GENETIC DISSECTION OF THE ONCOGENIC NEU^{YC} SIGNALLING PATHWAY

GENETIC DISSECTION OF SIGNALLING FROM

PHOSPHOTYROSINE RESIDUE 1201 OF THE

ONCOGENIC NEU RECEPTOR TYROSINE KINASE

By

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ABSTRACT

The ErbB2/Neu orphan receptor tyrosine kinase is amplified in 20-30% of breast and ovarian cancers and predicates poor patient prognosis. Five conserved tyrosine residues, autophosphorylated by Neu catalytic activity, "dock" adaptors and second messengers that activate discrete signalling pathways, most prominently the Ras/MAPK pathway, to regulate cell survival and proliferation.

Genetic analysis using *Drosophila* provides an efficient means for identifying evolutionarily conserved signalling components. Neu and *Drosophila* EGFR overexpression directs *Drosophila* tissue development synonymously. Consistent with biochemical evidence, genetic analysis of Neu signalling through individual pTyr revealed activating signals for 4 (Y1144, YB; Y1201, YC; Y1226/7, YD; Y1253, YE) of the 5 sites. Strong Ras-dependent signalling was mediated through adaptors Grb-2 (YB) and SHC (YD). In contrast to biochemical evidence, a strong Ras component was not genetically detected for YC or YE.

We have conducted two enhancer-suppressor screens to identify novel Ras and non-Ras requirements for YC signalling. For the first screen, a quantitative approach was designed to identify modification of an YC-specific wing notch phenotype. Thirty-two members of the Ras/MAPK signalling cassette were assessed. Sensitivity to Ras, Raf, MAPK and the Ras-related GTPase R-Ras was identified downstream of YC. The second screen, a large-scale mutagenesis, took advantage of an YC-induced rough eye phenotype. From over 19 000 screened flies, 11 enhancers and 6 suppressors were isolated. One strong suppressor has been genetically mapped to the dual transcription

factor and phosphatase *eyes absent*. Other promising Ras-dependent and Ras-independent modifiers await further mapping. Results from both screens point to YC as a unique pTyr that uses both Ras-dependent and Ras-independent outputs.

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LIST OF ABBREVIATIONS

A – adenine

APC – Adenomatous Polyposis Coli

al – aristaless

aos – argos

b – black

bbg – bigbang

C – cytosine

c – curved

ca – claret

cDNA – complementary DNA

co-IP – co-immunoprecipitation

CS-P – CantonS P-element free

csw – corkscrew

cu – curled

CyO – Curly of Oster

dab – disabled

Dac – dachshund

DER – *Drosophila* epidermal growth factor receptor

DN – dominant negative

DNA –deoxyribonucleic acid

dock – dreadlocks

dos – *daughter of sevenless*

dpp – *decapentaplegic*

Dsor – *Downstream suppressor of Raf*

drk – *downstream receptor tyrosine kinase*

e – *ebony*

ECM – extracellular matrix

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EMS – ethyl methane sulfonate

ErbB2 – avian erythroblastosis oncogene B 2/HER-2/Neu

ERK – extracellular signal-regulated kinase

Ey – *eyeless*

eya – *eyes absent*

FAK –focal adhesion kinase

G – guanine

GDP – guanine diphosphate

GDS – guanine nucleotide dissociation stimulator

GEF – guanine nucleotide exchange factor

GFP – green fluorescent protein

GMR – glass multimer reporter

Grb-2 – growth factor receptor-bound 2

GTP – guanine triphosphate

h – hairy

HER-2 – human epidermal growth factor receptor 2

JNK – Jun N-terminal kinase

ksr – kinase suppressor of Raf

MAPK – mitogen-activated protein kinase

MAPKK – mitogen-activated kinase kinase

MEK – mitogen-activated or extracellular-regulated kinase kinase

MG – midline glia

MMTV – mouse mammary tumour virus

Neu – neuroglioblastoma cell line derived oncogene/ErbB2/HER-2

NT – Neu transforming

NYPD – Neu tyrosine phosphorylation deficient

Pax – Paxillin

PCR – polymerase chain reaction

phl – pole hole

PI3'K – phosphatidylinositol 3' kinase

pr – purple

PTB – phosphotyrosine binding

pTyr – phosphotyrosine

px – plexus

R-Ras – Ras-related

rl – rolled

RNA – ribonucleic acid

RTK – receptor tyrosine kinase

RT-PCR – reverse transcriptase polymerase chain reaction

ru – roughoid

sev – sevenless

SH2 – src-homology 2

SHC – src homology 2 domain-containing

shv – shortvein

So – sine oculis

Sos – son of sevenless

sp – speck

Spi – Spitz

sr – stripe

st – scarlet

T – thymine

th – thread

tkv – thickvein

Toy – twin of eyeless

UAS – upstream activating sequence

Vn – Vein

CHAPTER 1 : INTRODUCTION

The human body is maintained in balance by the concerted action of the organs and tissues within the body. As the body ages, disruptions in normal behaviors accumulate, such as tissue or organ damage, and the balance is no longer maintained. When these disruptions persist or worsen, tissues and organs can fail, leading to death. Diseases and disorders also disrupt normal bodily function and lead to death. These can be caused by external factors, such as bacteria and viruses, or internal dysfunctions, such as autoimmune diseases and genetic disorders. Multiple Sclerosis is an example of an autoimmune disease. It disrupts the central nervous system by targeting and damaging the myelin sheaths surrounding the axons of neurons. This affects the ability of the neurons to conduct signals through the body and can present itself as any neurological symptom, from a cognitive disorder to loss of muscle control. Another example of an internal dysfunction that disrupts bodily functions is a set of diseases collectively known as cancer. Unlike Multiple Sclerosis or other diseases that are prevalent in particular sub-populations, cancers can affect a variety of people from any population. Cancers are characterized by the uncontrolled growth and differentiation of abnormal cells. These abnormal cells disrupt body function by infiltrating and destroying surrounding tissues and organs and eventually causing death.

What causes these cells to be abnormal and by what mechanisms do they infiltrate and destroy the surrounding tissue? The human body develops from the growth of hundreds of billions of cells. Cells undergo successive rounds of replication and growth before differentiating into their end fate, after which they no longer proliferate. To maintain tissue homeostasis, many cell types are re-generated during the course of a

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person's life. Abnormalities in these proliferative cells are often due to the accumulation

of multiple mutations in the genome, allowing for the unregulated proliferation of these

cells. These mutations cause the malignant transformation of normal cells into tumour

cells (Bargmann *et al.*, 1986; Bargmann and Weinberg, 1988b; Der *et al.*, 1982).

Mutations occur in two types of genes: 1) tumour suppressor genes; and 2)

protooncogenes (Harris *et al.*, 1969; Stanbridge, 1976; Stehelin *et al.*, 1976). Tumour

suppressor genes encode proteins that are required to control normal cell growth (Sherr,

2004). Loss-of-function mutations in these genes can make tumours malignant, resulting

in uncontrolled proliferation and cell growth. For example, familial colorectal cancer

involves the loss of Adenomatous Polyposis Coli (APC) function. As seen in these

cancers, those carrying germline mutations in *APC* have also acquired a somatic mutation

in *APC*, thereby inactivating protein function (Kinzler and Vogelstein, 1996; Levy *et al.*,

1994). Normally, APC downregulates the transcriptional coactivator function of β-

catenin. Loss of APC results in increased β-catenin function, which causes increased

transcription of target genes, such as the cell cycle regulatory gene *Cyclin D1*, thus

promoting cell cycle progression (Baeg *et al.*, 1995).

Protooncogenes encode proteins involved in promoting cell growth and

proliferation. Gain-of-function mutations in protooncogenes result in the formation of

oncogenes, which cause unchecked cell growth and proliferation. Additionally, localized

duplication or chromosomal translocation can also result in the formation of oncogenes.

In Burkitt's lymphoma, for example, the transcription factor c-Myc is overexpressed,

causing the unregulated expression of transcriptional targets, many of which are involved

in cell proliferation (reviewed by Meyer and Penn, 2008). Overexpression of c-Myc was identified as the result of a chromosomal translocation event, placing c-Myc under the transcriptional control of an immunoglobulin heavy-chain promoter.

The overproliferative and invasive nature of tumour cells is aided by the following properties also exhibited by these cells: 1) immortality; 2) a decreased requirement for growth factors; 3) modification of the extracellular matrix (ECM); 4) loss of cell-ECM and cell-cell interactions; and 5) loss of cell cycle control (Kufe *et al.*, 2003).

1.0.1 Immortality

When normal cells are established in cell culture, they divide a finite number of times. After each division, the genetic architecture of these cells is altered, such that the telomeric ends of chromosomes shorten after each division. Subsequent cell divisions are prevented once the telomeres reach a critical length, known as the Hayflick limit (Hayflick, 1965). Cancer cells, on the other hand, propagate indefinitely once established in culture with adequate nutrients. Cancer cells are able to maintain telomere length by elevated levels of telomerases, enzymes that function in lengthening the telomeres (reviewed by Bollmann, 2008). In this way, cancer cells are hypothesized to have the ability to divide indefinitely.

1.0.2 Decreased requirement for nutrients and growth factors

Cells require an adequate supply of nutrients and growth factors from the surrounding environment to regulate cellular functions. Compared to non-transformed cells, transformed cells have a reduced requirement for growth factors and nutrients in culture (Dulbecco, 1970). For example, mouse fibroblasts transformed by the SV40 virus

are able to grow in medium lacking specific growth factors, whereas untransformed cells do not (Holley and Kiernan, 1968). Cancer cells are able to produce their own growth factors to compensate for the inadequate supply of growth factors, and are able to respond to growth factors produced by neighboring cells (reviewed by De Luca *et al.*, 2008).

1.0.3 Modification of the ECM

The ECM plays a key role in regulating growth and movement of cells through any given tissue. Basement membranes are specialized ECM tissues that act as barriers to prevent overproliferation and cellular invasion (Yurchenco and Schittny, 1990). Human cancer cells and transformed cells produce a variety of enzymes, such as matrix metalloproteases, that degrade basement membranes, furthering the motility and invasiveness of malignant cells (Liotta and Kohn, 2001).

1.0.4 Cell-ECM and cell-cell adhesion

Structured tissue formation is a combination of physical interactions that occur between cells and between cells and ECM. Normal cell-to-cell and cell-to-ECM adhesion formation is mediated through the action of cadherins and integrins, respectively.

Cadherins are a group of calcium-dependent glycoproteins that are essential for adhesion between cells. Cadherins provide positional information during normal development through the establishment of adherens junctions. For example, E-cadherin signalling recruits β- and α-catenin, which influence actin cytoskeleton remodeling and adherens junctions (reviewed by Jeanes *et al.*, 2008). When E-cadherin signalling is altered, the number of adherens junctions is reduced and cells lose their adhesiveness, promoting invasion of adjacent tissues by the metastasis of tumour cells.

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Integrins are a family of cell surface receptors that form heterodimers from α and β subunits (Giancotti and Ruoslahti, 1999). Integrins and other signal transduction machinery cluster at contacts known as focal adhesions, linking the ECM to the intracellular cytoskeleton. The focal adhesion kinase (FAK) pathway participates in regulating anchorage-dependence and the mitogenic potency of proliferative cells. Dysregulation of integrin-dependent signalling occurs in tumour cells, promoting anchorage-independent growth and proliferation.

1.0.5 Loss of cell cycle control

Cells progress through four stages during their proliferation: G_1, S, G_2 and M. Under sub-optimal conditions during the G_1 phase, cells enter a quiescent stage, G_0, and do not carry out cell division. Once optimal conditions are re-established, cells can re-enter the cell cycle and commit to cell division. As a result of committing to divide, the cells must go through the S, G_2 and M phases and then return to G_1. Commitment decisions occur at cell cycle checkpoints, which regulate cell division. If a cell is prolonged at any checkpoint other than the G_1 checkpoint, the cell will die (reviewed by Shimada and Nakanishi, 2006). Checkpoints act as control mechanisms, inhibiting cells that have sustained genetic damage from replicating. Cells with damaged DNA or chromosomal breaks are arrested at the G_1 or G_2 cell cycle checkpoints, respectively. Cells are allowed to continue past these checkpoints once the damage has been repaired. In cancer cells, cell cycle checkpoints are poorly functioning or non-functional, allowing cells that have accumulated genetic mutations to divide freely.

1.1 Breast Cancer

In Canada, breast cancer is the most common cancer to afflict women. One in 9 women is expected to develop breast cancer and 1 in 28 is expected to die from it (Canadian Cancer Society and National Cancer Institute of Canada, 2008). The combined use of early detection through mammography screening and more effective adjuvant therapies following breast cancer surgeries prevent recurrence and eliminate remaining cancerous tissue. Several different forms of adjuvant therapies exist, and suitability is determined on a case-by-case basis. These include chemotherapy, radiation, hormone therapy and, more recently, targeted or biological therapy (reviewed by Perez and Baweja, 2008). The addition of biological therapies to the arsenal comes from the detection of abnormally high levels of the human epidermal growth factor receptor 2 (HER-2/ErbB2) oncogene in 18-20% of breast cancers (Payne et al., 2008). Positive HER-2 identification in the epithelial cells of breast cancer biopsies is associated with high grade tumours, lymph node involvement, a higher rate of disease recurrence and mortality. The HER-2 status of the tumour tissue is a predictive factor of response to systemic therapies, such as the anti-HER-2 antibody trastuzumab (Herceptin[TM]; Genentech, San Francisco, CA, USA) (Marmor et al., 2004).

HER-2 is vital to proper mammary gland development. There are two major stages of mammary gland development – ductal morphogenesis at puberty and alveolar morphogenesis during pregnancy. Epithelial cell proliferation during puberty is initiated by systemic hormones, leading to ductal growth and arborization of the fat pad. New ducts develop during pregnancy. The distal cells of these ducts differentiate into terminal

lobuloalveolar units. Lactation begins following the withdrawal of progesterone after pregnancy. Once lactation stops, involution leads to the destruction of alveolar units by apoptosis. In mice, ErbB2 expression and phosphorylation is detected during ductal development and alveolar morphogenesis. The signalling activity of HER-2/ErbB2 is reported to promote proliferation and differentiation of terminal alveolar units after birth (Jackson-Fisher et al., 2004). Transgenic mice expressing a dominant negative form of ErbB2 under the control of the mouse mammary tumour virus (MMTV) promoter show condensed alveolar structures and reduced milk secretion (Jones and Stern, 1999). Extensive side branching with terminal lobuloalveolar units is observed when expression of constitutively active rat ErbB2, Neu, is under the control of an endogenous promoter. This extensive branching is not observed in wildtype animals.

ErbB2 is important for tumour initiation and progression in mammary epithelial cells. Unlike other RTKs, ErbB2 functions cell autonomously and is more loosely regulated. (Liu et al., 2007; Schlessinger, 2004). Additionally, combined signalling from the ErbB2-ErbB3 heterodimer is more potent when compared to signalling activated by other RTKs (see section 1.3.2). Extremely high levels of ErbB2 are detected in a large number of breast carcinomas, conferring strong mitogenic and metastatic properties to these cells. Targeting HER-2/ErbB2 in biological therapies has successfully downregulated ErbB2 signalling, however, only a small subset of breast carcinomas are responsive to this treatment and resistance to these therapies is increasing (Chen et al., 2008b; reviewed by Perez and Baweja, 2008). Therefore, it is necessary to identify novel biological markers and therapeutic targets that are useful for a broader spectrum of breast

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carcinomas. Several different approaches have been used to characterize ErbB2/Neu

signalling, including large-scale proteomics-based screens and *in vitro* biochemical assays

(Dankort *et al.*, 1997; Marone *et al.*, 2004; Mukherji *et al.*, 2006; Schulze *et al.*, 2005).

These approaches are prone to identifying a large number of false-positives. The results

of these approaches have to be verified in an *in vivo* mammalian system, a process that is

very time-consuming. We have taken a genetic approach in the fruit fly, *Drosophila*

melanogaster, to identify novel components of Neu signalling directly in an *in vivo*

context.

1.2 ErbB Family of Receptor Tyrosine Kinases

The ErbB family of proteins are Type I transmembrane receptors with

intracellular kinase activity. The family consists of four members – EGFR (ErbB1/HER-

1), ErbB2 (Neu/HER-2), ErbB3 (HER-3) and ErbB4 (HER-4) – that function to relay

extracellular signals to the nucleus by activating intracellular signalling pathways (Prigent

and Lemoine, 1992). Three of the receptors bind specific ligands. EGFR binds

epidermal growth factor (EGF) and transforming growth factor (TGF)-α, while ErbB-3

and ErbB-4 bind ligands from the neuregulin family. ErbB2 is unique, as it has no known

extracellular ligand (Lonardo *et al.*, 1990). The structure of receptor monomers consists

of an extracellular ligand-binding domain, a transmembrane domain and an intracellular

kinase domain followed by a cytoplasmic tail regulatory domain (Yarden and Ullrich,

1988). Upon ligand binding, ErbB proteins undergo receptor dimerization and

transphosphorylation of key tyrosine residues in the regulatory domain. These

phosphorylated tyrosine (pTyr) residues dock a variety of intracellular adaptor proteins

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and second messengers that lead to the activation and crosstalk between numerous signalling pathways, resulting in diverse biological effects.

Crystal analysis of EGFR has revealed the structural basis for dimerization (Burgess *et al.*, 2003; Garrett *et al.*, 2002). The extracellular ligand-binding domain consists of four subdomains – 2 homologous large (L) subdomains and 2 cysteine-rich (CR) subdomains (Yarden and Ullrich, 1988). The subdomains are arranged in the following order: L1-CR1-L2-CR2. Crystallography of EGFR with and without a ligand shows two different conformations. In the unbound or tethered monomer form, the CR1 and CR2 subdomains physically interact, resulting in a closed conformation. In the ligand-bound or extended dimer form, ligands EGF or TGF-α bind a pocket between the L1 and L2 subdomains (Burgess *et al.*, 2003; Garrett *et al.*, 2002; Garrett *et al.*, 2003). Dimers form through the interactions of the CR2 dimerization arm. The tethered monomer form is also seen with ErbB3 and ErbB4 monomers (Bouyain *et al.*, 2005; Cho and Leahy, 2002).

The structure of the orphan ErbB2 receptor resembles the extended conformation of EGFR. ErbB2 monomers have the dimerization arm of CR2 exposed, ready for dimerization, and the L1 and L2 subdomains are in direct contact (Garrett *et al.*, 2003). This mimics the ligand binding conformation seen in other ErbBs. Furthermore, in ErbB2 the residues responsible for ligand binding are replaced with residues that inhibit ligand binding, providing an explanation as to why no extracellular ligand has been identified for ErbB2 (Burgess *et al.*, 2003). Therefore, the ErbB2 monomer is always

ready to form heterodimers with ligand-activated forms of other ErbB receptor monomers.

Activation of the intracellular kinase domain in RTKs occurs after ligand-induced dimerization and autophosphorylation of the activation loop of the catalytic domain (Hubbard and Till, 2000). Unlike the insulin growth factor receptor, the kinase domain in ErbB RTKs is in an active state and does not require phosphorylation of its activation loop (Burgess et al., 2003). Comparison of the activated insulin receptor activation loop and the EGFR activation loop revealed that the activation loop of EGFR exists in a constitutively active conformation. Although activation does not require phosphorylation of amino acid residues within the activation loop, other mechanisms to activate the catalytic domain may regulate function. Allosterically induced changes to the activation loop may be responsible for proper receptor function.

Unlike other ErbB proteins, ErbB3 has a non-functional kinase domain (Cho and Leahy, 2002; Garrett et al., 2003; Guy et al., 1994). It fails to bind ATP within the catalytic domain and is catalytically inactive due to critical amino acid substitutions (Siegel et al., 1999; reviewed by Citri et al., 2003). Activation of ErbB3 occurs through its heterodimerization partner. Chimeric, kinase active ErbB3 constructs fail to signal without heterodimerization, suggesting that ErbB3 is an obligate heterodimerization partner (Berger et al., 2004).

Identification of the structural basis for ErbB monomer dimerization and activation has also revealed the configuration responsible for the functional deficits of the ErbB2 and ErbB3 monomers. Despite these structural defects in ErbB2 and ErbB3

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monomers, heterodimerization of these monomers forms the most potent signalling dimer in the ErbB family of RTKs. The following explores the molecular basis of signalling potency of the ErbB2-ErbB3 heterodimer and its effect on tumorigenesis.

