

To generate somatic clones, male *ΔEP0719 P[ry^{+} neoFRT]40A / CyO* were crossed to *P[ry+ hsFLP]; 2XP[w^{+} GFP] P[ry^{+} neoFRT]40A / SM6a-TM6b* female. The progenies were heat shocked at 38C° for 45 min. at 1st instar larval stage and were dissected for imaginal discs during 3rd instar larva stage. Alternatively, the adult females of *P[ry+ hsFLP] / +; 2XP[w^{+} GFP] P[ry^{+} neoFRT]40A/ ΔEP0719 P[ry^{+} neoFRT]40A* were heat shocked at 37°C for 1 hour and the ovaries were dissected 3 days later. In the case of sar1 or CG6386, *P[ry^{+} neoFRT]82B ΔEP3575 / TM3*, or *P[ry^{+} neoFRT]82B ΔEP0863 / TM3* were crossed to *P[ry^{+} hsFLP]12, y^{1} w*; noc^{Sco}/CyO*, male offspring *P[ry^{+} hsFLP]12, y^{1} w*/Y; P[ry^{+} neoFRT]82B ΔEP3575/+*, or *P[ry^{+} hsFLP]12, y^{1} w*/Y; P[ry^{+} neoFRT]82B ΔEP0863/+* were crossed to *w^{-}; P[ry^{+} neoFRT]82B P[w^{+} GFP^{3-13-7}]* females. The progenies were dissected similarly as in the case of ΔEP0719 for imaginal discs.

To overexpress EP lines in mesoderm and trachea, EP1455 were crossed to *y w; btlGal4*, EP1413, EP1408 and EP1200 were crossed to *w; twiGal4*. The eggs from these crosses were fixed and *in situ* hybridization was performed to detect the overexpression.

4.3 Histochemistry

Embryo fixation

To fix the embryos, properly staged embryos were collected, dechorionated by 50%bleach, and washed in tap water. Embryos were fixed in 4% Formadehyde in PBS (Sambrook et al., 1989): heptane=1:1 solution at 37°C for 20 min. with vigorous shaking, followed by devitellinization with methanol: heptane=1:1 solution by vortexing for half minute. –20°C cold 80% ethanol was used instead of methanol for later on phalloidin staining. Embryos were washed several times in methanol, and stored in methanol at –20°C if not used immediately.

For staining embryos with anti-phosphotyrosine antibody, embryos were fixed with heat fixation protocol (Hunter, 2000).

For staining embryos with anti-phosphotyrosine and anti-tubulin staining, after dechorionation, embryos were fixed in –20°C cold fixative:heptane=1:1 for 20 min. The fixative contains 50mM NaEGTA, 8% Formadehyde and 66% methanol in water. After fixation, the embryos were devitellinized by vortexing for half minute in –20°C cold methanol:heptane=1:1 solution and then stored in –20°C cold methanol overnight before processing the staining.

***In situ* hybridization**

To generate probes, 1.5µg of each LD clones (table 4-1) were digested in 100µl solution by 100 units of EcoRI for 2 hours at 37°C, followed twice by extraction with phenol, once with chloroform, and then precipitated by 250µl Ethanol and 10µl 3M CH_3COONa (pH5.2). DNA was recovered and washed once in 75% Ethanol. 2µl DIG 10X RNA labeling mix (Roche), 2µl Transcription buffer, 2µl 0.1M DTT, 0.8µl RNasin (Promega), 2µl SP6 (for LDs on pOT2A vector) or T7 (for LD on pBluescriptSK- vector, pBSK) RNA polymerase were added to the dried DNA pellet, and made up to 20µl with H_2O, mixed well and incubated at 37°C for 2 hours. The reaction was stopped by incubation on ice for 5 min.; 80µl of pre-hybridization mix (50% Deionized formamide, 25% 20X SSC (pH 7.0), 0.5% tRNA 20mg/ml, 1% hsDNA 10mg/ml, 0.05% heparin 100mg/ml, 0.1% Tween 20 in water) were added to make a dilution of 1 in 5.