1.3 ErbB2/Neu Signalling and Cancer

1.3.1 Transformation by neu

The *neu* oncogene was originally identified as an ethylnitrosourea-induced mutant form of the gene responsible for transforming a rat neuroblastoma cell line (Schechter *et al.*, 1984). Oncogenic activity was identified as a single point mutation that results in a valine to glutamic acid substitution at position 664 in the transmembrane domain (neu^{NT}) (Bargmann *et al.*, 1986). Oncogenic *neu* displays constitutive activation and phosphorylation of carboxy terminal tyrosine residues (Bargmann and Weinberg, 1988a; Dankort *et al.*, 1997). neu^{NT} is transforming in mouse and Rat1 fibroblast cells and in mammary epithelial cells (Dankort *et al.*, 1997). Transformation potential is increased 3-fold in neu^{NT}-expressing cells that also carry a carboxy terminal deletion that removes most phosphorylated tyrosine residues (Akiyama *et al.*, 1991; Bargmann and Weinberg, 1988b; Siegel *et al.*, 1994). Additionally, several transgenic mouse models have demonstrated the role of neu^{NT} in tumour progression. Expression of neu^{NT} in mammary epithelia using the MMTV promoter resulted in the rapid formation of multifocal mammary tumours (Muthuswamy *et al.*, 1994). Expression of the *neu* protooncogene, in contrast, only formed focal mammary tumours after a long latency period (Andrechek *et al.*, 2000). In addition to mammary tumour formation, the tumorigenicity of *neu* is reported in other tissues, such as the skin and prostate.

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1.3.2 Mechanism of Tumorigenesis

Transformation by *neu*, as described above, involves the over-activation of Neu protein through mutation. A single activating mutation is not seen in human breast cancers overexpressing HER-2 (Hynes *et al.*, 1989; reviewed by Moasser, 2007). Increased signalling by HER-2 is due to over-amplification of the HER-2 gene, leading to overexpression of the resulting protein. Typically, there are approximately 24 000 copies of HER-2 protein and 2 copies of the HER-2 gene. In overexpressed tissues, a 100-fold increase is observed. The HER-2 protein numbers may increase up to approximately 2 400 000 molecules and the gene numbers may range from 50 to 100 copies (reviewed by Perez and Baweja, 2008).

High levels of HER-2/ErbB2 expression leads to ligand independent activation of the kinase domain and a shift in the population of ErbB heterodimers, promoting dimerization of ErbB2 with EGFR and ErbB3 (reviewed by Citri *et al.*, 2003). ErbB2 widens the spectrum of ligands bound by its heterodimerization partner and increases ligand binding affinity. ErbB2-containing heterodimers also increase the duration and complexity of the activated signalling network by interfering with the endocytic regulation of EGFR and by activating diverse pathways through ErbB3 activation. EGFR undergoes ubiquitin-mediated endocytic degradation after ligand-induced activation, while ErbB2 undergoes endocytic recycling back to the plasma membrane (Lenferink *et al.*, 1998; Waterman *et al.*, 1998; Duan *et al.*, 2003; Sorkin and Goh, 2008). Like ErbB2, heterodimers of EGFR and ErbB2 also undergo recycling over degradation, and therefore

have increased signalling potency and duration. Thus, EGFR expression and activity is increased when ErbB2 is overexpressed.

Signalling from heterodimerization partners ErbB2 and ErbB3 is extremely potent due to the combination of mitogenic and anti-apoptotic cues initiated from ErbB2 and ErbB3, respectively (reviewed by Citri *et al.*, 2003). Neuregulin-induced activation of ErbB3 recruits ErbB2 in its active conformation. ErbB2-ErbB3 activation results in auto- and transphosphorylation of conserved tyrosine residues that begin a series of protein-protein interactions. Specific stretches of conserved amino acids, or domains, facilitate protein-protein binding. Three prominent domains involved in signal transduction from the ErbB receptors are the Src-homology 2 (SH2), phosphotyrosine binding (PTB) and Src-homology 3 (SH3) domains (reviewed by Pawson, 2007). Activation of ErbB2 causes the activation of the Ras/MAPK signalling cassette by the recruitment and activation of SH2- and PTB-domain containing adaptor proteins, such as Grb-2 and SHC (Lai *et al.*, 1995; Li *et al.*, 1996; Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1993; van der Geer *et al.*, 1995). The guanine nucleotide exchange factor (GEF) Son of sevenless (Sos) is recruited via SH3-SH3 domain interactions, and then stimulates the exchange of GDP for GTP in the membrane-bound small GTPase Ras (Rogge *et al.*, 1991; Buday and Downward, 1993; Rozakis-Adcock *et al.*, 1993). The serine/threonine kinases Raf, MEK and MAPK are recruited in a scaffolding complex with Kinase suppressor of Raf (Ksr) and Connector Enhancer of Kinase Suppressor of Raf (CNK) (Roy *et al.*, 2002; Claperon and Therrien, 2007). Activation of Raf by Ras leads to Raf-dependent MEK phosphorylation, and subsequent diphosphorylation of MAPK by MEK

(Dickson *et al.*, 1992; Gardner *et al.*, 1993; Lange-Carter *et al.*, 1993; Biggs *et al.*, 1994;

Gardner *et al.*, 1994; Perrimon, 1994). Di-phospho-MAPK (dpMAPK) is then able to

translocate into the nucleus and modulate function of transcription factors, such as c-Myc

and Elk-1, leading to cell proliferation (Chuang and Ng, 1994). Intriguingly, ErbB

heterodimers appear to avoid coupling to effectors that can negatively regulate mitogenic

signals, such as Grb-2 and the Ras-specific GTPase-activating protein (RasGAP)

(reviewed by Citri *et al.*, 2003).

Increased signalling potency is the result of the activation of the phosphatidyl-

inositol 3' kinase (PI3'K)/protein kinase B (Akt) pathway (Siegel *et al.*, 1999; reviewed

by Citri *et al.*, 2003). ErbB2 does not associate with the p85 subunit of PI3'K, however,

ErbB3 contains several pTyr motifs that bind p85 (reviewed by Sergina and Moasser,

2007). This leads to the allosteric activation of the p110 catalytic subunit. p110 produces

the phosphatidylinositols phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P$_3$] from

phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$] and phosphatidylinositol-3,4-

bisphosphate [PI(3,4)P$_2$] obtained from the activity of 5'-inositol phosphatases. Akt is

recruited to the plasma membrane by its plekstrin homology domain and is

phosphorylated by phosphoinositide-dependent kinase (PDK)-1 (Anderson *et al.*, 1998;

Cho *et al.*, 2001). Phosphorylation of Akt causes its activation and translocation inside

the nucleus where it acts on targets that are regulators of apoptosis or cell growth. The

regulation of apoptosis is achieved by inhibiting pro-apoptotic molecules, thereby

contributing to cell survival. Additionally, cell cycle progression is promoted in cells

overexpressing ErbB3 by upregulation of Cyclin D1 and cyclin dependent kinases CDK4

and CDK6. This, along with mitogenic signals from ErbB2, results in the enhanced

proliferation and malignant transformation seen with the overexpression of the ErbB2-

ErbB3 heterodimer (reviewd by Citri *et al.*, 2003).

1.4 ErbB2/Neu Site-Specific Signalling in Mammals

ErbB2/Neu signalling activates multiple discrete pathways to mediate diverse

cellular processes that affect its transforming potential when overexpressed. These

pathways are advanced through the phosphorylation of five specific tyrosine residues –

Y1028 (YA), Y1144 (YB), Y1201 (YC), Y1226/7 (YD) and Y1253 (YE) – located in the

carboxy-terminal regulatory domain of the receptor (Hazan *et al.*, 1990; Segatto *et al.*,

1990; Akiyama *et al.*, 1991; Siegel *et al.*, 1994; Dankort *et al.*, 1997). Early studies

involving deletion or mutation of the autophosphorylation sites dramatically affected *neu*

transformation. These studies reported the importance of several different

autophosphorylation sites in *neu*-mediated transformation. The mutation of three of the

five tyrosine residues to phenylalanine was reported in one set of studies to dramatically

reduce *neu* transformation (Mikami *et al.*, 1992). In another set of studies, Tyr 1248 was

identified as the major autophosphorylation site of activated HER-2 and that mutation of

this residue to Phe decreased Neu tyrosine kinase and transformation activities (Akiyama

et al., 1991). Furthermore, restoration of this residue in a C-terminal deletion mutant

restored transformation. To identify the specific pTyr that confer *neu*-mediated

transformation and the discrete second messenger pathways they activate, a systematic

site-directed mutagenesis approach was employed (Dankort *et al.*, 1997). To begin with,

all 5 tyrosine residues were mutated to Phe in a catalytically active mutant allele of *neu*.

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In this *neu* phosphorylation deficient (*neu*^{NYPD}) mutant, *neu*-mediated transformation was greatly reduced. To identify individual pTyr function, single Tyr residues were "added back" to the *neu*^{NYPD} mutant, generating a series of add-back alleles corresponding to each of the five pTyr autophosphorylation sites. The restoration of four of these five pTyr sites (YB-YE) completely restored the transforming activity of *neu* in Rat1 fibroblasts (Dankort *et al.*, 1997). In contrast, the restoration of YA abolished all activity from the NYPD mutant. Transformation mediated from the four pTyr residues was found to be dependent on the activation of Ras (Dankort *et al.*, 1997; Dankort *et al.*, 2001b). Co-IP experiments identified Grb-2 as the mediator of YB-dependent transformation (Dankort *et al.*, 1997). Similarly, YD-mediated transformation was found to be dependent on Grb-2 activity; however, this interaction was mediated through direct binding of SHC. These observations identified the specific pTyr that associate with the adaptors Grb-2 and SHC, which were demonstrated in earlier experiments to form physical complexes with Neu. *In vivo* expression of activating YB and YD mutants in mouse mammary epithelia resulted in the rapid formation of focal tumours (Dankort *et al.*, 2001b). Furthermore, YB-expressing strains developed lung metastases at higher rates than YD strains, arguing that Grb-2- and SHC-dependent signalling play largely different roles in ErbB2/Neu-mediated tumorigenesis and metastasis (Dankort *et al.*, 2001b).

Signalling through the pTyr sites YC and YE are less clear. YC and YE function largely independently of Grb-2 and SHC (Dankort *et al.*, 2001b). A conserved stretch of amino acids surrounding the YC (ENPEYLVPR) and YE (ENPEYLGLD) autophosphorylation sites facilitate protein-protein interactions. The ENPEYL motif is

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not unique to YC and YE – it is present in vertebrate and invertebrate RTKs (Table 1.1).

This sequence contains the PTB domain-binding motif, NPXY, which suggested that

PTB-containing proteins mediated transformation from these autophosphorylation sites

(Dankort et al., 2001a). Indeed, the SHC PTB domain was shown to bind Y1196 of

ErbB2, a pTyr site analogous to sites YC and YE, with high affinity (Schulze et al., 2005;

Table 1.1). The SHC PTB domain was, however, the only PTB domain shown to bind

Y1196. Other proteins that bound to Y1196 did so through their SH2 domains, such as

Abl2 and NCK2, suggesting that YC and YE are capable of binding and activating both

PTB- and SH2-domain containing adaptors.

In vitro studies identified three binding partners for YE: Dok-R, a RasGAP

associating protein; and p150 and p34, two unidentified proteins. While Dok-R contains

a PTB domain and p150 associates with YE in a phosphorylation-dependent manner, only

p34 correlates with transformation (Dankort et al., 2001a). YC was shown to bind the

SH2 domain-containing protein Crk in vitro (Dankort et al., 2001b). YC-specific

signalling through Crk is hypothesized to result in MAPK activation through the Ras-

competitor Rap1A. Contrary to a previous study that demonstrated Ras-dependency in

YC-mediated transformation, Rap1A was ineffective against the transforming potential of

YC mutants (Dankort et al., 2001b). Together, these data argue for the involvement of

both Ras-dependent and Ras-independent pathways in Neu-mediated signalling. To

clarify the mechanism by which YC mediates Neu signalling, we have employed a

genetic approach using Drosophila melanogaster to identify novel signalling partners of

the YC autophosphorylation site.

Table 1.1: ENPEYL motif in RTKs

Receptor	pTyr	Motif	Function
Neu	1201YC	ENPEYLVPR	Low affinity SHC binding in Neu
	1253YE	ENPEYLGLD	(Kavanaugh *et al.*, 1995)
ErbB2	Y1196	ENPEYLTPQ	High affinity binding of SHC PTB domain (Schulze *et al.*, 2005)
DER	Y1308	DNPEYLLNA	NPXY peptides block SHC binding (Lai *et al.*, 1995)
Torso	Y698	ENKEYFDLL	
HER-1	Y1197	ENAEYLRV	

Adapted from Settle *et al.*, 2003

1.5 Transgenic Screens using *Drosophila melanogaster* as a Model Organism

Drosophila was first used approximately 100 years ago to study biological

processes. Mutant phenotypes were used to study biological processes without knowing

the identity of the gene mutated; a forward genetics approach. Forward genetics has been

pivotal in the generation and identification of novel mutated genes involved in a number

of developmental and biochemical processes, for example embryonic segment patterning,

eye patterning and signal transduction (Nusslein-Volhard and Wieschaus, 1980; Karim *et*

al., 1996; reviewed by St. Johnston, 2002). Over the years, a large number of powerful

tools have become available to assist the *Drosophila* researcher in a molecular biology

based, reverse genetics, approach to investigate gene function. This has permitted an

increase in the use of *Drosophila* to directly study the mechanisms underlying cancer.

Aside from the relatively short reproductive cycle (from egg to adult in approximately 11

days at room temperature) and the ease of culturing multiple strains and large sample

sizes, researchers are able to take advantage of examining the effects of conserved genes

underlying cancer progression in the context of the whole organism by modeling specific

cancers. For example, a *Drosophila* model of the cancer syndrome multiple endocrine

neoplasia type 2 (MEN2) was created by misexpression of the *Drosophila* orthologue of

the Ret receptor tyrosine kinase (dRet) in the developing adult eye (reviewed by Vidal

and Cagan, 2006). Ret is mutated to an activated form in most MEN2 patients.

Misexpression of activated dRet in the developing eye results in a rough eye phenotype.

This model was used to identify several genetic modifiers of the Ret-dependent eye

phenotype in an enhancer-suppressor screen, including the *Drosophila* orthologue for C-

terminal Src kinase (Csk), a tumour suppressor gene. In addition to successfully identifying genes involved in Ret-dependent signalling, a putative chemotherapy drug, anilinoquinazoline compound ZD6474, successfully inhibited the activity of dRet in the developing eye when fed to flies. This was the first demonstration that compound ZD6474 could prevent Ret-dependent overgrowth in intact epithelial tissue.

Similar studies have been performed on human genes transgenically expressed in *Drosophila*. Familial and sporadic colorectal cancers are associated with mutations in the human *Adenomatous Polyposis Coli* (*hAPC*) gene (Polakis, 1997). APC decreases Wnt signalling by downregulation of β-catenin. Transgenic *Drosophila* expressing truncated or full-length forms of *hAPC* were used in an enhancer-suppressor screen and in drug screening (Bhandari and Shashidhara, 2001). Consistent with its role in mammals, expression of hAPC was able to negatively regulate the function of the *Drosophila* ortholog of β-catenin, Armadillo, effectively inhibiting Wnt signalling during *Drosophila* development. Specifically, the β-catenin binding domain of hAPC was demonstrated to mediate negative regulation of Armadillo (Bhandari and Shashidhara, 2001). In addition, inhibition of Wnt signalling by a current anti-cancer drug was verified when fed to these transgenic flies (Bhandari and Shashidhara, 2001). These studies demonstrate the successful identification of conserved signalling pathway components using forward and reverse genetic screens in transgenic flies expressing human proteins or their *Drosophila* orthologues.

1.6 ErbB Receptor Tyrosine Kinase Signalling in *Drosophila*

1.6.1 Drosophila Epidermal growth factor receptor tyrosine kinase

The *Drosophila* EGFR (DER) is the only member of the ErbB family of Type I

RTKs in *Drosophila* (Shilo *et al.*, 1986). It is most similar to HER-1/EGFR. DER

signalling is important in a number of cell fate decisions regulating tissue growth,

patterning and differentiation, thus providing versatile tissue models for the study of

RTK-dependent signalling. DER activation occurs throughout development and can also

occur consecutively to direct the formation of a single tissue. DER signals are required

for the proliferation of progenitor cells in the developing wing imaginal disc during the

first and second instar larval stages. The wing imaginal disc begins as a group of 50 cells

in embryos, and divides exponentially during larval development to 50 000 cells in third

instar larvae. Proliferation is initiated by broad expression of the DER activating ligand

Vein. Vein mutants have very small late third instar discs, similar to those in late

second/early third instar larvae (Simcox *et al.*, 1996). In late third instar larvae/early

pupal stages, DER activation is then limited in presumptive vein primordium to initiate

wing vein formation through a combination of activating and inhibiting signals

(Schweitzer and Shilo, 1997).

Limiting the number of cells for mature pattern formation often involves initiation

of apoptotic signals. Cells required to survive to maturity can escape programmed cell

death by DER-dependent suppression of apoptosis. An example of this is seen in

embryonic midline glial (MG) cells. MG cells begin as a group of 12 cells per segment

that are eventually reduced to 3 cells by first instar larval stage (Sonnenfeld and Jacobs,

1995; Stemerdink and Jacobs, 1997; Lanoue and Jacobs, 1999; reviewed by Jacobs, 2000). The activation of DER signals is what distinguishes these 3 cells from those that undergo apoptosis. When DER activity is increased by activated Ras or ectopic Spitz expression, all 12 MG survive and differentiate to ensheath commissural axons.

DER activity is spatially restricted by 5 extracellular ligands: four activating ligands – Spitz, Keren, Gurken and Vein – and one inhibitory ligand – Argos. Processing and secretion of activating and inhibitory ligands provides an intricate level of DER regulation. The TGF-α ortholog Spitz (Spi) is the primary activating ligand of DER in most tissues (Shilo and Raz, 1991; Freeman, 1994; Tio et al., 1994; Stemerdink and Jacobs, 1997). Spi is generated as a transmembrane precursor that is processed within cells by the cooperative action of Star (S) and Rhomboid (Rho) (Bang and Kintner, 2000). S colocalizes with Spi in the ER membrane and translocates Spi from the ER to the Golgi where they sequentially bind Rho (Bang and Kintner, 2000; Hsiung et al., 2001; Tsruya et al., 2002). Spi is then cleaved in its transmembrane domain in a Rho-dependent manner and secreted from the cell (Urban et al., 2002; Tsruya et al., 2007; Yogev et al., 2008). The expression pattern of Rho is similar to that of DER-activated MAPK (Gabay et al., 1997). Rho is a limiting step in DER activation, as ectopic expression of Rho is sufficient to induce high levels of DER activation (Sturtevant et al., 1993; Golembo et al., 1996). Keren and Gurken, also TGF-α orthologues, are similarly regulated by S and Rho (Doroquez and Rebay, 2006).

Vein (Vn) is a neuregulin ortholog that has weak activating capacity (Schnepp et al., 1996). Vn is used in tissues that require low DER activation. Vn-dependent DER

activity is required during specification of muscle precursors and patterning of wing veins, as described earlier (Yarnitzky *et al.*, 1998; Martin-Blanco *et al.*, 1999; Wessells *et al.*, 1999).

Argos (Aos) is a secreted ligand that contains a cysteine-rich region similar to an EGF repeat (Sawamoto *et al.*, 1996). Aos functions in a feedback loop, with high levels of DER signalling potentiating *aos* expression (reviewed by Shilo, 2003). The model for the maintenance of constant levels of DER signalling is based on the assumption that Spi and Aos have different distributions. Experimental evidence shows DER signalling is maintained in cells close to the source of secreted Spi, even in the presence of Aos. In cells further away, Spi levels are lower and are therefore inhibited by higher Aos levels (Gabay *et al.*, 1997). The biochemical mechanism of Aos inhibition is accomplished by direct binding of Aos to Spi (Klein *et al.*, 2004; Klein *et al.*, 2008). The atypical EGF domain confers the inhibitory properties of Aos. A domain swap of the Aos EGF domain with those in Spi or Vn confers inhibitory properties to these ligands (Howes *et al.*, 1998; Schnepp *et al.*, 1998).

1.6.2 Structural and functional similarities between DER and Neu in Drosophila

Signalling pathways activated by DER are evolutionarily conserved, with DER primarily activating the Ras/MAPK signalling cassette (Shilo *et al.*, 1986; Kumar *et al.*, 1998; Zak and Shilo, 1990). Comparison of the primary protein structure between DER and Neu revealed a high degree of similarity. Specific domains differed in their similarity, with the highest degree of similarity occurring between the kinase domain (79%) and the lowest degree of similarity observed between the regulatory domains of

DER and Neu (31%) (Settle *et al.*, 2003). Closer examination of amino acid motifs

surrounding the conserved tyrosine residues in the regulatory region identified conserved

peptide recognition motifs. This evidence supported the introduction of *neu* expression in

Drosophila to study Neu-activated signalling pathways. Functional support for the study

was observed when *neu*^{NT} and *neu* add-back alleles were ectopically expressed in tissues

whose development is regulated by DER, such as embryonic midline glial cells,

developing compound eye and wing primordia (reviewed by Doroquez and Rebay, 2006).

Similar to DER overexpression, ectopically expressed *neu*^{NT} suppressed midline glial

apoptosis, as well as influenced pattern development in the developing eye and wing,

indicating the ability of Neu to signal in a manner akin to DER (Settle *et al.*, 2003).

1.6.3 Dimerization of Neu in Drosophila

Ligand binding to DER induces homodimerization of protein monomers. In

mammals, ErbB proteins are capable of heterodimerization, in addition to

homodimerization, since there are multiple family members. Neu signalling in

Drosophila may be the result of the combined effects from Neu homodimers and Neu-

DER heterodimers. One line of evidence suggests the Neu heterodimerizes with DER, as

the inhibitory add-back allele *neu*^{YA} suppressed eye patterning defects associated with

overexpression of DER (Hossain *et al.*, 2005). Contrary to this, kinase dead Neu failed to

attenuate suppression of midline glial apoptosis by constitutively active DER (Settle *et*

al., 2003). The constitutively active DER protein may signal independently of

dimerization, allowing for the continued suppression of midline glial apoptosis in the

presence of kinase dead Neu (Settle pers. comm.). Therefore, heterodimerization between DER and Neu may be occurring in transgenic flies.

1.7 Neu Site-Specific Signalling in *Drosophila*

As a starting point for a genetic approach to studying pathways associated with Neu, *neu* add-back alleles were analyzed by dosage-sensitive modifier genetics to identify which *Drosophila* adaptor proteins and second messengers function downstream of specific pTyr. Similar to mammalian studies, only Neu^{YD} functions through *Drosophila* SHC (Settle *et al.*, 2003). Similar to results from mammalian studies, Neu^{YB} functions through the *Drosophila* Grb-2 ortholog, Downstream of receptor kinase (Drk). Unlike the behavior observed in Rat1 fibroblasts, YB was not the only pTyr to be modified by Drk (Settle *et al.*, 2003). However, genes functioning upstream or downstream of *drk*, such as *Sos, dos, dab, csw* and *Ras1* all strongly suppressed YB (Settle *et al.*, 2003).

Inhibition of Ras by a neutralizing antibody repressed transforming potential from Neu add-back alleles YB, YC, YD and YE (Dankort *et al.*, 1997). Similar results were also observed with Rap1A-mediated Ras inhibition, except in the case of YC, suggesting Ras-dependent and independent mechanisms appear to govern YC-dependent transformation (Dankort *et al.*, 2001b). Transgenic flies expressing *neu^{YC}* were moderately sensitive to Ras function. Furthermore, mutations in genes that function upstream or downstream of Ras, such as *Sos* and *Raf*, do not strongly suppress Neu^{YC} output (Settle *et al.*, 2003). This supports the suggestion that Neu^{YC} may function with and without Ras activation. Therefore, we propose that Neu^{YC} signals through a non-canonical Ras pathway or in a Ras-independent manner.

1.8 Thesis Objectives and Outline

It is the objective of this thesis to identify both Ras1-dependent and Ras1-independent modifiers of neu^{YC}. This was accomplished by the following two aims:

1. To conduct an extensive dosage-sensitive modifier screen for known members of the Ras1 pathway modifiers that genetically modify neu^{YC}.

2. To identify novel genetic modifiers of neu^{YC} by performing a chemical mutagenesis screen.

The success of these screens was based on the assumption that loss of one functional copy of a downstream component of Neu^{YC} signalling will perturb the hyperactivated signalling pathway caused by the ectopic expression of neu^{YC}. This would lead to an enhancement or suppression that is easily identified in a phenotypic output, such as a notch phenotype in the wing or an ommatidial fusion phenotype in the compound eye.

The results from these experiments reveal a strong dependency on Ras GTPases for YC-specific output. In addition, novel Ras-dependent and Ras-independent genetic modifiers of neu^{YC} have been identified. Results from these experiments will be presented.

CHAPTER 2 : IDENTIFICATION OF DEPENDENCY ON THE SMALL GTPASE RAS IN NEU^{YC}-SPECIFIC SIGNALLING

2.1 Introduction

The ErbB2 orphan receptor, a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs), is characterized extracellular binding domain that is in a constitutively active state that renders the receptor incapable of ligand binding (reviewed by Citri *et al.*, 2003). ErbB2 transcription is amplified in 20-30% of breast and ovarian cancers, and is inversely correlated with patient survival. The overexpression of wild-type and constitutively active alleles of ErbB2 and Neu, the rat ortholog of ErbB2, in mammary epithelia result in the formation of metastatic tumours in transgenic mice (Siegel *et al.*, 1994).

Activation of Neu catalytic function results in the autophosphorylation of conserved tyrosine residues in the C-terminal cytoplasmic tail (Akiyama *et al.*, 1991; Mikami *et al.*, 1992). These phosphorylated tyrosine (pTyr) residues serve as docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Fantl *et al.*, 1992; Li *et al.*, 1996; van der Geer *et al.*, 1995). This activates discrete signalling pathways that regulate cell survival, proliferation and differentiation. For example, Neu activation is coupled to the activation of enzymes, such as PLCγ and Src kinase (Muthuswamy *et al.*, 1994; Peles *et al.*, 1991). Other proteins serve as adaptors, bridging Neu to downstream targets.

Biochemical analysis of Neu signalling through individual autophosphorylation sites revealed transformation of rat fibroblast cells by 4 (Y1144/YB, Y1201/YC, Y1227/YD, Y1253/YE) of 5 conserved pTyr (Dankort *et al.*, 1997). Transformation was mediated through the binding of Ras-specific adaptor proteins Grb-2 (YB), Crk (YC) and

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SHC (YD) (Dankort *et al.*, 1997; Dankort *et al.*, 2001a; Dankort *et al.*, 2001b). The

specific pTyr Y1144 binds the adaptor protein Grb-2 (Dankort *et al.*, 1997; Dankort *et al.*,

2001a; Dankort *et al.*, 2001b; Khoury *et al.*, 2001). Grb-2 is constitutively bound to the

guanine nucleotide exchange factor (GEF) Sos by SH3 domain interactions (Olivier *et al.*,

1993; Rozakis-Adcock *et al.*, 1993). Sos activation of Ras activates multiple effector

pathways, including the Raf/MAPK serine kinase cascade, the PI3'K/Akt/PKB pathway

and RalGDS (Bergmann *et al.*, 1998). Identification of a requirement for Ras

downstream of individual Neu pTyr was determined by competitive inhibition of Ras in

Rat1 fibroblasts using either anti-Ras neutralizing antibody or Rap1A inhibition of Ras.

In both assays, Ras was required downstream of YB, YD and YE.

Genetic analysis is an efficient tool for the identification of signalling pathways.

Model organisms, such as *C. elegans* and *Drosophila*, possess many adaptor and second

messenger proteins that are functionally conserved (Stern *et al.*, 1993). Previously we

have introduced constitutively active mutant *neu* alleles in *Drosophila* to genetically

dissect signalling pathways activated by individual Neu autophosphorylation sites (Settle

et al., 2003). Neu signalling is evolutionarily conserved, as overexpression of Neu directs

tissue development in a manner similar to *Drosophila* EGFR. Similar to the vertebrate

model, genetic modifier studies revealed YD function is dependent on SHC, Sos and Ras.

Although Grb-2 signalling was not specific to YB, YB signaled in a Sos- and Ras-

dependent manner.

In both model organisms, signalling activated by the third autophosphorylation

site, YC, appears to function in both a Ras-dependent and Ras-independent manner. In

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rat fibroblasts, Ras inhibition by anti-Ras neutralizing antibody inhibited YC-dependent DNA synthesis, however, Rap1A inhibition of Ras had no effect on YC-dependent foci formation (Dankort *et al.*, 1997; Dankort *et al.*, 2001b). In *Drosophila*, lack of a strong interaction between YC and reduced Ras1 function supports the idea that YC function may have Ras1-dependent and independent elements (Settle *et al.*, 2003).

To resolve the ambiguity surrounding signalling from the YC autophosphorylation site, we have generated transgenic *Drosophila* ectopically expressing the YC autophosphorylation site in the developing adult wing and eye using the UAS/GAL4 system (Brand and Perrimon, 1993). We establish a quantitative approach for identifying phenotypic modification of Neu signalling in the adult wing margin. Using this quantitative approach, we expand on the dosage sensitive modifier screen of Ras1-specific modifiers reported previously (Settle *et al.*, 2003). We demonstrate a role for the conserved Ras/MAPK pathway downstream of YC activation. Furthermore, we identify the Ras2 GTPase as a novel modifier of YC-specific signalling. This study presents the YC autophosphorylation site as a unique site in Neu RTK signalling that functions independently of strong Ras1 activation.

2.2 Materials and Methods

2.2.1 Drosophila stocks and transgenic lines

Flies were raised on standard agar-based medium supplemented with essential salts and yeast.   All crosses were performed at 25°C unless otherwise stated. Mutant strains $dshc^{111-40, BG}$ (S. Luschnig), dab^{M54-R1} (E. Giniger), $dos^{1.46}$ (M. Simon), $Ras1^{5703}$ (D. Montell), phl^{1} (M.P. Martin), $dock^{P1}$ (Y. Rao) and Crk^{ED107} (J. Locke) were generously donated. The drk^{10626} (#12378), Sos^{34Ea-6} (#2240), phl^{11} (#5733), rl^{1} (#386), $Df(2R)rl^{10a}$ (#742), $Df(2R)M41A10$ (#741), $Dsor^{G42}$ (#7131), $Dsor^{G79}$ (#7136), $Dsor^{S-1221}$ (#8496), $Dsor^{LH110}$ (#5545), $Ras1^{06677}$ (#11694), $Ras1^{A014}$ (#16011), $Ras1^{EY07538}$ (#16839) and $Pax^{EY11633}$ (#20812) strains were obtained from the Bloomington Stock Center in Indiana, USA. Bloomington Stock Center stock numbers are indicated in parantheses and are subject to change.

Transgenes used in this study include *sevenless-GAL4* (M. Therrien) and *big bang^{C96}-GAL4* (Gustafson and Boulianne, 1996; Kim *et al.*, 2006). UAS-$Ras1^{V12C40}$, UAS-$Ras1^{V12G37}$ and UAS-$Ras1^{V12S35}$ were donated by F. Karim. The following transgenes were ordered from the Bloomington Stock Center in Indiana: UAS-$Ras1^{V12}$ (#4847), UAS-$Ras1^{N17}$ (#4846), $Pax^{EY02020}$ (#15849), UAS-$Ras2^{V14}$ (#2025) and UAS-$mCD8::GFP$ (#5137).

neu add-back allele cDNAs were donated by William Muller [Department of Biochemistry, McGill University (Dankort *et al.*, 1997)], and were subcloned into the pUAST transformation vector (Brand and Perrimon, 1993). Transgenic lines were made in the *yw⁻* fly strain using standard protocols.

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2.2.2 Lethality Counts

Progeny were counted and compared to the expected genotypic ratio for each cross. Percent lethality was calculated by dividing the observed number of progeny from the expected number of progeny and multiplying by 100. The expected number of progeny was determined by using the average of the observed progeny number from the other genotypes produced by the cross.

2.2.3 Wing preparations

Adults were dehydrated using a graded ethanol series (70%, 80%, 90%, 95% and 100%). Clipped wings were placed in methyl salicylate prior to mounting in DPX resin (Aldrich, St. Louis, MO, USA, 31, 761-6).

2.2.4 Light photomicroscopy

Wings were photographed using a Nikon CoolPix 991 digital camera mounted on a Nikon SMZ1500 Stereomicroscope.

2.2.5 Wing measurements

Loss of wing perimeter in photographed wings was measured using ImageJ release 1.38x software (Abramoff et al., 2004; http://rsb.info.nih.gov/ij/; http://www.macbiophotonics.ca/imagej/). Using the *freehand lines* tool, the existing wing margin tissue and the whole wing margin were traced and measured. The length of notches was determined by subtracting the length of the existing wing margin tissue from the length of the whole wing margin. Statistical analysis was performed using the Minitab Statistical Software for Windows (release 13.20; www.minitab.com). Students'

T-Test was performed on wing notch data. Ectopic vein data analysis was performed using the non-parametric Mann-Whitney U-Test.

2.2.6 Environmental scanning electron microscopy

Adults were frozen at -4.0°C. Heads were separated and mounted in a 1:1 glue and graphite mixture. Adult compound eyes were visualized at 1.0 Torr on an Electroscan 2020 Environmental Scanning Electron Microscope (ESEM) at a magnification of 140x.

2.3 Results

2.3.1 Ectopic expression of neu^{YC} causes defects in adult tissues

To rapidly assay changes to Neu^{YC} signalling for genetic screening, neu^{YC} was misexpressed in cells of the wing and eye imaginal discs under the transcriptional control of the UAS-GAL4 system. Phenotypic analysis of neu^{YC} misexpression in the dorsal-ventral boundary of the developing wing and in the developing eye has been previously reported (Settle $et\ al.$, 2003). In agreement with the previous study, neu^{YC} misexpression in the wing margin generated notches along the margin and expanded or ectopic vein tissue near the wing margin (Figure 2.1B). Notches are presumably due to cell death during growth of the wing disc, and both notch and vein formation phenocopy $Drosophila$ EGFR overexpression in these tissues (Settle $et\ al.$, 2003).

Previous analysis of neu^{YC} ectopic expression in the eye using the glass multimer reporter (GMR) resulted in 98.7% lethality (Settle $et\ al.$, 2003). A rough eye phenotype, characterized by loss of regular patterning of ommatidia and bristles, and fusion of ommatidial units, was observed in the escapers. Therefore, we selected a GAL4 strain with a more specific temporal and spatial expression pattern in the developing eye to reduce lethality caused by neu expression. During larval stages, $sevenless$ expression is restricted to presumptive R1, R3, R4, R6 and R7 photoreceptor cells, four cone cell precursors and mystery cells in the eye imaginal disc (Banerjee $et\ al.$, 1987a; Banerjee $et\ al.$, 1987b; Tomlinson $et\ al.$, 1987). Although homozygous $p[sev\text{-}GAL4]\ p[UAS\text{-}neu^{YC}]$ flies are lethal, flies carrying one copy of $p[sev\text{-}GAL4]\ p[UAS\text{-}neu^{YC}]$ are viable. Misexpression of one copy of $p[sev\text{-}GAL4]\ p[UAS\text{-}neu^{YC}]$ resulted in flies that possessed

a rough eye phenotype, presumably due to ommatidial fusion (Figure 2.1D). The rough

eye was identified by the reduction in individual ommatidial units due to increased unit

fusion, a decrease in interommatidial bristles and a general loss of regular ommatidial

patterning.

2.3.2 Identification of Neu^{YC} signalling: a quantitative approach in the wing

For our dosage sensitive genetic screen, we chose to examine the wing phenotype

in the primary screen for modifiers, and examine the eye phenotype to validate the

genetic interactions. *neu*^{YC} generated wing phenotypes when ectopically expressed by the

enhancer trap *big bang*^{C96}-*GAL4* (*bbg*^{C96}). Bbg encodes a PDZ domain-containing

protein that is expressed throughout embryonic peripheral nervous system, the larval

peripheral nervous system and the dorsal-ventral boundary of wing and haltere imaginal

discs (Kim *et al.*, 2006). To assay the wing phenotype in an unbiased manner, we made

three assumptions. First, that notches and ectopic veins resulting from *bbg*^{C96}-directed

expression of *neu*^{YC} can form at any location along the margin. Secondly, that the location

of the notch or ectopic vein does not correlate with the signalling strength of Neu^{YC}. And

third, that the wing is vulnerable to further tissue loss once the integrity of the margin has

been compromised by notch formation. Based on these assumptions we have opted to

assay Neu^{YC} signal output quantitatively by either measuring the length of the notches

formed per wing or by counting the number of ectopic veins formed per wing. A

statistically significant ($P < 0.05$) increase or decrease in the average notch length or

ectopic vein occurrence per wing would indicate enhancement or suppression of the *neu*^{YC}

phenotype, respectively.

To determine whether quantifying the wing notch or vein phenotype was the better approach, hypomorphic alleles drk^{10626} (Grb-2), and phl^{11} (Raf) were tested for suppression of neu^{YC}-dependent notches and veins. Reduction in drk or phl was previously reported to mildly suppress the neu^{YC} wing phenotype (Settle *et al.*, 2003). Notches removed $10.95 \pm 0.95\%$ of the wing margin in $p[GAL4]bbg^{C96}p[UAS$-$neu^{YC}]$ flies, and ectopic vein tissue formed in 1.9 ± 0.19 locations per wing (Figure 2.1B). Suppression of neu^{YC}-dependent notch formation was observed when flies were haplosufficient for drk ($3.06 \pm 0.39\%$, $P = 0.00$) or phl ($5.20 \pm 0.77\%$, $P = 0.00$) (Figure 2.2A & B; Tables 2.3 & 2.4). The neu^{YC}-dependent ectopic vein phenotype was also suppressed in flies haplosufficient for drk (1.0 ± 0.10, $P = 0.00$). Loss of one copy of phl, however, did not suppress this phenotype (2.0 ± 0.21; $P = 0.33$). This inconsistency between neu^{YC} wing notch and vein phenotypes in phl^{11} ; $p[GAL4]bbg^{C96}$ $p[UAS$-$neu^{YC}]$ heterozygotes suggested that assessment of wing notches was a more sensitive method for identifying modification of Neu^{YC} signalling compared to documenting the occurrence of the ectopic vein phenotype.

To validate the genetic interactions observed in the wing, we tested the same alleles for suppression of neu^{YC} expression in the eye. Both drk^{10626} and phl^{11} reduced neu^{YC}-dependent ommatidial fusion, seen by recovery of detectable ommatidia and interommatidial bristles (Figure 2.2C, D).

Figure 2.1: Ectopic expression of *neu^{YC}* disrupts adult wing and eye patterning.

[A-B] Neu^{YC} signalling produces wing margin defects. Percent average margin loss (%

Avg. ML) is reported in the bottom left corner and average number of ectopic veins (Avg.

EV) is reported in the bottom right corner of each panel. 'n' indicates sample size. [A]

p[GAL4]bbg^{C96}/+ adult wings consist of 5 longitudinal veins and 2 transverse veins. No

wing notches or ectopic veins are detected ($0.00 \pm 0.0\%$; 0.00 ± 0.0, respectively). [B]

p[GAL4]bbg^{C96} p[UAS-neu^{YC}] / + adult wing. On average, $10.95 \pm 0.95\%$ wing margin

loss (asterisk) and 1.9 ± 0.19 (arrowhead) ectopic veins are detected per wing.

[C-D] Neu^{YC} produces defects in ommatidia patterning. Female compound eyes were

visualized by environmental scanning electron microscopy at 140x magnification. [C]

Ommatidial patterning in *p[sev-GAL4]/+* adult compound eyes. Individual ommatidial

units and bristles are regularly spaced. [D] Ommatidial patterning is disrupted in *p[sev-*

GAL4] p[UAS-neu^{YC}] / + adult compound eyes. The number of individual facets and

bristles are reduced. An increased number of facets are fused with neighboring facets. A

higher degree of fusion occurs in the anterior portion of the eye, accompanied by a greater

loss of interommatidial bristles. Towards the posterior of the eye, individual facets and

bristles are detectable but are irregularly spaced.

Calibration is 100 μm.

A
% Avg. ML   Avg. EV
0.00; n = 33   0.0; n = 33

B
% Avg. ML   Avg. EV
10.95; n = 56   1.9; n = 56

C

D

Figure 2.2: Suppression of Neu^{YC} signalling reliably detected by wing notch measurement.

[A-B] A reduction in the notch length and in the number of ectopic veins is detected in adult wings. The percent average margin loss is indicated in the bottom left corner and the average number of ectopic veins is indicated in the bottom right corner of the panels. Statistical significance was determined by Students' T-Test for wing margin loss and by the non-parametric Mann-Whitney U-Test for ectopic veins, with P < 0.05 indicating suppression of *neu^{YC}*. 'n' refers to sample size. [A] *drk^{10626}/+ ; p[GAL4]bbg^{C96} p[UAS-neu^{YC}]/+* flies have margin loss of 3.06 ± 0.39% (P = 0.00) and form 1.0 ± 0.10 (P = 0.00) ectopic vein per wing. Both phenotypes are reduced compared to *p[GAL4]bbg^{C96} p[UAS-neu^{YC}]/+* flies (Figure 2.1B). [B] In *phl^{11}/+ ; p[GAL4]bbg^{C96} p[UAS-neu^{YC}]/+* flies, a decrease in wing notch formation is observed, with each wing missing 5.20 ± 0.77% (P = 0.00) of the margin compared to 10.95 ± 0.95% (Figure 2.1B). Suppression of ectopic vein phenotype in not detected in these flies. Each wing possesses 2.0 ± 0.21 (P = 0.33) ectopic veins, similar to 1.9 ± 0.91 ectopic veins in control heterozygotes. [C-D] Genetic interactions identified by notch lengths verified in the compound eye. Ommatidial fusion was suppressed in [C] *drk^{10626}/p[sev-GAL4] p[UAS-neu^{YC}]* and [D] *phl^{11}/+ ; p[sev-GAL4] p[UAS-neu^{YC}]/+* eyes. Individual facets and bristles are detected but lack typical arrangement.

Calibration is 100 μm.