Table 4-1. LD clones used for generating antisense RNA probe:

Clone ID	vector	Represent genes
LD45231	pOT2A	*CG6386*
LD46744	pOT2A	*tim10*
LD29226	pOT2A	*CG4266*
LD40388	pOT2A	*CG14217*
LD12312	pBSK	*CG2829*
LD21322	pOT2A	*ago (CG15010)*
LD30271		
LD39266	pOT2A	*sar1(CG7073)*
LD24714	pOT2A	*CG3542*

Sequence information is available in flybase.

The fixed embryos were rehydrated in PBST (0.3%Tween 100 in PBS), followed by heat treatment at 90°C for 5 min. in PBST, and several washes afterwards. Embryos were pre-hybridized for 1-2 hours at 56°C in pre-hybridization buffer, followed by over night hybridization with 1:1000 further dilution of the probe in pre-hybridization buffer at 56°C. The free probe was washed away by 1X100%, and 2X50% pre-hybridization mix in PBST for 20 min. each at 56°C. Embryos were washed in PBST for one hour at room temperature. Anti-DIG antibody conjugated with AP (1:1000, Roche) was added after washing and incubated for 1 hour at room temperature. After the free antibody was washed away, the signals were visualized by adding 1% NBT (10mg/ml Nitro blue tetrazolium in 70% Dimethylformamide) and 1% X-phosphate solution (10mg/ml 5-bromo-4-chloro-indoxylphosphate in Dimethyformamide) in staining buffer (100 mM Na_2CO_3, pH9.5 or 50mM Tris HCL, pH9.5, 2mM $MgCl_2$, 0.1% Tween20).

Antibody staining

The fixed embryos were rehydrated in PBST, followed by 1 hour blocking at room temperature using 5%BSA in PBST. The liquid phase was taken off, and the first antibody

was added. The reaction was left at 4°C overnight. Embryos were washed with PBST at room temperature for 1 hour, followed by another hour second antibody (biotin labeled) reaction at room temperature. After the second antibody was washed away, prepared ABC mix (ABC kit, Linaris Biologische Produkte GmbH) (solution A or B: PBST=1:50, the diluted solution A and B were mixed and rotated at room temperature for 30 min.) was added. The reaction was left at room temperature for 30 min. After the reagent was washed away, the antibody was detected by adding 20% DAB (1mg/ml stock) and 2% H_2O_2 (0.3% stock). To remove the peroxidase, 3% H_2O_2 was added for 20 to 30 min.. After the peroxidase was washed away, a second round antibody staining was processed as described above.

First antibodies used were mouse 2A12 (provided by N. Patel) for visualizing the trachea, used at 1:20 dilution, rabbit-anti-eve antibody (provided by M. Frasch) for visualizing the heart cell precursors, used at 1:2000 dilution, and rabbit-anti-β-gal antibody (Organon teknika corporation) to distinguish the homozygous mutant, used at 1:1000 dilution. Second antibodies were biotin coupled goat-anti-mouse antibody (Dianova) and goat-anti-rabbit antibody (Dianova). Both were used at 1:500 dilution in 1%BSA.

For phalloidin staining, embryos were incubated in 1:500 dilution of FITC coupled phalloidin for 10min. at room temperature. After washing away the solution, embryos were mounted directly in the vectashield (Linaris Biologische Produkte GmbH). For fluorescence staining, embryos were stained with mouse-anti-phosphotyrosine antibody (1:20) and/or mouse-anti-tubulin (1:200) antibody overnight at 4°C. After the washing step the next day, embryos were proceeded for FITC coupled goat-anti-mouse antibody staining at room temperature for 1 hour. After the washing step, the embryos were mounted in the vectashield. For the propidium iodide staining, embryos were digested with 0.4µg/µl RNase at room temperature for one hour, and stained with 1:1000 dilution of propidium iodide (PI) for 4 min., wash away the PI afterwards.

Embedding stained embryos in Araldite

Stained embryos were dehydrated in 30%, 50%, 70%, 90%, and 2X 100% (absolute ethanol treated with molecular Sieves, Sigma M-2010, 1/8 inch pellets) ethanol for 15min. each. After dehydration, 100% acetone (treated same as the ethanol) was added for 15min., followed by a mixture of 50% acetone (treated) and 50% Araldite (57.4g CY212 Araldite with 48g HY964 Hardener were mixed thoroughly first, and then 2ml 2,4,6-Tris(dimethylaminomethyl)phenol was added and mixed. Serva). The mixture containing embryos was transferred into a shallow plastic lid, and acetone was allowed to evaporate under the hood overnight.