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2.3.3 Ras GTPases function downstream of neu^{YC}

To closely examine the role of the Ras subfamily of GTPases in Neu^{YC} signalling, loss- and gain-of-function alleles of *Ras* were tested for suppression or enhancement of the *neu^{YC}*-dependent phenotypes in the wing and eye. The results of these experiments are summarized in Table 2.1. The *Drosophila* genome contains two *Ras* genes – *Ras85D* (*Ras1*) and *Ras64B* (*Ras2*) (Neuman-Silberberg *et al.*, 1984). *Ras1* is the *Drosophila* orthologue of 3 mammalian *Ras* genes – *H-Ras*, *K-Ras* and *N-Ras*. Ras1 was originally identified as a downstream component of the Sev RTK. Reduction in the dose of *Ras1* impaired Sev signalling during R7 specification, and overexpression of constitutively active *Ras1* rescued R7 photoreceptor loss in *sev* mutants (Fortini *et al.*, 1992). We have tested four hypomorphic alleles of *Ras1* for suppression of *neu^{YC}*. Flies heterozygous for *p[GAL4]bbg^{C96} ; p[UAS-neu^{YC}]* and either *Ras1^{06677}* or *Ras1^{EY07538}* had reduced notch length to 1.27 ± 0.47% and 5.22 ± 0.74% of the wing margin, respectively. In contrast to this, we have observed an enhancement of *neu^{YC}* wing notch formation by two *Ras1* alleles identified as hypomorphs. *Ras1^{A014}* and *Ras1^{5703}* increased notch lengths to 15.99 ± 1.0% and 17.00 ± 0.80% of the wing margin, respectively. Differences observed between *Ras1* alleles and observed between the eye and wing tissues were most likely inherent to the region of *Ras1* disrupted by the P-element insertions or may have been due to secondary insertions in the genetic background.

The kinase domain of Neu retains weak signalling capabilities in the absence of activating transphosphorylation (Dankort *et al.*, 1997; Settle *et al.*, 2003). The effects of Ras1 on kinase signalling may be sufficient to explain the modification of YC

Table 2.1: Hypomorphs of *Ras1* modulate Neu^{YC} signalling

Modifier Alleles	Mammalian Orthologue	Function	% Average Margin Loss	Modification	
				Wing	Eye
neu^{YC}	ErbB2	RTK	10.95; ($n = 56$)		
Ras1^{5703}	H-Ras, K-Ras, N-Ras	GTPase	17.00; $P = 0.00$ ($n = 164$)	+	+
Ras1^{06677}	H-Ras, K-Ras, N-Ras	GTPase	1.27; $P = 0.00$ ($n = 52$)	-	-
Ras1^{A014}	H-Ras, K-Ras, N-Ras	GTPase	15.99; $P = 0.00$ ($n = 77$)	+	-
Ras1^{EY07538}	H-Ras, K-Ras, N-Ras	GTPase	5.22; $P = 0.00$ ($n = 67$)	-	-

("+" = enhancement; "-" = suppression)

phenotypes, and it is possible that Neu^{YC} also functions independently of Ras1. This is supported by the observation that $Ras1^{A014}$ and $Ras1^{06677}$ also suppressed the kinase domain eye phenotype, while the other *Ras1* alleles, all moderate modifiers of *neu*^{YC}, did not affect the kinase domain phenotype (Figure 2.3).

To further delineate the role of *Ras1* in *neu*^{YC} function, we sought to overexpress a number of transgenic *Ras1* activating mutations. The introduction of two *UAS* transgenes under the control of a single *GAL4* regulatory element may reduce the expression of *neu*^{YC} by reducing the GAL4 protein population available to a single UAS site. We ectopically expressed one copy of *p[UAS-mCD8::GFP]* in the background of *p[GAL4]bbg*^{C96} *p[UAS-neu*^{YC}*]* heterozygotes to control for this possibility. Having both *p[UAS-mCD8::GFP]* and *p[UAS-neu*^{YC}*]* under the transcriptional control of the *p[GAL4]bbg*^{C96} regulatory element reduced the *neu*^{YC}-induced wing notch phenotype to $6.56 \pm 0.72\%$ from $10.95 \pm 0.95\%$ in *p[GAL4]bbg*^{C96} *p[UAS-neu*^{YC}*]* heterozygous flies.

Overexpression of constitutively active *Ras1* transgene, *UAS-Ras1*^{V12}, strongly enhanced *neu*^{YC} in the wing ($31.80 \pm 1.6\%$; Figure 2.4). The *neu*^{YC}-dependent rough eye was also enhanced in the 5.6% surviving *p[sev-GAL4] p[UAS-neu*^{YC}*] / p[UAS-Ras1*^{V12}*]* female heterozygotes (Figure 2.5H). Consistent with the hypothesis that *Ras1* acts downstream of *neu*^{YC}, ectopic expression of dominant negative *UAS-Ras1*^{N17} strongly suppressed the *neu*^{YC}-dependent wing notches ($0.12 \pm 0.11\%$) and mildly suppressed facet fusion in the eye (Figures 2.4 and 2.5I). Although *UAS-Ras1*^{V12} overexpression strongly enhanced the catalytic domain eye phenotype to the extent that it resembled that of *neu*^{YC}, it did not cause lethality in *neu*^{NYPD}-expressing heterozygotes (Figure 2.5N). *UAS-*

$Ras1^{N17}$, however, is epistatic to the catalytic domain (Figure 2.5O). Therefore, although

Neu^{YC} appears to signal in a Ras1-dependent manner, Ras1 activation does not appear to

be the primary pathway for transducing signals from Neu^{YC}.

Ras1 connects to a number of different effectors to influence cell survival, gene

expression and cytoskeleton remodeling (Vojtek and Der, 1998). We have, therefore,

examined 3 *Ras1* effector domain mutants to identify the effector pathways activated by

neu^{YC}. Results are summarized in Figure 2.4. $Ras1^{V12S35}$, $Ras1^{V12C40}$ and $Ras1^{V12G37}$

couple to the Raf/MAPK, PI3K and RalGDS second messenger systems, respectively

(Karim and Rubin, 1998). Overexpression of $UAS-Ras1^{V12S35}$ and $UAS-Ras1^{V12G37}$

strongly enhanced neu^{YC}-induced wing notches ($12.15 \pm 0.90\%$ and $14.70 \pm 1.4\%$,

respectively) and overexpression of $UAS-Ras1^{V12C40}$ mildly enhanced neu^{YC}-dependent

wing notches ($8.76 \pm 0.72\%$), when compared to $p[GAL4]bbg^{C96} p[UAS-neu^{YC}] / p[UAS-mCD8::GFP]$. All three Ras1 effector domain mutations also enhanced the neu^{YC}-

dependent eye phenotype, as well as the kinase domain-dependent eye phenotypes

(Figure 2.5J-L, P-R). Enhancement of the neu^{YC} phenotypes by $Ras1^{V12S35}$ appeared to be

additive; overexpression of $Ras1^{V12S35}$ in $p[sev-GAL4]$ $p[UAS-neu^{YC}]$-expressing flies

resulted in 100.0% lethality in males and 36.5% lethality in females. Gender differences

observed in $p[sev-GAL4]$ $p[UAS-neu^{YC}]$-expressing flies are most likely due to X

chromosome dosage compensation in males. An increase in *sev* transcription would lead

to an increase in $p[sev-GAL4]$ transcription, heightening the potency of the GAL4 (Zhang

and Oliver, 2007). Lethality was not observed in flies heterozygous for $p[sev-GAL4]$ and

the Ras1 effector domain mutations. Taken together, this suggests that the Raf/MAPK

Figure 2.3: Kinase domain is suppressed by loss of *Ras1*.

Electron micrographs of adult compound eyes taken at 140X. [A] Control eyes ectopically expressing *neu^{NYPD}* using *sev-GAL4*. Mild ommatidial fusion, minor bristle loss and bristle duplication are visible in these eyes. The [B] *Ras1^{5703}* and [E] *Ras1^{EY07538}* alleles do not modify *neu^{NYPD}* rough eye. Suppression of *neu^{NYPD}* mild eye phenotype is seen in flies also heterozygous for [C] *Ras1^{A014}* and [D] *Ras1^{06677}*, indicated by a decrease in ommatidial fusion and bristle patterning defects.

Calibration is 100 μm in panels and 25 μm in the insets.

Figure 2.4: Ras1 effector domain mutants enhance *neu^{YC}* wing notches.

Bar graph comparing the effects of the Ras1 effector domain mutations on *neu^{YC}* wing

margin loss. Horizontal and vertical axes indicate mutant strains and the average wing

margin loss in percentage, respectively. The legend to the right indicates that mutant

strains crossed to *bbg^{C96}* are in light red, while mutant strains crossed to *p[GAL4]bbg^{C96}*

p[UAS-neu^{YC}] are in dark red. Standard errors bars are shown for data set.

Overexpression of a second UAS construct, *p[UAS-mCD8::GFP]*, in *p[GAL4]bbg^{C96}*

p[UAS-neu^{YC}] flies produces wing margin loss of 6.56 ± 0.72% per wing. *Ras1^{V12}*

produces a wing notch phenotype when overexpressed by *p[GAL4]bbg^{C96}* (11.87 ±

0.97%). *Ras1^{V12}* strongly enhances *neu^{YC}* average wing notch length to 31.80 ± 1.6%.

Ras1^{N17} completely suppresses *neu^{YC}* wing notches (0.12 ± 0.10%). Overexpression of

Ras1 effector domain mutations *V12G37*, *V12C40* and *V12S35* do not produce wing

notches in *p[GAL4]bbg^{C96}* heterozygous flies, however, enhance *neu^{YC}* margin loss (14.7

± 1.5%, 8.76 ± 0.72% and 12.15 ± 0.90%, respectively). Similar to *Ras1* alleles, *Ras2^{V14}*

does not result in margin loss when misexpressed in the wing margin, but does enhance

neu^{YC} margin loss to an average of 17.40 ± 2.4% per wing. Enhancement and suppression

of *neu^{YC}* by *Ras1* alleles is more than additive, since overexpression of *Ras1^{V12}* and

Ras2^{V14} results in lethality of *neu^{YC}*-expressing flies, suggesting a role for *Ras1*

downstream of *neu^{YC}* (see text).

Overexpression of *Ras* Modifies *neu^YC* in the Wing Margin

Figure 2.5: Ras1 effector domain mutants enhance neu^{YC} and neu^{NYPD} eye phenotypes.

Female compound eyes visualized at 140x on a environmental scanning electron microscope are shown. Reduction in *neu* add-back allele expression due to expression of a second UAS construct is controlled for by ectopic expression of *p[UAS-mCD8::GFP]* in control flies. [A] Ectopic expression of *UAS-mCD8::GFP* by *sev-GAL4* does not modify eye morphology. [B-F] Overexpression of *Ras1* gain-of-function alleles by *sev-GAL4* produces a range of rough eye phenotypes in the adult eye, due to fusion of ommatidia and defects in bristle patterning. Overexpression of [B] *Ras1^{V12}*, [C] *Ras1^{N17}* and [F] *Ras1^{V12S35}* produces moderate fusion of ommatidia and bristle loss. [D] *Ras1^{V12G37} ; sev-GAL4* and [E] *Ras1^{V12C40} ; sev-GAL4* heterozygous flies possess mild ommatidia fusion and minor defects in bristle patterning. [G] Modification of eye development in *p[sev-GAL4] p[UAS-neu^{YC}] / p[UAS-mCD8::GFP]* heterozygotes results in moderate fusion of individual ommatidium. [H] Overexpression of *Ras1^{V12}* strongly enhances facet fusion in *neu^{YC}*-expressing flies. This is observed by an increase in fusion and flattening of eye surface, and fewer bristles within the fused tissue. [J] *Ras1^{V12G37}*, [K] *Ras1^{V12C40}* and [L] *Ras1^{V12S35}* are moderate to strong enhancers of *neu^{YC}*-dependent ommatidial fusion, respectively. [I] *Ras1^{N17}* allele mildly suppresses the fusion phenotype in *neu^{YC}* heterozygotes. [M] *neu^{NYPD}* expression results in a mild rough eye phenotype, characterized by loss or duplication of interommatidial bristles and mild ommatidial fusion in the posterior eye. [N] Overexpression of *Ras1^{V12}* strongly enhances ommatidial fusion throughout the eye, with the greatest fusion occurring in the anterior

portion of the eye.　[O] The expression of $Ras1^{N17}$ masks the neu^{NYPD}-dependent rough

eye, suggesting that signalling through the catalytic domain of Neu is dependent on Ras1.

Overexpression of [P] $Ras1^{V12G37}$ and [Q] $Ras1^{V12C40}$ enhances the neu^{NYPD} eye

phenotype.　[R] Like $Ras1^{N17}$, overexpression of $Ras1^{V12S35}$ masks the neu^{NYPD} rough eye,

also suggesting that signalling from the catalytic domain of Neu is dependent on Ras1.

Calibration is 100 μm.

pathway is activated by Neu^{YC}-dependent Ras1 activation, but that this is not the only signalling pathway activated by Neu^{YC}.

The second *Drosophila Ras* gene, *Ras2*, is orthologous to mammalian R-Ras (Mozer *et al.*, 1985; Brock, 1987). Although *Ras2* is expressed in the developing eye disc like *Ras1*, constitutively active *Ras2, Ras2^{V14}*, did not rescue the missing R7 photoreceptor cell phenotype of *sevenless* mutants, suggesting Ras2 is not a downstream target of Sev RTK (Fortini *et al.*, 1992). Overexpression of *Ras2^{V14}* was lethal in 100% of male and 80% of female *p[sev-GAL4] p[UAS-neu^{YC}]* heterozygous flies. All female *p[sev-GAL4] p[UAS-neu^{YC}]/p[UAS-Ras2^{V14}]* survivors had an enhanced rough eye phenotype (Figure 2.6I). Similarly, 75% lethality was observed in *p[UAS-Ras2^{V14}]/+ ; p[GAL4]bbg^{C96} p[UAS-neu^{YC}]/+* flies. Wing notches were enhanced (17.40 ± 2.4%) in the surviving progeny. Lethality was also observed in *p[UAS-Ras2^{V14}] ; p[GAL4]bbg^{C96}* flies (66.6%), however, wings from these control flies had no notches at the wing margin. *big bang* encodes a PDZ protein that is expressed throughout embryonic and larval development (Kim *et al.*, 2006). The lethality observed in *bbg^{C96}*-directed expression of *Ras2^{V14}* is likely due to earlier expression of *Ras2^{V14}* during embryogenesis or early larval stages, and not a result of enhanced Neu^{YC} signal output in the wing.

To determine whether Ras2 is specific to Neu^{YC} signalling, modification of the kinase domain, *neu^{YB}* and *neu^{YE}* by Ras2 was tested. Ectopic expression of *Ras2^{V14}* masked the kinase domain-dependent rough eye phenotype (Figure 2.6F & G). *Ras2^{V14}* enhanced the rough eye phenotypes of both *neu^{YB}* and *neu^{YE}* (Figure 2.6H & J). Unlike *neu^{YC}*, lethality was not observed in these flies. This suggests *Ras2* is not specific to the

53

YC pTyr. Results of ectopic expression studies can be misleading, since transcription by

the UAS-GAL4 system generates an over-abundance of target gene expression. This can

lead to aberrant behaviour of the target gene product. Therefore, these genetic

interactions may be a secondary effect due to the over-abundance of *Ras2* expression by

the UAS-GAL4 system. Without a loss-of-function mutation, we are unable to determine

if Ras2 is directly involved in the Neu pathway or if it acts in a pathway parallel to Neu.

2.3.4 Identification of adaptor proteins that mediate Neu^{YC} signalling

Following identification of Ras1 as a downstream component of Neu^{YC}, we next

asked if adaptor proteins known to be involved in the Ras1 pathway are specific to *neu*^{YC}.

Hypomorphic alleles of a number of Ras1-specific adaptors were tested for suppression of

neu^{YC}. Results are summarized in Table 2.2. The majority of adaptors tested, like *dock*

and *Dab*, appeared to function in a tissue-specific manner as they only suppressed one of

the overexpression phenotypes. As mentioned earlier, loss of one copy of *drk* reduced

wing notch length and ommatidial fusion. Consistent with Drk activation of the GEF Sos,

wing notches and rough eyes were suppressed in *neu*^{YC}-expressing flies with one

functional copy of *Sos*.

2.3.5 Members of the Raf/MAPK pathway suppress Neu^{YC} signalling

Lethality and enhancement phenotypes observed in *p[sev-GAL4] p[UAS-neu*^{YC}*]*

flies overexpressing *Ras1*^{V12S35} suggests a strong involvement of the Raf/MAPK pathway.

We, therefore, investigated the roles of *Raf* (*phl*), *MEK* (*Dsor*) and *MAPK* (*rl*) in *neu*^{YC}

signalling (Table 2.3). Two hypomorphic alleles of *phl* suppressed *neu*^{YC}-dependent wing

notch and rough eye phenotypes. A genetic null of the *rl* gene, *rl*^{10A}, suppressed *neu*^{YC}

phenotypes. The rl^l allele enhanced neu^{YC} phenotypes and also enhanced neu^{YD} rough

eye phenotype (Table 2.3 and data not shown, respectively). This is unexpected, as rl^l

suppresses activated *Ras1* and *Ras2*. Furthermore, rl^l mutants lack most R7 cells and

have a reduced number of other photoreceptor cells in adult eyes (Hilliker, 1976; Peverali

et al., 1996). Together, this data suggests that Rl functions to positively transduce signals

from Neu^{YC}, but that the region affected by the rl^l mutation may negatively regulate this

signalling. Two *Dsor* alleles, $Dsor^{G79}$ and $Dsor^{S-1221}$, suppressed wing notches, but

enhanced ommatidial fusion. Tissue specific roles for *Dsor* may contribute to the

conflicting interactions with neu^{YC} in wing and eye tissues. Therefore, we conclude

Neu^{YC} activates the canonical MAPK phosphorylation cassette to positively transduce

signals from activated Neu RTK.

Figure 2.6: *Ras2* enhancement of Neu pTyr signalling.

Adult compound eyes visualized by environmental scanning electron microscopy at 140x magnification. *p[UAS-mCD8::GFP]* is misexpressed in control eyes [A-E] to account for reduction in *neu* add-back allele expression by *p[sev-GAL4]*. [A] Flies heterozygous for *p[UAS-mCD8::GFP]* and *p[sev-GAL4]* possess wild-type eyes. *p[sev-GAL4] p[UAS-mCD8::GFP]* flies also heterozygous for [B] *p[UAS-neu^{NYPD}]*, [C] *p[UAS-neu^{YB}]*, [D] *p[UAS-neu^{YC}]* and [E] *p[UAS-neu^{YE}]* show varying degrees of ommatidial fusion. [F] Overexpression of *Ras2^{V14}* results in an increase in size of facets and a decrease in the total number of facets. [G] A comparable phenotype is observed in *p[UAS-neu^{NYPD}]* ; *p[Ras2^{V14}]* / *p[sev-GAL4]* flies. [H-J] An increase in ommatidial fusion in the anterior of the eye is observed when *Ras2^{V14}* is overexpressed in *neu^{YB}*- [H], *neu^{YC}*- [I] and *neu^{YE}*- [J] expressing flies.

Calibration is 100 μm.

Table 2.2: Specific adaptors suppress *neu*^{YC}

Modifier Alleles	Mammalian Orthologue	Function	% Average Margin Loss (T-test)	Modification	
				Wing	Eye
neu^{YC}	ErbB2	RTK	10.95; ($n = 56$)		
D-Shc$^{111-40}$	SHC	SH2/SH3 adaptor	13.09; $P = 0.08$ ($n = 98$)	NE	+
drk^{10626}	GRB-2	SH2/SH3 adaptor	3.06; $P = 0.00$ ($n = 109$)	-	-
Dab^{M54-R1}	Dab	SH2/SH3 adaptor	7.34; $P = 0.00$ ($n = 98$)	-	+
dock^{P1}	Nck	SH2/SH3 adaptor	7.93; $P = 0.02$ ($n = 60$)	-	NE
Crk^{ED107}	Crk	SH2/SH3 adaptor	12.40; $P = 0.30$ ($n = 65$)	NE	-
dos$^{1.46}$	Gab-1	PH/SH2/SH3 adaptor	10.74; $P = 0.88$ ($n = 54$)	NE	-
Sos^{34Ea-6}	Sos	GNEF	6.58; $P = 0.00$ ($n = 217$)	-	-

("+" = enhancement; "-" = suppression; "NE" = no effect)

Table 2.3: Raf/MAPK pathway signal downstream of Neu^{YC}

Modifier Alleles	Mammalian Orthologue	Function	% Average Margin Loss (T-test)	Modification	
				Wing	Eye
neu^{YC}	ErbB2	RTK	10.95; ($n = 56$)		
phl^{1}	Raf; MAPKKK	Kinase	5.72; $P = 0.00$ ($n = 177$)	-	-
phl^{11}	Raf; MAPKKK	Kinase	5.20; $P = 0.00$ ($n = 50$)	-	-
Dsor^{G42}	MAPKK	Kinase	8.70; $P = 0.07$ ($n = 71$)	NE	NE
Dsor^{G79}	MAPKK	Kinase	3.71; $P = 0.00$ ($n = 68$)	-	+
Dsor^{S-1221}	MAPKK	Kinase	3.34; $P = 0.00$ ($n = 50$)	-	+
Dsor^{LH110}	MAPKK	Kinase	9.18; $P = 0.69$ ($n = 55$)	NE	NE
rl^{10a}	MAPK	Kinase	6.00; $P = 0.00$ ($n = 69$)	-	-
rl^{1}	MAPK	Kinase	21.08; $P = 0.00$ ($n = 141$)	+	+

("+" = enhancement; "-" = suppression; "NE" = no effect)

2.4 Discussion

In this study, we report that the expression of neu^{YC} generates phenotypes similar to those previously reported in *Drosophila* wing margin and compound eye development (Settle *et al.*, 2003). neu^{YC} expression modifies development of adult tissues in a manner similar to endogenous *Drosophila* EGFR. Phenotypes produced in both tissues are moderate; therefore, we can use these phenotypes as a baseline for the identification of enhancers and suppressors of neu^{YC}. A reduction in component levels by loss of one genetic copy has been sufficient to identify novel regulators of signal transduction pathways (Karim *et al.*, 1996; Therrien *et al.*, 2000). Indeed, haplosufficiency of components have identified modifiers of the Neu signalling pathway in *Drosophila* (Settle *et al.*, 2003). We have used dosage sensitive genetics to screen for Ras-dependent modifiers that positively regulate signals from Neu^{YC}.

We applied a quantitative measure to identify changes to the wing notch phenotype in our dominant modifier screen. This provides a sensitive and accurate approach to identifying suppressions and enhancements. The technique was validated by the identification of *Grb-2-* and *Raf*-dependent suppression of neu^{YC}, as previously reported in the wing margin (Settle *et al.*, 2003). Other adaptors that function downstream of Neu^{YC}, such as Dab, Nck and Sos, were also identified using this approach (Settle *et al.*, 2003). We were also able to detect a greater magnitude of suppression by reduced Grb-2, previously identified as a moderate suppressor of neu^{YC}. Other modifiers were observed to have the opposite effect on Neu^{YC} signal output. Both DShc and Dos reduction had no effect on Neu^{YC} compared to the suppressing effects reported

60

previously. We observed contradictory results with the hypomorphic allele $Ras1^{5703}$.

This may be intrinsic to the $Ras1^{5703}$ strain. It is likely this strain carried more than one

P-element insertion (Spradling *et al.*, 1999). Loss of one of the P-element insertions

could contribute to the change in behaviour observed with neu^{YC}.

Neu^{YC} is positively regulated by Ras1. Strong sensitivity of Neu^{YC} to Ras1

reduction appears to be due to the combined effects of both Neu^{YC}- and kinase domain-

dependent signalling. The dominant negative allele $Ras1^{N17}$ is epistatic to signalling from

the kinase domain, suggesting that the kinase domain functions independently or

upstream of Ras1.

Strong sensitivity to reduced Grb-2 function suggests Ras1 activation by Neu^{YC}

may be Grb-2 dependent, although Grb-2 is not required for Neu^{YC} transformation in rat

fibroblasts (Dankort *et al.*, 1997). Our observations are further supported by the moderate

suppression of neu^{YC} by reduced *Sos*, an activator of Ras1 (Lowenstein *et al.*, 1992;

Buday and Downward, 1993). The Crk adaptor protein binds to Neu^{YC} *in vitro* (Dankort

et al., 2001a). We observed mild Crk-dependent modification of Neu^{YC} signalling in a

tissue-specific manner. Since *Grb-2* or *Ras1* haplosufficiency is sufficient to completely

suppress neu^{YB} but not neu^{YC}, additional Ras1-independent effects of YC are likely.

Surprisingly, constitutively active Ras2 augments Neu^{YC} signalling. In addition,

we also observed that Ras2 enhances signal output from other pTyr, including the

catalytic domain of Neu, suggesting that parallel activity of Ras2 is not specific to Neu^{YC}.

Ras2 is not a downstream target of canonical RTK signalling in *Drosophila* and by itself

did not modify wing margin morphology in our assay (Fortini *et al.*, 1992). Little else is

known regarding the function of Ras2 in *Drosophila*. Mammalian R-Ras binds Ras

effectors Raf kinase, PI3'K and RalGDS *in vitro* (Spaargaren *et al.*, 1994; Herrmann *et*

al., 1996). It most potently activates PI3'K, but also activates Raf and RalGDS to a lesser

degree (Herrmann *et al.*, 1996; Marte *et al.*, 1997).

The combination of R-Ras and H-Ras activation is believed to regulate cell

adhesion. R-Ras was shown to promote integrin activation by antagonizing H-Ras-

mediated integrin suppression without directly inhibiting H-Ras (Sethi *et al.*, 1999).

Studies suggest R-Ras promotes focal adhesion formation, and that targeting of R-Ras to

focal adhesions is critical for its ability to regulate integrin function (Furuhjelm and

Peranen, 2003; Kwong *et al.*, 2003). Neu/ErbB2 cooperates with FAK signalling to

contribute to the metastatic properties of Neu signalling (Benlimame *et al.*, 2005).

Additionally, Neu^{YC}-expressing cells in mammary tumour explants are highly motile and

have smaller mature focal adhesions that are reduced in number (Northey *et al.*, 2008).

R-Ras involvement in Neu^{YC} signalling may potentiate the metastatic nature of this pTyr

output. Involvement of R-Ras in Neu^{YC}-dependent signalling will be resolved with the

use of loss-of-function or dominant negative R-Ras alleles.

In conclusion, we show Neu^{YC} is positively regulated by *Drosophila* Ras1 and

Ras2 GTPases. Ras1 activation by Neu^{YC} most likely occurs through Grb-2 and Sos;

however, the Ras1 pathway does not appear to be the sole pathway activated by Neu^{YC}.

Ras2 may be a direct target of Neu^{YC} or it may be activated in parallel to Neu activation.

Future studies using chemical mutagenesis or loss-of-function analysis will seek to

identify a Ras1-independent mechanism involved in the regulation of Neu^{YC}.

CHAPTER 3 : DOMINANT ENHANCER-SUPPRESSOR SCREEN TO IDENTIFY NOVEL MODIFIERS OF NEU^{YC}-SPECIFIC SIGNALLING

3.1 Introduction

Chemical mutagenesis is a method widely used in *Drosophila* for the *de novo* synthesis of DNA lesions. The purpose of generating genetic lesions is to create mutant alleles that can then be screened to identify novel genes of interest. Several chemicals are well characterized for their effects in *Drosophila*, including ethyl methane sulfonate (EMS), methyl methane sulfonate, ethylnitrosourea and triethylmelamine. A full list of chemical mutagens that affect *Drosophila* can be found at the Environmental Mutagen Information Center at the Oak Ridge National Laboratory (Ashburner *et al.*, 2005).

The use of EMS has become a standard method for *Drosophila* mutagenesis since its introduction by Lewis and Bacher (1968). It is a highly effective mutagen that is easy to administer to flies. EMS is an alkylating agent that produces GC to AT transitions (Pastink *et al.*, 1991; Bentley *et al.*, 2000). Alkylation of guanine residues produces the abnormal base O-6-ethylguanine. O-6-ethylguanine can mispair with thymine, and over successive rounds of replication, the GC pair can become an AT pair. Disruption of functional gene products is the result of point mutations in the form of missense or nonsense mutations within the coding sequence or at splice sites. It was widely believed that transitions were the only lesion created by EMS; however, this is not the case. Aberrations, such as transversions, duplications and deletions, are also possible, albeit at lower frequencies. The proportion of aberrations to point mutations following EMS treatment is dependent on the concentration used and the extent to which females are allowed to store sperm before laying (Grigliatti, 1998).

EMS alkylation affects only one strand of the DNA helix (Grigliatti, 1998). This triggers DNA repair mechanisms during spermatogenesis and even in embryos well after first cleavage (Pastink *et al.*, 1991; Bentley *et al.*, 2000). DNA repair in embryos generates a high percentage of mosaics in the F1 generation. This leads to the identification of a high number of false positives in screens where the phenotype is assessed in the F1 generation. An extra generation is often included in the mating protocol to remove the flies carrying somatic mutations from the candidate pool. Mosaicism is not a problem in screens where the phenotype is scored later (Grigliatti, 1998; reviewed by Bokel, 2008).

Several techniques are available for the administration of chemical mutagens. The three main techniques are by feeding adults, by fluid injection and by vapour exposure. The chosen route is dependent on the suitability to the chemical by its characteristics and by its effectiveness when administered to different developmental stages (Ashburner *et al.*, 1989).

Adult feeding is the preferred method for administering chemical mutagens, as it is a very simple and straightforward technique. The chemical is dissolved in a sucrose solution to increase palatability. Flies are starved for 12 hours prior to exposure. Tissue or filter paper is saturated with the chemical solution. Flies are then exposed to the mutagen for 12-24 hours. One problem with feeding is maintaining accurate dosimetry. Individuals vary in the amount they feed. Additionally, flies can also take up the chemical by vapour exposure or by direct contact with the solution (Ashburner *et al.*, 1989).

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Fluid injection uses needles drawn from 1mm outer diameter glass capillary

tubing and have tip diameters of 100μm (Ashburner *et al.*, 1989). Tips have a 45° bevel

and a sharp point. Adults are injected between abdominal sternites. Injection is still

preferred where mutagens are unstable in solution or food media. Fluid injection also

suffers from problems with dosimetry, as loss of fluid can occur after injection.

Vapour or gas exposure of adult flies results in the uptake of the desired mutagen

by its spiracles or trachea (Ashburner *et al.*, 1989). Adults may, however, close spiracles

in noxious environments, thereby affecting the effective dose of the mutagen.

EMS is normally administered to adults by feeding, popularized by Lewis and

Bacher (1968). At an average concentration of 25mM, new mutations are induced on

average once every 150 kb (reviewed by Bokel, 2008). This corresponds to

approximately 50 lesions within open reading frames per chromosome, or it corresponds

to the observation of 1 to 2 lethal mutations per chromosome. This varies greatly,

however, as mutations in large genes, such as *dumpy*, have been recovered at greater than

20 times this rate (Ashburner *et al.*, 1989; reviewed by St. Johnston, 2002).

EMS mutagenesis is used in a number of breeding programs to identify different

types of mutations. This includes, but is not limited to, sex-linked recessive mutations,

autosomal recessive mutations and dominant modifier screens (Grigliatti, 1998). In

screens for sex-linked recessive mutations and autosomal recessive mutations, the

identification of mutations based on lethality or a visible phenotype occurs in the F2 or F3

generations, once the mutation has been made homozygous. Dominant modifier screens

are used to modulate a phenotype that is caused by a sensitized genetic background.

Sensitization of the genetic background can be achieved by removing an element or by hyperactivation of a specific signalling pathway. Screening for mutations occurs in the F1 generation. Recovery is prone to a high number of false-positives due to mosaicism. A second generation of mating is included to eliminate individuals that are mosaic for modifiers from the pool of candidates.

The study of Neu^{YC} signalling involves the identification of downstream modifiers. In this chapter, a dominant modifier screen using EMS mutagenesis was conducted in an attempt to identify and isolate novel modifiers involved in Ras1-dependent and independent Neu^{YC} signalling. After screening approximately 19 100 flies for enhancement or suppression of a *neu^{YC}*-dependent phenotype, 17 modifiers were isolated. Modifiers were analyzed for their specificity to the YC pTyr and to the Ras1 GTPase. One modifier that suppressed general Neu signalling was identified as a target of MAPK phosphorylation, providing validity for our screen. In addition to an analysis of these findings, suggestions for improvement will also be discussed.

3.2 Materials and Methods

3.2.1 Stock Maintenance

Drosophila stocks were maintained in a common room at $21.0 \pm 2.0°C$, unless

otherwise noted. The room was set-up to follow a natural light/dark cycle. Stocks were

changed every 2 weeks and treated with filter paper soaked with 0.5% tedion (4-

chlorophenyl 2,4,5-trichlorophenyl sulfone dissolved in acetone) biannually. Shelves

housing flies were kept clean periodically with a 70% ethanol solution and a solution of

50% benzyl benzoate dissolved in ethanol annually. Polycarbonate vials (Fisher

Scientific Canada, Ottawa, ON, AS519) and 16x100mm borosilicate tubes (VWR

International, Mississauga, ON, 47729-576) were used to house the flies. Vials were

plugged with rayon rope (Fisher Scientific Canada, Ottawa, ON, 1264041). Flies were

raised on an agar-based medium containing (final concentrations) 10% sugar, 0.8%

potassium sodium tartrate, 0.1% potassium dibasic phosphate and 0.05% each of sodium

chloride, calcium chloride, magnesium chloride and ferric sulphate. The solution was

then autoclaved and combined with a 5% autoclaved solution of dry active Baker's yeast

in ddH_2O. The mixture was allowed to partially cool to 55°C and treated with 0.7%

tegosept dissolved in ethanol and 0.5% acid mix (41.8% propionic acid and 5% o-

phosphoric acid).

3.2.2 Fly Strains

Transgenes used in this study include *sevenless-GAL4* (M. Therrien) and *big*

bang^{C96}-GAL4 (Gustafson and Boulianne, 1996; Kim *et al.*, 2006). *p[UAS-neu]* add-back

alleles used are described in Chapter 2. Meiotic mapping strains $al^1 dp^{ov1} b^1 pr^1 c^1 px^1 sp^1$

(#156), $al^l dp^{ovl} b^l p^l Bl^l c^l px^l sp^l$ (#214), $ru^l h^l th^l st^l cu^l sr^l e^s ca^l$ (#576) and

$ru^l h^l th^l st^l cu^l sr^l e^s Pr^l ca^l$ (#1711), deficiencies and P-element mapping strains (listed in

Appendix A) were obtained from Bloomington Stock Center (Bloomington, IN, USA), .

Transgenes *UAS-mCD8::GFP* (#5137), *UAS-eya.B.I* (#5676) and *UAS-eya.B.II* (#5675),

and mutant strains $eya^{cli-IID}$ (#3280), *In(2L)eya, eya^l* (#3631) and *eya^2* (#2285) were

ordered from Bloomington Stock Center, as well. Bloomington Stock Center stock

numbers are indicated in parantheses and are subject to change.

Crosses were carried out at 25°C in polycarbonate vials, for large-scale crosses,

and 16x100mm borosilicate tubes for smaller-scale crosses. After 4 days, parents were

transferred to a new polycarbonate vial or borosilicate tube containing fly media. In most

cases, reciprocal crosses were conducted.

3.2.3 Penetrance Calculations

Percent penetrance of EMS modifiers on the neu^{YC} eye phenotype was calculated

by counting the number of progeny with the modified phenotype, dividing it by the total

number of progeny of that genotype and multiplying by 100. A minimum of 20 eyes

were looked at under the ESEM.

3.2.4 Wing Preparations

Adults were anaesthetized with CO_2 gas, placed in 10x75mm borosilicate tubes

and dehydrated using a graded ethanol series (70%, 80%, 90%, 95% and 100%). Clipped

wings were placed in methyl salicylate prior to mounting in DPX resin (Sigma-Aldrich,

St. Louis, MO, USA, M6752).

3.2.5 Environmental Scanning Electron Microscopy

Adults were frozen at -4°C. Heads were separated and mounted in a 1:1 glue and graphite mixture. Eyes were visualized at 1.0 Torr on an Electroscan 2020 Environmental Scanning Electron Microscope (ESEM).

3.2.6 Light Photomicroscopy

Wings were photographed using a Nikon CoolPix 991 digital camera mounted on a Nikon SMZ1500 Stereomicroscope.

3.2.7 Wing Measurements

Loss of wing perimeter was measured using ImageJ release 1.38x software (Abramoff et al., 2004; http://rsb.info.nih.gov/ij/; http://www.macbiophotonics.ca/imagej/), as described in Chapter 2. Statistical analysis using Students' T-Test and Mann-Whitney U-Test was performed using the Minitab Statistical Software for Windows (release 13.20; www.minitab.com).

3.2.8 Administration of Ethyl Methane Sulfonate

Isogenized CS-P virgin males aged 3-5 days old were starved for 5-6 hours. Ethyl methane sulfonate (EMS) was dissolved in a 1.0% sucrose solution using a 22 gauge needle to a final concentration of 16.8 mM. Males were transferred to polycarbonate vials containing 2 layers of filter paper covering the bottom. A volume of 0.550 mL of EMS solution was administered to the adult males through the rayon rope using a 1.0 cc syringe. Males were allowed to feed overnight, and the following morning they were transferred to polycarbonate vials containing fresh fly media. Males were allowed to recover for 6-8 hours, during which time they were successively transferred 3-4 times to

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new polycarbonate vials with fly media. Ten males were then crossed to 20 *yw⁻; p[sev-GAL4] p[UAS-neu^{YC}]/CyO* virgin females. Parents were removed from the vial after 4 days. Crosses were conducted at 25°C.

3.3 Results

3.3.1 Titration of chemical mutagen ethyl methane sulfonate

To identify novel modifiers of *neu^{YC}*, we chose to conduct a dominant mutagenesis screen using the chemical mutagen EMS to generate random point mutations within the *Drosophila* genome. The induction rate for point mutations, or dosage, is dependent on the concentration of EMS. Increasing EMS concentrations induce an increasing number of point mutations within an organism (Grigliatti, 1998). High EMS concentrations can generate deletions and duplications, as well as cause organismal lethality. In genetic screens, induction of 25-40% X-linked lethal mutations is ideal, as it equals roughly one mutation per autosome, on average. Standard protocols for generating point mutations for genetic screens in *Drosophila* cite using an EMS concentration of 25 mM. This typically yields 30% X-linked lethal mutations, or 60% autosomal lethal mutations, as an autosome is roughly double the size of an X chromosome.

The effective dosage of EMS varies between fly strains and laboratory conditions. To identify the EMS concentration that corresponds to an average of one lethal mutation per chromosome in isogenized *Canton-S* (*CS-P*) males, I performed a sex-linked lethality assay to monitor the percent of induced X-linked lethal mutations at 3 different concentrations of EMS (Figure 3.1). In this assay, males mutagenized with EMS were crossed to virgins bearing an X chromosome balancer, in this case *FM7j*. The F1 daughters were allowed to freely mate with their brothers and after a few days were placed individually in tubes to lay fertilized eggs. The F2 males will inherit their X chromosome from their mothers, who now carry one copy of the parental X chromosome

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Figure 3.1: Genetics scheme for sex-linked lethality assay.

Adult males 3-5 days old were fed varying concentrations of EMS overnight and were crossed to virgin *FM7j* females the following day. Females in the F1 progeny were allowed to freely mate with their brothers for several days and then placed in individual vials to lay eggs. After a few days, females were removed from the vials. The F3 progeny for each brood were scored for the presence or absence of B^+-carrying males. Presence of B^+ males is indicative of a viable X chromosome mutation, whereas broods with only B^- males have a lethal X chromosome mutation. Therefore, the percent of broods that lack B^+ males is equivalent to the percent of X-linked lethals.

SEX-LINKED LETHALITY ASSAY

EMS
↓

P: ♂ + ; + ; +   X   yw⁻, FM7j ; + ; + ♀

F1:   ♀ $\dfrac{+^*}{\text{yw}^-, \text{FM7j}} ; \dfrac{+^*}{+} ; \dfrac{+^*}{+}$ X   yw⁻, FM7j ; + ; + ♂

F2:   Look for males absent for FM7j

The % of broods where all males carry FM7j is equivalent to the % of X-linked lethals

that has been mutagenized by EMS (called X^*). The F2 males can therefore be $FM7j/Y$ or

X^*/Y. A percentage of X^* lines will carry a newly induced lethal mutation, therefore

only $FM7j/Y$ male flies will be present. Thus, the percentage of lines with no Bar^+ F2

sons is the percentage of newly induced X-linked lethals.

Use of the standard concentration of EMS, 25 mM, resulted in 100% lethality in

the F1 generation (Table 3.1). At 16.8 mM, 34.4% of broods carried a newly induced X-

linked lethal mutation. Since an autosome is roughly double the size of an X

chromosome, this equates to approximately 55.4% autosome lethal, which corresponds to

roughly one mutation per chromosome on average (Grigliatti, 1998). Ideally, less than

20% multiple lethals are induced per autosome at the appropriate dosage. Based on the

dosage of 16.8 mM, we expect approximately 13.4% multiple autosome lethals.