4.4 PCR detection of imprecise excisions

Isolation of genomic DNA

Flies were homogenized in 500µl of the homogenization buffer (100mM Tris-HCL, pH9.0; 100mM EDTA; and 1% SDS). The resulting slurry was incubated at 70˚C for 30 min and cooled down on ice. 70µl of 8M CH_3COOK was added and the mixture incubated on ice for 30 min. The mixture was then extracted twice with phenol/chloroform and once with chloroform. The genomic DNA was precipitated with 2 volumes of ethanol for 5 min at room temperature and recovered by centrifugation. The genomic DNA was dissolved in 100µl water, and an equal volume of 5M LiCl was added, and the mixture was incubated on ice for 15 min. The tRNA and proteins were precipitated by centrifugation. The genomic DNA in the supernatant was precipitated with 0.1 volume of 3M CH_3COONa (pH5.2) and 2.5 volumes of ethanol for 10 min at –80˚C and recovered. The pellet was washed with 80% ethanol, air dried, and dissolved in TE (pH8.0). The DNA concentration was measured using the spectrophotometer, and the final concentration adjusted to 500ng/µl.

Genomic PCR

Table 4-2. The annealing and extension temperatures for different primer pairs.

Primer pairs	Annealing temperature	Extension temperature
3542-5/3542-3	52°C	70°C
3542-5/3542f3	52°C	70°C
5'sar1/3'sar1R	68°C	68°C
5863/3863R	68°C	68°C
5'1135/3'ULR	68°C	68°C

The sequence and genomic position of these primer pairs can be found in appendices.

The PCRs were carried out in an UNO thermoblock (biometra). A 50µl amplification reaction contained 1µg genomic DNA, 200µM of each dNTP, 15pmol of each primers (Eurogentec), 10x buffer and 1.8U DNA polymerase (Expand high Fidelity PCR system from Roche). The Mg^{2+} concentration was adjusted to final concentration of 3.5mM. Primers that were used in each case are indicated in appendices. The PCR programme included a denaturation step of 3 min at 94˚C, followed by 30 cycles: 10 seconds at 94˚C, 30 seconds at annealing temperature (see table 4-2) and 2 to 8 min. (depending on the length of the product, roughly 1 min. for 1Kb DNA) at extension temperature, ending with a 10 min extension at 72˚C. One-fifth of the reaction was analyzed by electrophoresis on a 1% Agarose gel.

PCR sequencing
The PCR sequencing was performed by ABI-Sequencer (Model 373 & 377) using BIG DYE TERMINATOR KIT (ABI prism® from PERKIN-ELMER). Genomic PCR products were purified by MicroSpin^{TM} S-400HR columns (Amersham Pharmacia Biotech Inc). 120ng genomic PCR product, 10pmol primer and 4µl BIG DYE Mix were used in the reaction. The final volume was adjusted to 20µl per reaction.

4.5 Construction of transgenic flies

Molecular cloning
LD28410 (fz4 in pOT2A, see flybase for sequence) was digested by NheI and SmaI, and then ligated to pUASp vector (Rorth, 1996) that had been digested by NotI and blunted, and then digested by XbaI.

Microinjection
Microinjection protocol follows the method developed by Spradling and Rubin (Rubin et al., 1982; Spradling, 1986; Spradling et al., 1982).

Establishing the lines
Injected flies were first crossed to w^- flies. The male offspring with red or orange eyes were crossed individually to *w^-; al^1 dp^{ov1} b^1 pr^1 Bl^1 c^1 px^1 sp^1/SM1* and *w*; TM3, Sb^1 Ser^1/TM6B* females at the same time. The male offspring with coloured eye and SM1 or TM3 chromosome were crossed to females with coloured eye and SM1 or TM3 respectively. The homozygous or balanced progenies were collected for establishing the lines.

4.6 Analysis of protein-protein interactions

Molecular cloning
Two restriction sites, SmaI and XhoI were generated by PCR using LD21322 as template. The two primers used were 5'ULSMAI-5'-cctgtacccgggtatggaaagaggatgcccagc-3', and 3'ULXHOI-5'-ttaggtgacactatagaactcg-3'. The PCR product was digested by SmaI and XhoI overnight at room temperature, and then ligated to pACT2 (Durfee et al., 1993) vector digested by SmaI and XhoI. Two positive clones were sequenced and none of them had mutation generated by PCR.

Yeast two-hybrid
The recipes for media and methods for yeast two-hybrid can be found in the website http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html. PACT2 -ago and pGBDU-dof (Battersby, 2001) were cotransformed into yeast PJ69-4A (gift from Phillip James) according to frozen yeast transformation method (Dohmen et al., 1991) using UL^-

plates for selection. Transformed yeast were plated on ULH$^-$ plates. As negative controls, pACT2-ago were cotranformed with pGBDU vector, or pGBDU-dof with pACT2 vector, or just the two vectors together.

4.7 Microscopy and image analysis

Non-fluorescence photo images were taken using Axiophot Photomikroscop (Zeiss) with the ProgRes 3008 (Kontron Elektronik) camera, and processed in photoshop (Adobe systems). Fluorescent photo images were taken under same series of microscope with Quantic (Photometrics) camera and processed in IPlab (Scanalytics), or confocal microscopy system from leica. The pictures used in figures are edited in photoshop and canvas 8.0 (Deneba system).

4.8 DNA sequence analysis

The insertion sites of the EP elements and the annotations of the Drosophila genome sequence are found in flybase: http://flybase.bio.indiana.edu/ and http://www.fruitfly.org/annot/. Protein homologue search was performed in http://www.ncbi.nlm.nih.gov/BLAST/.
DNA sequence alignments were analyzed at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html, and processed at http://www.ch.embnet.org/software/BOX_form.html.

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1. The sequence information of the imprecise excisions of CG3542 and the primers used to identify the excisions and for sequencing. The splicing information is available in the Flybase. The EP insertion site is marked by '↓'. The transcription begins with the mark 𝔾, and ends at the end of the sequence. The start codon is marked by ATG, and the stop codon is marked by TAG. Forward primers are marked in red and reverse in blue. The names of the primers are indicated next to the sequence. Sequence between '[]' is deleted in #3.3, between '()' is deleted in #9.4, between '{}' is deleted in #35.1, between '**' is deleted in #18.2, between '!!' is deleted in #9.3, and marked with 'aqua' colour is deleted in #15.5.

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CTTGGGGTCC TCTCGCATCA GAGCAATGTC TTTGGTGCCA CATATCTTCT GAGCCACACG
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GACCAGCTGA CAGGCGTTGA GTCAAAGCCG ACATCGACAT CGTGTGGCAC TCTCTAGCTC 3542-3
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GGGTCCCACG GGCATTCCCA GAGCAATGCG CAACTCCTCG GAGAGATCTC CGGGCTTCTT
TTCCTTAAGG CGTGTCTCAA ACTCCTTGCC CTCGT

2. The sequence information of the imprecise excisions of sar1 and the primers used to identify the excision. These primers are also used for sequencing. The EP insertion site is marked by '↓'. The information for splicing is available in the Flybase. The two primers used for detecting the excision by PCR are indicated in red (forward primer) and blue (reverse primer). The names of the primers are labeled next to the sequence. 5'sar1 is also used for sequencing the excisions. The transcription starting site is labeled by 𝕋, while the start codon is labeled by ATG. The sequence between '[]' is deleted in #71, '{}' is deleted in #28.