3.3.2 EMS mutagenesis identifies modifiers of neu^{YC}

Genetic screens have been successfully utilized to identify novel components of

signalling pathways in *Drosophila*, such as KSR and CNK (Karim *et al.*, 1996; Therrien

et al., 2000). Conservation of signalling pathways has lead to the identification of

orthologous components involved in vertebrate signalling pathways (reviewed by

O'Kane, 2003). To take advantage of this powerful tool, we have conducted a dominant

F1 mutagenesis screen to identify novel modifiers of neu^{YC}. Isogenized *CS-P* males were

fed 16.8 mM EMS and were subsequently crossed to female virgins ectopically

expressing neu^{YC} in the developing eye disc (Figure 3.2). F1 male and female progeny

were selected for enhancement or suppression of the neu^{YC}-dependent rough eye

phenotype in both eyes. Enhancement was identified by increased balding and

Table 3.1: Sex-linked lethality assay identifies optimal EMS concentration

EMS Concentration (mM)	Total # of Broods	# Viable	# Lethal	% X-Linked Lethals	% Single X-Linked Lethals	% Single Autosome Lethals	% Multiple Autosome Lethals
12.5	148	109	39	26.4	22.4	44.8	8.0
16.8	131	86	45	34.4	27.7	55.4	13.4
25.0	0	0	0	0	0	0	0

discolouration of the eye surface, whereas suppression was identified by the formation of

discrete ommatidial units evenly across the eye surface and reduction in ommatidial unit

fusion. These were then back-crossed to $p[sev\text{-}GAL4]$ $p[UAS\text{-}neu^{YC}]$-expressing flies.

Modified F2 males were also back-crossed to $p[sev\text{-}GAL4]$ $p[UAS\text{-}neu^{YC}]$-expressing

flies. Back-crossing the selected F1 and F2 progeny served 2 purposes: the first was to

discard mutations that were not heritable, and the second was to isolate the mutation by

determining chromosomal linkage by segregation analysis. Chromosomal linkage in

CyO^+ progeny was determined by phenotypic ratio. For example, mutations on the

second chromosome modified all F2 progeny, whereas mutations on the third

chromosome were present in a 1:1 ratio of modified to unmodified neu^{YC}-dependent eye

phenotypes in F2 progeny. The mutation was then maintained over the appropriate

balancer.

A total of 19 081 F1 progeny were screened for enhancement or suppression of

neu^{YC} in the compound eye. From these, 271 suppressing mutations and 197 enhancing

mutations were identified. In the F2 generation, 41 suppressors and 40 enhancers verified

for their original neu^{YC} modification. Of these verified mutations, 6 suppressors and 11

enhancers were successfully isolated and maintained. The low recovery rate for

mutations can be attributed to the high expressivity of neu^{YC} in the eye. Mutations that

modified neu^{YC} beyond its phenotypic range were first identified in single flies. During

successive rounds of mapping, the number of neu^{YC}-expressing flies also carrying the

modifying mutation increased, and the modified phenotype overlapped with the broad

range of the neu^{YC}-induced rough eye phenotype, therefore, making it difficult to recover mutations that modify neu^{YC}.

The segregation of mutations from $p[sev\text{-}GAL4]$ $p[UAS\text{-}neu^{YC}]$ identified 1 suppressor mutation linked to the X chromosome (Figure 3.3C), 1 suppressor and 5 enhancers linked to the second chromosome (Figures 3.3B and 3.4B-F, respectively), and 4 suppressors and 6 enhancers on the third chromosome (Figures 3.3D-G and 3.4G-L, respectively). Allelism was determined by sorting mutations on recombined chromosomes into lethal complementation groups. Second chromosome modifiers were grouped into 4 lethal complementation groups. $E(neu^{YC})3\text{-}1$ and $E(neu^{YC})3\text{-}3$, on the second chromosome, failed to complement. Likewise, mutations on the third chromosome were grouped into 9 lethal complementation groups. $E(neu^{YC})42AJ$ and $E(neu^{YC})45J$ were the two mutations that failed to complement.

The identification of non-complementing mutations suggests saturation of the genome in our screen, if one assumes a single mutation rate for all genes (Pollock and Larkin, 2004). Furthermore, estimations based on a Poisson distribution using 16.8 mM EMS indicated saturation would be achieved after mutating 15 000 animals. However, saturation is unlikely in our screen. The number of alleles per locus identified during mutant screens rarely follows a Poisson distribution (Pollock and Larkin, 2004). Moreover, due to the low efficiency of the screen and observer bias, we were only able to recover approximately one quarter of the mutations that were heritable. It is reasonable to assume that all modifiers of neu^{YC} in *Drosophila* have not been identified during the course of our screen and that future more efficient screens may successfully identify other

Figure 3.2: EMS mutagenesis genetics scheme.

This scheme describes the mutagenesis screen for the X, second and third chromosomes. Isogenized *CS-P* males aged 3-5 days old were fed 16.8 mM EMS overnight. After recovery the following day, the males were crossed to *p[sev-GAL4] p[UAS-neu^{YC}]/CyO* virgins and the F1 females and males collected showed enhancement or suppression of the *neu^{YC}*-dependent rough eye phenotype. Selected progeny were then back-crossed to *p[sev-GAL4] p[UAS-neu^{YC}]/CyO* virgins to verify the modification and increase the number of mutants. F2 males showing modification were back-crossed to *p[sev-GAL4] p[UAS-neu^{YC}]/CyO* virgins to identify chromosomal linkage. Males carrying a mutation on the X chromosome will pass this chromosome to daughters; therefore, only *CyO$^+$* females in the F3 generation will show modification of the rough eye phenotype. Mutations on the second chromosome segregate such that all straight-winged progeny show the modified phenotype and, therefore, carry the mutation of interest. For mutations on the third chromosome, a 1:1 ratio of unmodified YC rough eye to modified YC rough eye for straight winged flies. Mutations were then isolated using the appropriate balancer and maintained in balanced stocks.

EMS MUTAGENESIS SCHEME

EMS

P: ♂ + ; + ; +   X   yw⁻ ; $\dfrac{\text{sev-GAL4 UAS-neu}^{YC} \text{ ; +}}{\text{CyO}}$ ☿

F1/F2:   ♂ yw⁻ ; $\dfrac{+^*}{\text{sev-GAL4 UAS-neu}^{YC}}$; $\dfrac{+^*}{+}$ X   yw⁻ ; $\dfrac{\text{sev-GAL4 UAS-neu}^{YC} \text{ ; +}}{\text{CyO}}$ ☿

F3: If mutation is on the 2nd chromosome: If mutation is on the 3rd chromosome:

All modified YC phenotype   1 Unmodified YC phenotype : 1 Modified YC phenotype

Balance on the 2nd   Balance on the 3rd

Figure 3.3: Suppressing mutations identified in EMS screen.

[A] Heterozygous flies expressing *p[sev-GAL4] p[UAS-neu^{YC}]* /+ generate a rough eye phenotype, characterized by ommatidial fusion, reduced interommatidial bristles and loss of typical eye patterning. [B-G] Suppressors of *neu^{YC}* identified. Percentage in bottom right hand corner indicates penetrance of mutation on *neu^{YC}* rough eye phenotype. Suppression of rough eye phenotype is detected by a reduction in ommatidial fusion, an increase in the number of interommatidial bristles and the return of ordered patterning. [B] *Su(neu^{YC})3* is a strong suppressor of the *neu^{YC}* rough eye phenotype on the second chromosome. Suppression of *neu^{YC}* is 100% penetrant. [C] *Su(neu^{YC})77* is a moderate X-linked suppressor that is also 100% penetrant. Suppressors [D] *Su(neu^{YC})93*, [E] *Su(neu^{YC})99*, [F] *Su(neu^{YC})138* and [G] *Su(neu^{YC})162* are moderate to weak suppressors of *neu^{YC}*. They are 50%, 75%, 50% and 50% penetrant, respectively. Moderate to weak suppression of *neu^{YC}* by these four modifiers overlaps with *neu^{YC}* expressivity.

Figure 3.4: Enhancers of *neu*^{YC} identified by EMS mutagenesis.

[A] Ectopic expression of *p[sev-GAL4] p[UAS-neu*^{YC}*]* in heterozygous flies produces a rough eye phenotype, as previously described. Enhancement of the rough eye phenotype is characterized by an increase in ommatidial fusion that extends posteriorly, with the fused area becoming flatter, and fewer interommatidial bristles present in fused tissue. [B-L] Mutations that enhance the *neu*^{YC} rough eye. [B] *E(neu*^{YC}*)3-1* and [C] *E(neu*^{YC}*)3-3* are moderate enhancers of *neu*^{YC} on the second chromosome with a penetrance of 80% and 75%, respectively, on *neu*^{YC} ommatidia fusion. [D] *E(neu*^{YC}*)6J*, [E] *E(neu*^{YC}*)50* and [F] *E(neu*^{YC}*)69* are moderate to strong enhancers of *neu*^{YC} on the second chromosome. They are 40%, 65% and 100% penetrant, respectively. On the third chromosome, [G] *E(neu*^{YC}*)5*, [H] *E(neu*^{YC}*)32* and [I] *E(neu*^{YC}*)32J* are weak to moderate enhancers of *neu*^{YC} that are 40% and 55% penetrant, respectively. Strong enhancers of *neu*^{YC} are [J] *E(neu*^{YC}*)42AJ*, [K] *E(neu*^{YC}*)45J* and [L] *E(neu*^{YC}*)46*, with penetrances of 100%, 75% and 60%, respectively.

genes specific to YC pTyr output.

3.3.3 Identification of neu^{YC}-specific mutations

Signalling through individual docking sites within an RTK is often redundant, as activation of individual pTyr can converge on a single signalling cascade (Dankort *et al.*, 1997; Dankort *et al.*, 2001a). It is, therefore, likely that novel modifiers of *neu^{YC}* may modify other pTyr within the docking domain of Neu. Mutations were tested for the ability to modify the pTyrs YB, YD and YE, and *neu^{NYPD}*, the phosphorylation-deficient allele of *neu* (Figures 3.5-3.12). Signals from Neu^{YB} and Neu^{YD} both converge on the Ras1/MAPK signalling cassette through the recruitment and activation of different adaptor proteins upstream of Sos GEF (Dankort *et al.*, 1997; Dankort *et al.*, 2001a; Dankort *et al.*, 2001b; Settle *et al.*, 2003). *Su(neu^{YC})3* suppressed ommatidial patterning defects in both *neu^{YB}*- and *neu^{YD}*- expressing flies (Figures 3.5B and 3.6B, respectively). This suggests that the gene encoded by *Su(neu^{YC})3* functions in a pathway common to *neu^{YB}*, *neu^{YC}* and *neu^{YD}*, and that this shared pathway is most likely the Ras1/MAPK pathway. *Su(neu^{YC})77*, *Su(neu^{YC})99* and *Su(neu^{YC})138* did not suppress *neu^{YB}*, but did suppress *neu^{YD}* in the developing eye, suggesting these modifiers are also involved in canonical Ras1 signalling, but that this is distinct from the Neu^{YB} pathway (Figures 3.5C-E and 3.6C-E). Ommatidial fusion is mildly enhanced in *p[sev-GAL4] p[UAS-neu^{YB}]/E(neu^{YC})3-1* and *p[sev-GAL4] p[UAS- neu^{YB}]/E(neu^{YC})3-3* flies, but not in *p[sev-GAL4] p[UAS-neu^{YD}]/E(neu^{YC})3-1* and *p[sev-GAL4] p[UAS-neu^{YD}]/E(neu^{YC})3-3* flies, suggesting *E(neu^{YC})3-1* and *E(neu^{YC})3-3* are involved in *neu^{YC}* and *neu^{YB}*-specific pathways (Figures 3.7B-C and 3.8B-C). *E(neu^{YC})45J* appears to be specific to *neu^{YD}* as

Figure 3.5: Suppressing modifiers of *neu^{YC}* modify *neu^{YB}*.

[A] Ectopic expression of *neu^{YB}* by *sev-GAL4* results in a moderate ommatidia fusion phenotype. Individual facets are large, less in number and are disarrayed. [B] *Su(neu^{YC})3* mildly suppresses *neu^{YB}*, seen by a mild restoration of ommatidial patterning. [C] *Su(neu^{YC})77*, [D] *Su(neu^{YC})93*, [E] *Su(neu^{YC})99*, [F] *Su(neu^{YC})138* and [G] *Su(neu^{YC})162* do not suppress *neu^{YB}* in the eye.

Figure 3.6: Suppression of *neu^{YD}* by EMS modifiers.

[A] Strong rough eye phenotype is produced in *p[sev-GAL4] p[UAS-neu^{YD}]* flies.

Suppression is characterized by a decrease in fused ommatidia and an increase in

interommatidial bristles and ordered patterning of individual facets and. Varying degrees

of suppression of *neu^{YD}* is seen with suppressors of *neu^{YC}*. Strong suppression is seen

with [B] *Su(neu^{YC})3* and [C] *Su(neu^{YC})77*. [D] *Su(neu^{YC})99* moderately suppresses *neu^{YD}*.

[E] *Su(neu^{YC})138* most mildly suppresses *neu^{YD}*.

well as *neu^YC*, as it suppressed the *neu^YD*-dependent rough eye phenotype (Figure 3.8H).

The signalling output from the YE pTyr is currently unknown. The amino acid

motifs surrounding pTyrs YC and YE, however, are identical, suggesting that these 2

pTyr may recruit a common output pathway, or recruit shared adaptor proteins or second

messengers to activate downstream pathways (Dankort *et al.*, 1997; Settle *et al.*, 2003).

Modifiers were tested for their ability to modulate *neu^YE* signalling. None of the

suppressing mutations affected *neu^YE*-dependent rough eye phenotype (Figure 3.9).

Several enhancers, however, did enhance the *neu^YE* phenotype (Figure 3.10). *neu^YB*-

specific modifiers *E(neu^YC)6J*, *E(neu^YC)42AJ*, *E(neu^YC)3-1* and *E(neu^YC)3-3* enhanced the

neu^YE eye phenotype (Figure 3.10B-D, J), suggesting shared pathway components are

involved in *neu^YC*, *neu^YB* and *neu^YE* output. Similarly, *E(neu^YC)45J*, a strong suppressor of

neu^YD, strongly enhanced *neu^YE* (Figure 3.10K). Three modifiers, *E(neu^YC)50*, *E(neu^YC)5*

and *E(neu^YC)32* only enhanced *neu^YE* in addition to enhancing *neu^YC*. This supports the

hypothesis of common components between the YC and YE pTyrs (Figures 3.10E, G-H).

The phosphorylation-deficient allele of *neu*, *neu^{NYPD}*, still retains weak signalling

abilities by recruiting adaptor and second messengers directly to the kinase domain of the

receptor (Peles *et al.*, 1991; Kim *et al.*, 2005). Misexpression of *neu^{NYPD}* in the eye by

sev-GAL4 resulted in a mild rough eye phenotype that is characterized by the loss or

duplication of interommatidial bristles, mild facet fusion and mild loss of ommatidial

organization (Figure 3.11A and 3.12A). *neu^YD*-specific modifiers *Su(neu^YC)3*,

Su(neu^YC)77 and *Su(neu^YC)99* suppressed the *neu^{NYPD}* eye phenotype (Figure 3.11B, C and

E). *Su(neu^YC)162* also suppressed *neu^{NYPD}* rough eye, suggesting these four suppressors

are involved in signalling downstream of the Neu kinase domain (Figure 3.11G).

Likewise, neu^{YE}-specific enhancers $E(neu^{YC})3$-1, $E(neu^{YC})50$, $E(neu^{YC})32$, $E(neu^{YC})42AJ$

and $E(neu^{YC})45J$ enhanced the catalytic domain phenotype, suggesting involvement in

kinase domain signalling (Figure 3.12B, E, H, J and K).

3.3.4 Modulation of Ras1 by EMS modifiers identifies Ras1-specificity

Activation of the Ras1 GTPase occurs when Ras-bound GDP is switched for GTP

by a group of proteins known as guanine nucleotide exchange factors (GEFs) (Bourne *et

al.*, 1991). In *Drosophila*, Sos is the only GEF known to activate Ras1 (Self *et al.*, 2001;

Tian and Feig, 2001). Activation of Ras1 leads to the activation of the Ser/Thr kinase

Raf. Neu pTyrs YB and YD activate Raf by Sos-mediated Ras1 activation (Dankort *et

al.*, 1997; Dankort *et al.*, 2001a; Dankort *et al.*, 2001b; Settle *et al.*, 2003). Mutations that

modify these *neu* alleles, most likely mutate genes involved in the canonical

Ras/Raf/MAPK pathway. To identify novel genes that modulate Neu signalling through

a non-canonical Ras1 pathway or in parallel to the canonical Ras1 pathway, mutations

were examined for the modification of constitutively active and dominant negative *Ras1*

alleles, $Ras1^{V12}$ and $Ras1^{N17}$, respectively. The mutations that act downstream of Ras1

will suppress $Ras1^{V12}$ and enhance $Ras1^{N17}$, since mutations in genes that act immediately

upstream of Ras1 will have minimal effect on signalling pathways activated by the over-

abundance of $Ras1^{V12}$, and minimal effect on signalling pathways inhibited by $Ras1^{N17}$.

Only two mutations were found to modify both $Ras1^{V12}$ and $Ras1^{N17}$ rough eye

phenotypes. $Su(neu^{YC})99$ and $E(neu^{YC})69$ both appear to depend on *Ras1*, as they

Figure 3.7: Enhancers of *neu^YC^* modify *neu^YB^*.

[A] Flies heterozygous for *p[sev-GAL4] p[UAS-neu^YB^]* show moderate a rough eye phenotype. Enhancement is detected by an increase in facet fusion and decrease in the number of interommatidial bristles. [B] *E(neu^YC^)3-1*, [C] *E(neu^YC^)3-3*, [D] *E(neu^YC^)6J* and [J] *E(neu^YC^)42AJ* mildly enhance *neu^YB^*. An increase in facet fusion in the middle of the eye is seen in *E(neu^YC^)3-1*, *E(neu^YC^)6J* and *E(neu^YC^)42AJ*. Ommatidial units are slightly larger in *E(neu^YC^)3-3*, and they appear less in number, indicative of increased fusion. No enhancement of *neu^YB^* is seen with [E] *E(neu^YC^)50*, [F] *E(neu^YC^)69*, [G] *E(neu^YC^)5*, [H] *E(neu^YC^)32*, [I] *E(neu^YC^)32J*, [K] *E(neu^YC^)42AJ* or [L] *E(neu^YC^)46*.

Figure 3.8: Modulation of *neu^{YD}* by enhancers of *neu^{YC}*.

[A] *p[sev-GAL4] p[UAS-neu^{YD}]* heterozygous flies have a strong ommatidial fusion

phenotype in the anterior eye. All second chromosome enhancers, [B] *E(neu^{YC})3-1*, [C]

E(neu^{YC})3-3, [D] *E(neu^{YC})50* and [E] *E(neu^{YC})69* fail to modify the rough eye phenotype.

Third chromosome mutations [F] *E(neu^{YC})5*, [G] *E(neu^{YC})32* and [I] *E(neu^{YC})46* also fail

to modify *neu^{YD}*. [H] *E(neu^{YC})45J* suppresses *neu^{YD}* rough eye phenotype.

Figure 3.9: Identified suppressors do not modify *neu^{YE}* in the eye.

[A] *p[sev-GAL4] p[UAS-neu^{YE}]* expression results in a moderate rough eye phenotype.

All suppressing mutations [B] *Su(neu^{YC})3*, [C] *Su(neu^{YC})77*, [D] *Su(neu^{YC})93*, [E]

Su(neu^{YC})99, [F] *Su(neu^{YC})138* and [G] *Su(neu^{YC})162* do not affect *neu^{YE}* expression.

Figure 3.10: Enhancing mutations affect _neu_^{YE} rough eye phenotype.

[A] _p[sev-GAL4] p[UAS-neu_^{YE}_]_ heterozygotes possess a moderate rough eye phenotype in adults. [B] _E(neu_^{YC}_)3-1_ and [E] _E(neu_^{YC}_)50_ strongly enhance _neu_^{YE} ommatidial fusion [C] _E(neu_^{YC}_)3-3_ and [D] _E(neu_^{YC}_)6J_ weakly enhance fusion of anterior ommatidia in _neu_^{YE} heterozygotes. [F] _E(neu_^{YC}_)69_ does not enhance _neu_^{YE} phenotype. Third chromosome modifiers [G] _E(neu_^{YC}_)5_, [J] _E(neu_^{YC}_)42AJ_ and [K] _E(neu_^{YC}_)45J_ all strongly enhance fusion phenotype. [H] _E(neu_^{YC}_)32_ mildly enhances _neu_^{YE}. [I] _E(neu_^{YC}_)32J_ and [L] _E(neu_^{YC}_)46_ do not modify _neu_^{YE}. Enhancement of _neu_^{YE} by modifiers suggests common components between _neu_^{YC} and _neu_^{YE} outputs.

Figure 3.11: Modification of NYPD pTyr output.

[A] *p[UAS-neu^{NYPD}] ; p[sev-GAL4]* heterozygotes possess a mild rough eye phenotype characterized by disorganized ommatidial patterning, missing and doublets of interommatidial bristles. Suppression of the *neu^{NYPD}* phenotype results in re-establishment of proper bristle and ommatidial patterning. Suppressors [B] *Su(neu^{YC})3*, [C] *Su(neu^{YC})77*, [E] *Su(neu^{YC})99* and [G] *Su(neu^{YC})162* all suppress the rough eye. [D] *Su(neu^{YC})93* and [F] *Su(neu^{YC})138* fail to modify catalytic domain-dependent phenotype.