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tcgctagcga gacaacaacc actcttgatt tggctcgctt cgctctagca gagttatcca
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AAAACGAGCT GGATTCGCTG CTCACGGATG AGGCGCTGTC CAACTGCCCC GTGCTCATAT
TGGGCAACAA AATCGATAAG CCCGGCGCGG CTAGCGAGGA TGAGCTGAGA AACGTGTTCG
GACTGTATCA GCTAACAACC GGCAAGGTGA GCACCAAAAC CACCTCCCTC CCTCCCTCCC
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GATTTGCCCG GCCGTCCTCT GGAATTGTTC ATGTGCTCCG TGCTGAAGCG ACAGGGCTAC
GGCGAGGGTT TCCGTTGGCT GGCGCAGTAT ATCGATTAAG TCAGCATTAG CAACCACCAC
CAGCACCATA TTTTCAAGAA CACCACTCAA CACTCAAAAC CGAAAACTTG GCTACAAAAT
TTCCAAAAAT GATTAGAGAC CGCAGAAAGA ACAGAACCCA AGGGATGTGA ACCCAGCCCA
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GAAACTTTTA ATAATAATTA TGAGTAACAA ATTATAGCAA ATTAAAGGTG ATTTATTTAA
GAGCATGTGC TTTCAACGAA CGCTGTTTCG CCAATAAAAT GTAGCCACCT ACCTTTATTT
CAAGTTTATG AATTGTAAAT GTCGCTATTG ATTTCCTAAA TAGTTTACTT TGGCCGCAAA
ACATTATGTA CATCCCTATA AGGATTCGAC CTCCGATAAA TGGTACACCT TAGTTTAGCT
AAAAATTTTA AGTTGCATGT CATCGATGTA CTTTGTTTCT TTTATACAAT ACGAAAGGGA

3. The sequence information of the imprecise excisions of CG6385 and the primers used to identify the excision. These primers are also used for sequencing. The predicted splicing information is available in the Flybase. The translation start and stop codon are indicated by ATG and TAG respectively. The insertion site is indicated by '↓'. The sequences of primers used for detecting the excisions are indicated in red (forward primer) and blue (reverse primer), and their names are indicated next to the sequence. The sequence between '[]' are deleted in #43, between '{}' are deleted in #53.

CCGCAATATT ACCATTTCTG TTCAATCGAT TTTTCTGCTG CCTGTAAACT GCGCCTGCTT
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TGCAACACGA AACCGGAGCG TGCGAGTTGT TTCTACGGAA TTCGGTTTCG ACTACTTGAA
ATTTTTTCGG GGAAAAGACG CCGTCAGCGG ACGCCAGGGG TGGAACGGAA TATATCGATG
TGTGGCCAAT CGATATGCCC TCACCCCTAG ATGGTAGCTT CCTTTTGTGG CCGCACATTG
CCAAGTGGCG CCGTTTTGCA GAGCTGTCTA TCGTAATATA TCCTTGAAAT CATAATCATC
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TTGCTGCTGC TACTGCTGCT GGCATTCGTG GAACTGCTGG GCTCGGGCCG GTAGAAGGGT
```

4. The sequence information of the imprecise excisions of *archipelago* (*ago*) and the primers used to identify the excision. These primers are also used for sequencing. The full sequence and the information for splicing is available in the Flybase. The transcription starts at 𝔸. The EP insertion is marked by '↓'. The primers are marked in red (forward primer) and blue (reverse primer). The sequence between '[]' is deleted in excision #17.

```
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AACTATTTGA ATATGGTCAT ACACTTCTTT AGTATAAAAA TTATTTGGAA GGCTTTTCCT
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```

Abstract

The FGF signal is important for cell survival, proliferation, differentiation and migration. It is not clear how the FGF signalling cascade is regulated. In order to identify genes downstream of FGF signalling, a gain-of-function (GOF) screen was conducted using the Drosophila compound eye as a model system. The screen was based on the observation that expression of an activated form of the FGF receptor and Dof together in the Drosophilia eye ('GMR>λ-fgfr, dof'), produces a rough eye phenotype, whereas expressing either alone has no effect. This implies that at least some of the components in the signalling cascade are present during Drosophila eye development. When one copy of *raf*, *rhoA* or *rhoGEF* was mutated, the rough eye phenotype of 'GMR>λ-fgfr, dof' flies was enhanced. In the screen, 'GMR>λ-fgfr, dof' flies were crossed to the EP collection and the progeny were screened for the modification of the rough eye phenotype. In total, 24 suppressors and 26 enhancers were obtained, which may affect 38 annotated genes. 8 lines did not have any interactions with other signalling pathways that I have tested, which are the PDGF and EGF signalling pathways, and pathways involving Cdc42 or Rac1. Nor were they found in any of the published GOF screens. In order to investigate the interaction of the candidates with the endogenous FGF signalling pathway, imprecise excisions of three candidates – EP719, EP3575 and EP863 - were generated and the mutant phenotypes were studied.

Zusammenfassung

Der Fibroblasten-Wachstums-Faktor FGF-Signalweg ist wichtig für das Überleben der Zelle, die Proliferation, die Differenzierungn und die Zellwanderung. Die Regulation der FGF-Kaskade ist noch nicht vollständig aufgeklärt. Mit dem Ziel neue Gene zu identifizieren, die sich „downstream“ des FGF-Rezeptors befinden, wurde ein „Misexpressions-Screen“ durchgeführt, in dem das Facettenauge von Drosophila melanogaster als Modell System diente. Die Untersuchung basierte auf der Erkenntnis, dass die Expression einer ständig aktiven Form des FGF-Rezeptors zusammen mit dem Molekül „Downstream of FGF (DOF)“ [`GMR>λ-fgfr, dof´] im Auge einen „Rauhen Augen“ Phänotyp erzeugt. Die Expression eines der beiden Moleküle alleine hingegen erzeugt keinen Effekt. Dies deutet darauf hin, daß zumindest einige Komponenten der FGF-Signalkaskade während der Entwicklung des Auges von Drosophila melanogaster vorhanden sind. Die Mutation einer Kopie der Gene raf, rhoA oder rhoGEF führte zu einer Verstärkung des Augenphänotyps der `GMR>λ-fgfr, dof´-Fliegen. Im Screen wurden die `GMR>λ-fgfr, dof´-Fliegen mit den Stämmen der EP-Sammlung gekreuzt. Die Nachkommenschaft wurde auf Veränderungen des Augenphänotyps untersucht. Insgesamt wurden 24 Suppressoren und 26 Enhancer gefunden, welche 38 beschriebene Gene beeinflussen könnten. Acht dieser Kandidaten zeigen keinerlei Interaktion mit anderen untersuchten Signalwegen. Zu diesen gehören der PDGF-, der EGF und solche Signalwege in denen die Proteine Cdc42 oder Rac1 beteiligt sind. Sie wurden auch nicht in anderen, publizierten Misexpressions-Screens gefunden. Um die mögliche Interaktion von Kandidaten mit dem endogenen FGF-Signalweg zu untersuchen, wurden unpräzise Exisionen von drei Kandidaten -EP719, EP3575 und EP863- generiert und die mutanten Phänotypen studiert.

Abbreviations:

Abb:	Full name
ago	archipelago
Atk or PKB	protein kinase B
bnl	branchless
btl	breathless
csw	corkscrew
dco	double time or discs overgrown
dock	dreadlock
dof	downstream of FGF
dos	daughter of sevenless
dpp	decapentaplegic
drk	downstream of receptor tyrosine kinase
dsh	dishevelled
Dsor1	Drosophila MEK (MAPKK)
dsp1	dorsal switch protein 1
EGF	epidermal growth factor
FBP	formin binding protein
FGF	fibroblast growth factor
fmi	flamingo
fz	frizzled
gap1	GTPase activating protein
GOF	gain-of-function
htl	heartless
IR	insulin receptor
KDN	dominant negative form of ksr
ksr	kinase suppressor of ras
LOF	loss-of-function
MAPK	mitogen-activated protein kinase
msp300	muscle specific protein 300
NGF	nerve growth factor

PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PDZ	post synaptic density/disc-large/zo-1
PH	pleckstrin homology
PI3K	phospholinositide 3-kinase
PLCγ	phospholipase Cγ
PTB	phosphotyrosine binding domain
PtdIns(4,5)P_2	phosphatidylinositol-4,5-phosphotate
rl	rolled, Drosophila MAPK
robo	roundabout
RTK	receptor tyrosine kinase
ser	serine
SH2	src homolog 2
SH3	src homolog 3
sos	son of sevenless
thr	threonine
tou	toutatis
VEGF	vascular endothelial growth factor

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