Figure 3.12: *neu^{NYPD}* **rough eye phenotype modified by** *neu^{YC}* **enhancers.**

[A] *p[UAS-neu^{NYPD}]* ; *p[sev-GAL4]* expression results in a mild rough eye phenotype.

Enhancement results in an increase in ommatidial fusion and disorganization, and

increased loss of interommatidial bristles. Modifiers [B] *E(neu^{YC})3-1*, [E] *E(neu^{YC})50*,

[H] *E(neu^{YC})32* and [J] *E(neu^{YC})42AJ* all enhance *neu^{NYPD}* rough eye. No effects are seen

with [C] *E(neu^{YC})3-3*, [D] *E(neu^{YC})6J*, [F] *E(neu^{YC})69*, [G] *E(neu^{YC})5*, [I] *E(neu^{YC})32J*,

[K] *E(neu^{YC})45J* and [L] *E(neu^{YC})46*.

both suppressed $Ras1^{V12}$ and enhanced $Ras1^{N17}$ (Figures 3.13E, 3.14E, 3.15F and 3.16F, respectively). $Su(neu^{YC})99$ suppressed signalling from neu^{YD} and the catalytic domain of Neu. Therefore, $Su(neu^{YC})99$ is most likely involved in the canonical Ras1/MAPK pathway downstream of Neu^{YD} and the Neu kinase domain. $E(neu^{YC})69$ did not modify any other pTyr alleles. Thus, $E(neu^{YC})69$ may be a novel or non-canonical Ras1-activating modifier specific to neu^{YC}.

Several of the modifiers affected only one of the Ras1 alleles, but not both. We reasoned that mutations that modify only $Ras1^{V12}$ are involved downstream of Ras1, while genes that modify only $Ras1^{N17}$ are most likely involved upstream of Ras1. $Su(neu^{YC})77$, $Su(neu^{YC})138$ and $E(neu^{YC})6J$ are most likely involved in canonical Ras1/Raf/MAPK pathway, as $Su(neu^{YC})77$ and $Su(neu^{YC})138$ are specific to neu^{YD}, and $E(neu^{YC})6J$ is specific to neu^{YB}. $Su(neu^{YC})77$ and $Su(neu^{YC})138$ suppressed the $Ras1^{V12}$ phenotype and did not affect $Ras1^{N17}$, suggesting that these mutations function downstream of $Ras1$ or in a parallel pathway (Figures 3.13E and F, and 3.14E and F, respectively). $E(neu^{YC})6J$ enhanced $Ras1^{V12}$ eye phenotype (Figure 3.15D). Additionally, $E(neu^{YC})6J$ also enhanced neu^{YE}, suggesting $E(neu^{YC})6J$ modulates $Ras1$ function as a shared component of the neu^{YC}, neu^{YB} and neu^{YE} pathways. $Su(neu^{YC})162$ and $E(neu^{YC})45J$ only enhanced the dominant negative $Ras1$ allele (Figures 3.13G, 3.14G, 3.15K and 3.16K). In addition to enhancing $Ras1^{N17}$, $Su(neu^{YC})162$ also suppressed the neu^{NYPD} phenotype, but not other pTyr phenotypes. Ras1 positively mediates signalling from the Neu catalytic domain, as loss of Ras1 suppressed the neu^{NYPD}-dependent eye phenotype and constitutively active Ras1 enhanced the neu^{NYPD}-dependent eye phenotype

(Chapter 2 Figures 2.3 and 2.5). Therefore, $Su(neu^{YC})162$ may function in Ras1

activation or negatively regulate Ras1. $E(neu^{YC})45J$ also enhanced neu^{YD} and neu^{YE}

phenotypes, suggesting that $E(neu^{YC})45J$ may be activated by YD and YE pTyrs to

modulate Ras1 activation by functioning upstream of Ras1 or by negatively regulating

Ras1 function.

Mutation $E(neu^{YC})50$ suppressed dominant negative $Ras1^{N17}$ but failed to modify

$Ras1^{V12}$ (Figure 3.15E and 3.16E). In addition, $E(neu^{YC})50$ enhanced neu^{YE} and neu^{NYPD}

rough eye phenotypes, suggesting that $E(neu^{YC})50$ acts downstream of Ras1 or parallel to

Ras1 in a pathway shared by both YC and YE pTyr and the kinase domain of Neu.

As stated earlier, $Su(neu^{YC})3$ is not specific to neu^{YC}, as it also suppressed neu^{YB}

and neu^{YD}, both potent activators of Ras1. However, $Su(neu^{YC})3$ did not modify $Ras1^{V12}$

or $Ras1^{N17}$ (Figures 3.13B and 3.14B). This suggests that $Su(neu^{YC})3$ is too far

downstream in the pathway to effectively modify Ras1. Similarly, neither $Ras1^{V12}$ nor

$Ras1^{N17}$ were affected by $Su(neu^{YC})138$, suggesting that the role of $Su(neu^{YC})138$ in

Neu^{YC} and Neu^{YD} signalling is independent of Ras1 activation (Figure 3.13F and 3.14F).

$E(neu^{YC})3-1$, $E(neu^{YC})3-3$, $E(neu^{YC})5$, $E(neu^{YC})32$, $E(neu^{YC})32J$, $E(neu^{YC})42AJ$ and

$E(neu^{YC})46$ also did not modify $Ras1^{V12}$ or $Ras1^{N17}$ (Figure 3.15B-C, G-J and L, and

3.16B-C, G-J and L). These modifiers may represent intersecting points in the YC, YB,

YE and NYPD pathways that function independently of Ras1 activation or are too weak

to modify the $Ras1$ alleles.

Figure 3.13: Suppressors modify activated *Ras1*.

[A] Fusion of constitutively active *Ras1^{V12}* to the *sevenless* promoter results in a moderate

eye fusion phenotype. Suppression of ommatidial fusion is seen with [C] *Su(neu^{YC})77*,

[D] *Su(neu^{YC})93* and [E] *Su(neu^{YC})99* mutations. No modification is observed in [B]

p[sev::Ras1^{V12}] / Su(neu^{YC})3, [E] *p[sev::Ras1^{V12}] ; Su(neu^{YC})138* and [F] *p[sev::Ras1^{V12}]*

; Su(neu^{YC})162 heterozygotes

Figure 3.14: Suppressors modify dominant negative *Ras1*.

[A] Dominant negative *Ras1* allele, *p[UAS-Ras1^{N17}]*, results in a moderate rough eye

when driven by *p[sev-GAL4]* in the developing eye. Enhancement of *Ras1^{N17}* results in a

decrease in facet number, presumably due to increased programmed cell death. This

produces a smaller eye and disruption in bristle patterning. Both [E] *Su(neu^{YC})99* and [G]

Su(neu^{YC})162 enhance *Ras1^{N17}*. *Ras1^{N17}* is not modified by [B] *Su(neu^{YC})3*, [C]

Su(neu^{YC})77, [D] *Su(neu^{YC})93* and [F] *Su(neu^{YC})138*.

Figure 3.15: Enhancers modify activated *Ras1*.

[A] A moderate rough eye phenotype is seen in *p[sev::Ras1^{V12}]* heterozygotes visualized under environmental scanning electron microscope. Increased ommatidial fusion in seen in [D] *E(neu^{YC})6J/p[sev::Ras1^{V12}]* flies. Reduced fusion is observed in [F] *E(neu^{YC})69/p[sev::Ras1^{V12}]* flies. *Ras1^{V12}* expression is not modified in [B] *E(neu^{YC})3-1*, [C] *E(neu^{YC})3-3*, [F] *E(neu^{YC})69*, [G] *E(neu^{YC})5*, [H] *E(neu^{YC})32*, [I] *E(neu^{YC})32J*, [J] *E(neu^{YC})42AJ*, [K] *E(neu^{YC})45J* and [L] *E(neu^{YC})46* heterozygotes.

Figure 3.16: Enhancers modify dominant negative *Ras1* allele.

[A] Moderate rough eye phenotype is produced in *p[UAS-Ras1^{N17}] ; p[sev-GAL4]* heterozygotes. Suppression produces a larger eye with increased facet number, regular ommatidial spacing and bristle patterning. Enhancement produces a smaller eye with decreased facet number due to increased apoptosis and increased ommatidial and bristle pattern defects. Suppression of *Ras1^{N17}* is observed in flies also heterozygous for [E] *E(neu^{YC})50*. [F] *E(neu^{YC})69* and [K] *E(neu^{YC})45J* enhance *Ras1^{N17}*-dependent phenotype. *Ras1^{N17}* is not modified by [B] *E(neu^{YC})3-1*, [C] *E(neu^{YC})3-3*, [D] *E(neu^{YC})6J*, [G] *E(neu^{YC})5*, [H] *E(neu^{YC})32*, [I] *E(neu^{YC})32J*, [J] *E(neu^{YC})42AJ* or [L] *E(neu^{YC})46*.

3.3.5 Identification of tissue- and promoter-specific mutations

Our mutagenesis screen assayed phenotypic changes in the ectopic expression of

$p[UAS\text{-}neu^{YC}]$ by the promoter-specific GAL4 driver *sev-GAL4*. *sev* expression is limited

to presumptive R1, R3, R4, R6 and R7 photoreceptor cells, cone cells and mystery cells

in the developing eye disc (Banerjee *et al.*, 1987a; Banerjee *et al.*, 1987b; Tomlinson *et*

al., 1987). Modification of the neu^{YC} phenotype can occur in one of three different ways:

1) by mutating genes involved in the neu^{YC} signalling pathway; 2) by mutating genes that

regulate the expression of the GAL4 regulatory element that controls neu^{YC} expression;

and 3) by mutating genes that regulate development of the tissue being assayed. To

ensure our identified modifiers truly modify neu^{YC}, and not the *sev* promoter or eye

development, the modifiers were re-verified for their interaction with neu^{YC} in the adult

wing margin using a different $p[UAS\text{-}neu^{YC}]$ insertion. Ectopic expression of $p[UAS\text{-}$

$neu^{YC}]$ by the enhancer trap $p[GAL4]bbg^{C96}$ drives expression of neu^{YC} in the

dorsoventral boundary of the developing wing. Adult wing margins of neu^{YC}

heterozygotes contain notches, which correspond to increased apoptosis, resulting from

high levels of RTK signalling at the dorsoventral boundary. By measuring the relative

wing notch lengths, we can quantitatively ascertain enhancement or suppression of this

phenotype (Chapter 2, Figure 2.1-2.2). Ectopic expression of neu^{YC} in yw^{-} ;

$p[GAL4]bbg^{C96}$ flies resulted in 12.59 ± 1.1% loss of tissue along the wing margin. All

suppressing mutations, except $Su(neu^{YC})77$, were observed to suppress neu^{YC} in the wing

(Figure 3.17). Enhancers $E(neu^{YC})3\text{-}1$ and $E(neu^{YC})3\text{-}3$ strongly enhanced neu^{YC} notches

(35.00 ± 0.93% and 37.37 ± 1.2%, respectively, Figure 3.17). Identified enhancers

$E(neu^{YC})6J$, $E(neu^{YC})32J$, $E(neu^{YC})50$, and $E(neu^{YC})69$ suppressed wing notch formation,

suggesting these modifiers possess tissue-specific roles (Figure 3.17). Four enhancers,

$E(neu^{YC})32$, $E(neu^{YC})42AJ$, $E(neu^{YC})45J$ and $E(neu^{YC})46$, did not modify the wing notch

phenotype, suggesting that these mutations specifically affect eye development or the

sevenless promoter (Figure 3.17).

3.3.6 Meiotic recombination mapping delimits locations of EMS mutations

To identify the mutated genes that modified neu^{YC}, we undertook a two part

mapping strategy involving rounds of rough and fine mapping. Rough mapping can

resolve the location of the mutation to within 300 kb (Zhai *et al.*, 2003), while the results

of fine mapping can range from identifying the gene mutated to pinpointing the exact

location of the point mutation created by EMS mutagenesis. Our rough mapping strategy

began by identifying the cytological position of the mutations as defined by a set of

recessive markers that span the second and third chromosomes – $al^{1}b^{1}pr^{1}c^{1}px^{1}sp^{1}$ on the

second chromosome and $ru^{1}h^{1}th^{1}st^{1}cu^{1}sr^{1}e^{s}ca^{1}$, or *rucuca*, on the third chromosome.

Meiotic recombination mapping takes advantage of the cross-over events between non-

sister chromatids during meiosis in females. Each cross-over, or recombination, event

produces two unique recombinant chromosomes. When mapping a mutation of interest,

females are generated that are heterozygous for the mutation of interest and the

corresponding multiply marked chromosome. Recombinant chromosomes from these

females will consist of some combination of the recessive markers and the mutation of

interest. The combination of markers observed with the mutation of interest can reveal

the location of the mutation to within two markers (Figure 3.18). For example, suppose

Figure 3.17: Tissue- and promoter-specific roles for EMS mutations.

Bar graph representing the modification of neu^{YC} wing perimeter loss by mutations that modify neu^{YC} in the developing eye. Wild-type wings do not possess any notches in the wing margin. Ectopic expression of $UAS\text{-}neu^{YC}$ in the dorsoventral boundary of the developing wing disc by $p[GAL4]bbg^{C96}$ results in an average margin loss of $12.59 \pm 1.1\%$ (n = 61) per wing in a yw^{-} background. Suppression of notches occurs in flies also heterozygous for $Su(neu^{YC})3$ ($2.67 \pm 0.56\%$, n = 51), $Su(neu^{YC})93$ ($7.56 \pm 0.87\%$, n = 58), $Su(neu^{YC})99$ ($8.12 \pm 0.71\%$, n = 63), $Su(neu^{YC})138$ ($6.75 \pm 0.77\%$, n = 69) and $Su(neu^{YC})162$ ($9.43 \pm 0.89\%$, n = 69). Similarly, enhancers $E(neu^{YC})5$ ($8.76 \pm 0.92\%$, n = 72), $E(neu^{YC})6J$ ($6.23 \pm 0.68\%$, n = 85), $E(neu^{YC})32J$ ($6.88 \pm 0.71\%$, n = 83), $E(neu^{YC})50$ ($8.36 \pm 0.73\%$, n = 85) and $E(neu^{YC})69$ ($5.54 \pm 0.65\%$, n = 73) suppress margin loss. Both $E(neu^{YC})3\text{-}1$ and $E(neu^{YC})3\text{-}3$ affect wing morphology. Wings appear scalloped in $E(neu^{YC})3\text{-}1/+$ and $E(neu^{YC})3\text{-}3/+$ flies, resulting in margin loss of $28.7 \pm 1.3\%$ (n = 60) and $45.4 \pm 1.9\%$ (n = 29), respectively. Wing margin loss in $E(neu^{YC})3\text{-}1$; $p[GAL4]bbg^{C96} p[UAS\text{-}neu^{YC}]$ heterozygotes is enhanced compared to both parental controls ($35.00 \pm 0.93\%$, n = 56, P = 0.00). In $E(neu^{YC})3\text{-}3$; $p[GAL4]bbg^{C96} p[UAS\text{-}neu^{YC}]$ heterozygotes, wing margin loss is enhanced compared to neu^{YC}-expressing flies ($37.37 \pm 1.2\%$, n = 58, P = 0.00), but is suppressed in comparison to $E(neu^{YC})3\text{-}3$ heterozygotes ($45.4 \pm 1.9\%$, P = 0.00). No modification of neu^{YC}-induced wing morphology was seen with $Su(neu^{YC})77$ ($14.0 \pm 1.0\%$, n = 64), $E(neu^{YC})32$ ($14.14 \pm 0.80\%$, n = 75), $E(neu^{YC})42AJ$ (13.35 ± 0.61, n = 124), $E(neu^{YC})45J$ ($12.43 \pm 0.96\%$, n =

42) and $E(neu^{YC})46$ (10.0 ± 1.4%, n = 57), suggesting these mutations modify neu^{YC} by affecting compound eye development or the *sev* promoter.

EMS Mutations Modify *neu*^{YC} Wing Notch Formation

Figure 3.18: Meiotic recombination mapping genetics scheme.

The following scheme represents the genetics used to begin the mapping process for unknown mutations on the second chromosome as an example. Meiotic recombination mapping takes advantage of the production of individual recombinants during gamete formation, where cross-over events occur between two nonsister chromatids that carry the mutation of interest on one chromatid and the multiple recessive markers on the other. Virgins of the balanced dominant mutation, M, are crossed to homozygotes of a recessive multiply marked chromosome. For second chromosome mutations, the chromosome carried the markers *aristaless* (al^1), *black* (b^1), *purple* (pr^1), *curved* (c^1), *plexus* (px^1) and *speck* (sp^1). Female virgins heterozygous for M and $al^1 b^1 pr^1 c^1 px^1 sp^1$ are collected and crossed to $al^1 b^1 pr^1 Bl^1 c^1 px^1 sp^1 / CyO$ males. The dominant mutation *Bristle* (Bl^1) helps distinguish this chromosome from the recombinant chromosomes. Recombinant flies are identified by loss of one or more visible recessive markers in F2 males, such as $al^1 M / al^1 b^1 pr^1 Bl^1 c^1 px^1 sp^1$ and $b^1 pr^1 c^1 px^1 sp^1 / al^1 b^1 pr^1 Bl^1 c^1 px^1 sp^1$, for example. Dominant mutation M may not have a visible phenotype, therefore, recombinant males are crossed to $p[sev\text{-}GAL4]\ p[UAS\text{-}neu^{YC}] / CyO$. Recombinants that modify neu^{YC} have M present and are maintained in balanced stocks. Meiotic recombination mapping for third chromosome mutations is similar, but uses the *rucuca* and *ruPrica* chromosomes.

MEIOTIC RECOMBINATION MAPPING

$$yw^- ; \frac{M}{CyO} ; + \qquad X \qquad al^1b^1pr^1c^1px^1sp^1$$

⚥ $\dfrac{M}{al^1b^1pr^1c^1px^1sp^1}$ X $\dfrac{al^1b^1pr^1Bl^1c^1px^1sp^1}{CyO}$ ♂

Recombinants

♂ $\dfrac{al^1M}{al^1b^1pr^1Bl^1c^1px^1sp^1}$

...

♂ $\dfrac{b^1pr^1c^1px^1sp^1}{al^1b^1pr^1Bl^1c^1px^1sp^1}$

X

$yw^- ; \dfrac{sev\text{-}GAL4 \; UAS\text{-}neu^{YC}}{CyO} ; +$ ⚥ ⟶ Verify presence of dominant mutation M

$w^- ; \dfrac{Sco}{CyO} ; +$ ⚥ ⟶ Establish broods

the dominant modifying mutation M is located in the cytological band 36B3 on the second chromosome. Using the multiply marked second chromosome $al^1 b^1 pr^1 c^1 px^1 sp^1$, we are able to generate recombinants that carry M and some of the markers from the $al^1 b^1 pr^1 c^1 px^1 sp^1$ chromosome. Single recombinants carrying M, for example $al^1 b^1 M$ and $M pr^1 c^1 px^1 sp^1$, are selected by verifying the modified phenotype. Based on these recombinants, we can define M to be between the markers b^1 and pr^1, which are located at 34D1 and 38B3, respectively.

Using this approach, we defined chromosomal positions for several second and third chromosome mutations identified during our screen (Figure 3.19). Weak suppressors $Su(neu^{YC})93$, $Su(neu^{YC})99$ and $Su(neu^{YC})162$ were not mapped due to low penetrance and expressivity that overlapped with neu^{YC}. The strong suppressor $Su(neu^{YC})3$ was mapped to a region between 21C1 and 34D1 on the left arm of the second chromosome. $E(neu^{YC})6J$ and $E(neu^{YC})50$ also mapped to between bands 21C1 and 34D1. All 3 mutations complemented their lethality phenotype, suggesting three different genes are mutated within this region. $E(neu^{YC})3-1$ and $E(neu^{YC})3-3$ failed to complement and mapped to the same location on the right arm of the second chromosome, between bands 58E4 and 60C2, supporting allelism. The eye specific mutations $E(neu^{YC})46$ and $E(neu^{YC})32$ were mapped to regions 66D10-72D1 and 73A3-86D4, respectively, on the third chromosome. $E(neu^{YC})42AJ$ and $E(neu^{YC})45J$, which were non-complementing and appeared to be tissue-specific, mapped to adjacent third chromosome regions 90E4-93D1 and 93C7-99C2, respectively. Failure to complement may not be due to allelism, but due to position-effect variegation or second site non-complementation.

Figure 3.19: Schematic representation of meiotic recombination mapping results.

[A] Second chromosome map of mutations. Recessive markers used and cytological

positions are indicated. The positions of second chromosome modifiers are shown above

the chromosome. $Su(neu^{YC})3$, $E(neu^{YC})6J$ and $E(neu^{YC})50$ were all resolved to a region

between the 2L markers al^{1} and b^{1}. $E(neu^{YC})3\text{-}1$ and $E(neu^{YC})3\text{-}3$ were isolated to the

telomeric end of 2R, between px^{1} and sp^{1}. The location of $E(neu^{YC})69$ was limited to two

regions on the second chromosome: the first region spans the centromere and is between

pr^{1} and c^{1}, and the second region is between px^{1} and sp^{1} at the telomeric end of 2R.

[B] Map of third chromosome mutations. Third chromosome recessive markers are

depicted along the length of the chromosome with their relative cytological positions

underneath. Locations of mutations are shown above the chromosome. $E(neu^{YC})46$ was

mapped to a region between *hairy* (h^{1}) and *thread* (th^{1}). $E(neu^{YC})32$ is between *scarlet*

(st^{1}) and *curled* (cu^{1}) in the centromeric region. $E(neu^{YC})42AJ$ and $E(neu^{YC})45J$ are in

adjacent regions limited by the markers *stripe* (sr^{1}) and *claret* (ca^{1}). $E(neu^{YC})32J$ spans

this region at the telomeric end of 3R as well. Mutations whose locations were not

resolved to between two markers, such as $E(neu^{YC})5$ and $Su(neu^{YC})138$, were resolved to

larger regions based on the recombinants generated and those that verified for the

presence of the mutation of interest.

Calibration is approx. 10^{6} nucleotides.

A

$Su(neu^{YC})3$
$E(neu^{YC})6J$
$E(neu^{YC})50$

$E(neu^{YC})69$

$E(neu^{YC})69$
$E(neu^{YC})3-1$
$E(neu^{YC})3-3$

al^1 — 21C1
b^1 — 34D1
pr^1 — 38B3
c^1 — 52D3-D7
px^1 — 58E4-E8
sp^1 — 60C1-C2

~10⁶ nt

B

$E(neu^{YC})5$

$Su(neu^{YC})138$

$E(neu^{YC})32J$

$E(neu^{YC})42AJ$ $E(neu^{YC})45J$

$E(neu^{YC})46$

$E(neu^{YC})32$

ru^1 — 61F8
h^1 — 66D10
th^1 — 72D1
st^1 — 73A3
cu^1 — 86D3-D4
sr^1 — 90E4-F1
e^S — 93C7-D1
ca^1 — 99C2

~10⁶ nt

A limitation to mapping by meiotic recombination is that resolving the position of the mutation is dependent on the type of recombinants generated during meiosis and which of these recombinants positively verify for the mutation of interest. For example, $E(neu^{YC})69$ was mapped to two distinct regions on the second chromosome – 38B3-51D7 and 58E4-60C2 – while $E(neu^{YC})32J$ was mapped to 90E4-99C2, a large region spanning 3 markers on the third chromosome.

Based on these results, select strong modifiers – $Su(neu^{YC})3$, $E(neu^{YC})3-1$, $E(neu^{YC})69$ and $E(neu^{YC})6J$ – were chosen for continued rough and fine mapping using a deficiency or a P-element mapping strategy. $Su(neu^{YC})3$ has been mapped to a single locus that validates our mutagenesis screen. $E(neu^{YC})3-1$, $E(neu^{YC})3-3$, $E(neu^{YC})69$, and $E(neu^{YC})6J$ have been partially mapped (Appendix G). Identification of these genes in the future will provide other avenues for the dissection of Neu^{YC} signalling.

3.3.7 Second chromosome mutation Su(neu^{YC})3 maps to the gene eyes absent

$Su(neu^{YC})3$ is a strong suppressor of neu^{YC} in both eye and wing tissues. Additionally, $Su(neu^{YC})3$ suppressed neu^{YB}, neu^{YD} and neu^{NYPD} phenotypes, suggesting that $Su(neu^{YC})3$ functions in the canonical Ras1/MAPK signalling pathway. However, since neither constitutively active Ras1 nor dominant negative Ras1 were modified by $Su(neu^{YC})3$, it is likely that $Su(neu^{YC})3$ functions too far downstream to affect Ras1 function.

$Su(neu^{YC})3$ is a lethal mutation on the second chromosome, between bands 21C1 and 34D1 on the left arm. Within this region are the genes *dock* and *Sos*, which encode proteins that act upstream of *Ras1*. Lethal alleles of *dock* and *Sos* complemented

Su(neu^{YC})3 lethality, supporting the hypothesis that *Su(neu^{YC})3* is downstream of *Ras1*.

To continue mapping *Su(neu^{YC})3*, we chose to map the lethality of *Su(neu^{YC})3* by using

deficiencies that uncover the region between 21C1 and 34D1. Two deficiencies,

Df(2L)BSC6 and *Df(2L)BSC7*, failed to complement *Su(neu^{YC})3*. *Df(2L)BSC6* and

Df(2L)BSC7 overlap in the region 26D10-26F7. A smaller deficiency that uncovers

26D7-26E3, *Df(2L)BSC354*, also failed to complement *Su(neu^{YC})3* lethality. Moreover,

Df(2L)BSC7 and *Df(2L)BSC354* suppressed *neu^{YC}*-induced ommatidial fusion, similar to

suppression by *Su(neu^{YC})3* (Figure 3.20B-D). This indicated that the mapping of the

lethality corresponded to modification of *neu^{YC}*. All three deficiencies overlap in a 7kb

region that is within the bands 26D10 and 26E3, suggesting *Su(neu^{YC})3* is within this

region. Genes within this region include part of the uncharacterized gene *CG31637* and

the gene *eyes absent* (*eya*), a dual functioning serine/threonine phosphatase and

transcription factor that is a direct target of MAPK phosphorylation (Hsiao *et al.*, 2001; Li

et al., 2003; Tootle *et al.*, 2003). *eya$^{cli-IID}$*, a loss-of-function allele, failed to complement

Su(neu^{YC})3. Moreover, *eya$^{cli-IID}$* suppressed the *neu^{YC}* rough eye phenotype (Figure

3.21B), indicating that *Su(neu^{YC})3* mutates the *eya* open reading frame. Homozygous

mutants of alleles *eya^{1}* and *eya^{2}* have an eyeless phenotype due to the absence of eye

progenitor cells in the developing eye field. Heterozygous *eya^{1}* and *eya^{2}* flies have

normal eyes. Unlike *eya$^{cli-IID}$*, in *neu^{YC}* heterozygotes, haplosufficiency due to *eya^{1}* or

eya^{2} mildly enhanced ommatidial fusion (Figure 3.21C-D). This is most likely inherent

to the nature of the mutations, since *eya^{1}* and *eya^{2}* are the result of small deletions in the

eye-specific promoter region of *eya*.

Figure 3.20: Deficiencies that uncover *Su(neu^{YC})3* suppress *neu^{YC}* in the eye.

[A] *p[sev-GAL4] p[UAS-neu^{YC}]* heterozygotes with rough eye phenotype. [B] Suppression of *neu^{YC}* rough eye when flies are also heterozygous for *Df(2L)BSC7*, which uncovers the region 26D10 to 27C1. [C] Suppression of rough eye by *Df(2L)BSC354*. *Df(2L)BSC354* uncovers the region 26D7 to 26E3. [D] *Su(neu^{YC})3* suppression of ommatidial fusion. Both deficiencies suppress *neu^{YC}* to the same degree as *Su(neu^{YC})3*. The overlapping region between *Df(2L)BSC7* and *Df(2L)BSC354* partially uncovers the gene *CG31637* and fully uncovers *eyes absent*.

Figure 3.21: *eyes absent* **modifies** *neu*^{YC} **in the eye.**

[A] Rough eye phenotype in *p[sev-GAL4] p[UAS-neu*^{YC}*]/+* compound eyes. [B]

Suppression of *neu*^{YC}-dependent facet fusion by *eya*$^{cli-IID}$ compared to *p[sev-GAL4]*

p[UAS-neu^{YC}*]* heterozygotes. *neu*^{YC} ommatidial fusion is mildly enhanced by [C]

In(2L)eya, eya^{1} and [D] *eya*^{2} mutations, indicated by increased flattening of fused tissue.

eya encodes two alternative splice variants, Type I and Type II. Overexpression of the Type I splice variant, p[UAS-eya.B.I], and the Type II splice variant, p[UAS-eya.B.II], in the developing eye enhanced the neu^{YC} phenotype, consistent with a positive role for eya in Neu^{YC} and MAPK activated signal transduction (Figure 3.22E-F). eya expression has not been reported in the wing imaginal disc (Bonini et al., 1998; Leiserson et al., 1998). neu^{YC} margin loss in adult wings was suppressed by eya$^{cli-IID}$, eya^1 and eya^2 mutations. Complete suppression was observed in eya^1 heterozygous flies (0.04 ± 0.04%; Figure 3.23B-D). Surprisingly, overexpression of eya transcripts also suppressed neu^{YC} notch formation (Figure 3.24E & F). This suppression is not due to the dilution of GAL4 protein over the two UAS targets, UAS-neu^{YC} and UAS-eya. Overexpression of Type I transcript reduced wing margin loss to 3.74 ± 0.78% (P = 0.00) and Type II transcript reduced margin loss to 0.46 ± 0.26% (P = 0.00) compared to 6.56 ± 0.73% wing margin loss in neu^{YC}-expressing flies. Whether Eya functions in a regulatory feedback loop in the wing as it does during eye development awaits further testing. We can deduce that eya functions as a positive mediator of YC pTyr output in the eye and that it may also have a role downstream of RTK signalling during wing development.

In summary, we have screened 19 081 flies for enhancement or suppression of neu^{YC}. Eya, a MAPK target, has been identified as a downstream component of neu signalling, providing strength to our screen. Furthermore, several novel YC-specific and Ras1-specific modifiers are currently being mapped (Summarized in Table 3.2). E(neu^{YC})69, Su(neu^{YC})93, Su(neu^{YC})162, E(neu^{YC})50 and E(neu^{YC})32J are the most

promising neu^{YC}-specific modifiers. $E(neu^{YC})69$ is a strong neu^{YC}-specific enhancer that is $Ras1$ dependent. $Su(neu^{YC})93$ and $Su(neu^{YC})162$ are moderate suppressors that are $Ras1$ dependent. $E(neu^{YC})50$ is also a moderate enhancer that also modifies neu^{YE} and $Ras1$. $E(neu^{YC})32J$ is the only YC-specific modifier identified that is independent of $Ras1$. These alleles will provide new opportunities towards characterizing YC signalling output.

Figure 3.22: Overexpression of *eyes absent* modifies *neu^{YC}*.

p[UAS-mCD8::GFP] is misexpressed in [A] *p[sev-GAL4]* heterozygotes and [D] *p[sev-GAL4] p[UAS-neu^{YC}]* heterozygotes to account for the reduction in *neu^{YC}* expression by *p[sev-GAL4]*. Overexpression of [B] *p[UAS-eya.B.I]* and [C] *p[UAS-eya.B.II]* produces a mild phenotype in the eye characterized by a few missing bristles. In *p[sev-GAL4] p[UAS-neu^{YC}]* heterozygotes [E] *p[UAS-eya.B.I]* and [F] *p[UAS-eya.B.II]* mildly enhance ommatidial fusion and flattening of eye surface compared to *neu^{YC}* rough eye.

Figure 3.23: *eya* **mutations modify** *neu*^{YC} **in the wing margin.**

Suppression of *neu*^{YC} margin loss in flies also haplosufficient for *eya*. Average margin

loss and standard error in percent are reported in the bottom right corner. A P < 0.05

indicates statistical significance as determined by Students' T-Test. 'n' indicates sample

size. [A] Ectopic expression of *p[UAS-neu*^{YC}*]* by *p[GAL4]bbg*^{C96} produces an average

margin loss of 12.59 ± 1.1% in *yw*$^-$ background. [B] *eya*$^{cli-IID}$ reduces wing margin loss to

an average of 7.73 ± 1.4% per wing. Complete suppression of wing notches is seen with

loss of *eya*, in [C] *In(2L)eya, eya*^{1} heterozygous flies (0.04 ± 0.04% average notch

length). Near complete suppression of wing notches is seen in [D] *eya*^{2}*/+ ;*

p[GAL4]bbg^{C96} *p[UAS-neu*^{YC}*]/+* heterozygotes, with 1.51 ± 0.36% margin loss per wing.

Figure 3.24: Overexpression of *eya* suppresses *neu^{YC}* wing notches.

p[UAS-mCD8::GFP] is misexpressed in [A] *p[GAL4]bbg^{C96}* heterozygotes and [D]

p[GAL4]bbg^{C96} p[UAS-neu^{YC}] heterozygotes to account for the reduction in *neu^{YC}*

expression by *p[GAL4]bbg^{C96}*. Average margin loss and standard error in percentage is

reported in the bottom right corner. Significance is determined by the Mann-Whitney U-

Test, with $P < 0.05$. 'n' indicates sample size. [A] Ectopic expression of *p[UAS-

mCD8::GFP]* does not affect wing morphology. Misexpression of *eya* using [B] *p[UAS-

eya.B.I]* and [C] *p[UAS-eya.B.II]* do not produce wing notches at the margin. [D]

Control *neu^{YC}* flies have reduced average wing notch length of $6.56 \pm 0.73\%$ per wing.

Misexpression of [E] *p[UAS-eya.B.I]* and [F] *p[UAS-eya.B.II]* suppress margin loss to

$3.74 \pm 0.78\%$ and $0.46 \pm 0.26\%$, respectively.

Table 3.2: Summary of genetic modification by EMS mutations

	Viability	*YB*	*YD*	*YE*	*NYPD*	*Ras1^{V12}*	*Ras1^{N17}*	*bbg^{C96}; neu$_{YC}$*
Su(neu^{YC})3	L	-	---	N	---	N	N	--
Su(neu^{YC})77	V	N	---	N	-	--	N	N
Su(neu^{YC})93	V	N		N	N	-	N	-
Su(neu^{YC})99	L	N	--	N	---	--	+++	-
Su(neu^{YC})138	V	N	-	N	N	-	N	-
Su(neu^{YC})162	L	N		N	---	N	++	-
E(neu^{YC})5	L	N	N	+++	N	N	N	-
E(neu^{YC})6J	V	+		+	N	+	N	-
E(neu^{YC})32	V	N	N	+	++	N	N	N
E(neu^{YC})32J	V	N		N	N	N	N	-
E(neu^{YC})42AJ	V	+		+++	++	N	N	N
E(neu^{YC})45J	V	N	-	+++	+	N	+	N
E(neu^{YC})46	V	N	N	N	N	N	N	N
E(neu^{YC})50	V	N	N	+++	+++	N	-	-
E(neu^{YC})69	L	N	N	N	N	-	+++	-
E(neu^{YC})3-1	L	+	N	+++	+++	N	N	++
E(neu^{YC})3-3	L	+	N	+	N	N	N	++

('L' = lethal; 'V' = viable; '+' = mild enhancement; '++' = moderate enhancement; '+++' = strong enhancement; '-' = mild suppression; '--' = moderate suppression; '---' = strong suppression; 'N' = no modification)

3.4 Discussion

This chapter reports the results from conducting an EMS mutagenesis screen to identify novel modifiers involved in the Neu^{YC} signalling pathway. Following isogenization of the *CS-P* starter strain, a concentration of 16.8 mM was identified to generate 34.4% single X-linked lethal mutations, a dose well within the optimal range of 25-40% that corresponds to approximately one mutation per chromosome. This differs from the widely used standard concentration of 25 mM. The effective EMS concentration can differ depending on the fly strain, administration method and general lab surroundings (Grigliatti, 1998; Bokel, 2008). Using 25 mM, F1 progeny failed to survive. We, therefore, conducted our mutagenesis screen using 16.8 mM of EMS to generate mutations in genes that modify *neu*^{YC}.

Saturation of mutagenesis screens is desirable, as this indicates that all the genes within the pathway of interest have been mutated at least once. In this screen, saturation of the genome was not achieved, as only one third of the identified mutations were isolated. The low efficiency of the screen can be attributed to factors that centre on screen design. For instance, male and female adult flies exhibit differential expressivity of the *neu*^{YC}-dependent rough eye phenotype due to effects of dosage compensation on *sev* expression (Appendix C3). Identification of enhancers in females and suppressors in males was therefore difficult. Expression of *neu*^{YC} showed high expressivity, which was also problematic for identifying a threshold for enhancement or suppression of the rough eye phenotype (Appendix C3). Furthermore, strains expressing *neu*^{YC} were prone to accumulating suppressing mutations in the background (Appendix C4). This confounded

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the effectiveness of the screen and the background required "cleaning" every few months. Efficiency of future screens involving neu^{YC} may be improved by controlling neu^{YC} expression using a promoter that is not affected by dosage compensation, such as GMR in the eye, or shortvein (shv)/decapentaplegic (dpp) or thickvein (tkv) in wing vein development. Transgenic flies carrying a promoter fusion construct of GMR-1::neu^{YC} were generated; however, flies carrying a single copy of the construct produced a very mild eye phenotype that was not easily scored under the light microscope [data not shown]. Increasing the number of GMR-binding sites within the vector would increase neu^{YC} expression, thereby providing a moderate phenotype that is visible under the light microscope and susceptible to detectable modification.

We have hypothesized that Neu^{YC} has both Ras1-dependent and Ras1– independent outputs. Ten modifiers [$Su(neu^{YC})3$, $Su(neu^{YC})93$, $Su(neu^{YC})99$, $Su(neu^{YC})138$, $Su(neu^{YC})162$, $E(neu^{YC})3-1$, $E(neu^{YC})3-3$, $E(neu^{YC})6J$, $E(neu^{YC})50$ and $E(neu^{YC})69$] are involved in canonical Ras1 pathway signalling as they are sensitive to either $Ras1^{V12}$, $Ras1^{N17}$ or the neu add-back alleles YB, YD or NYPD. Only two modifiers, $E(neu^{YC})5$ and $E(neu^{YC})32J$, do not appear to be involved in the Ras1 pathway. Yet, we cannot rule out the possibility that these two modifiers may be involved in Ras1 signalling. As shown in Chapter 2, Neu^{YC}-dependent signalling is mediated by Ras1, and $E(neu^{YC})5$ and $E(neu^{YC})32J$ may be weak loss-of-function alleles that do not significantly affect $Ras1^{V12}$ or $Ras1^{N17}$. In addition to altering signalling from Neu^{YC}, $E(neu^{YC})5$ also alters Neu^{YE} signalling. Although Neu^{YE} was reported as being insensitive to Ras1 in *Drosophila*, Neu^{YE}-dependent transformation was inhibited by Rap1A and α-Ras

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antibody-mediated inhibition of Ras in Rat1 fibroblasts (Dankort *et al.*, 1997; Dankort *et al.*, 2001a; Dankort *et al.*, 2001b). Identification of these mutations by further mapping and molecular characterization will resolve their role in relation to Ras1.

Four of the Ras1-dependent modifiers – $Su(neu^{YC})93$, $Su(neu^{YC})162$, $E(neu^{YC})6J$ and $E(neu^{YC})50$ – modified $Ras1^{V12}$, indicating their requirement downstream of *Ras1*. $Su(neu^{YC})3$, $Su(neu^{YC})138$, $E(neu^{YC})3-1$, and $E(neu^{YC})3-3$ are also required downstream of *Ras1*. Although they failed to modify the Ras1 alleles, YB and/or YD outputs were modified, indicating a role in canonical Ras1 signalling. $Su(neu^{YC})99$ and $E(neu^{YC})69$ appear to be active upstream of *Ras1*, as they are sensitive to both $Ras1^{V12}$ and $Ras1^{N17}$. A second neu^{YC} insertion was suppressed in the wing by $Su(neu^{YC})3$, $E(neu^{YC})6J$, $E(neu^{YC})32J$, $E(neu^{YC})50$ and $E(neu^{YC})69$. Differential effects of enhancers with neu^{YC} inserts suggest tissue-specific roles for these modifiers in regulating Neu^{YC} signalling. Another explanation is the possibility of other mutations within these strains modifying neu^{YC} or confounding the identified modifier.

Four modifiers [$E(neu^{YC})3-1$, $E(neu^{YC})3-3$, $E(neu^{YC})6J$ and $E(neu^{YC})69$] have been partially mapped (Appendix G). Of these, $E(neu^{YC})69$ is the most promising neu^{YC-} specific modifier. $E(neu^{YC})69$ is most likely a mutation in a novel gene that functions downstream of Ras1 in response to YC pTyr activation, as it failed to modify other pTyr, including the kinase domain of Neu. Completion of mapping and further characterization awaits $E(neu^{YC})69$.

One modifier [$Su(neu^{YC})3$] has been genetically identified. $Su(neu^{YC})3$ appears to be a mutation in the *eyes absent* gene. Eya is a phosphorylation target of the Ser/Thr

kinase MAPK (Hsiao *et al.*, 2001). This is consistent with suppression of YB, YD and

NYPD pTyr outputs by $Su(neu^{YC})3$, as all three activate the Ras/MAPK pathway. Eya is

a dual function transcription factor and protein tyrosine phosphatase (Tootle *et al.*, 2003).

First identified for its role in compound eye development, *eya* is an essential retinal

determination gene involved in the specification of the eye field. In the eye primordium,

loss of *eya* in progenitor cells induces programmed cell death and produces an eyeless

phenotype in adults, suggesting *eya* positively mediates cell proliferation (Bonini *et al.*,

1998). Consistent with this, Neu^{YC}-dependent eye fusion is reduced in the presence of

eya$^{cli-IID}$, and overexpression of both Eya isoforms enhanced rough eye formation.

Surprisingly, the eye-specific mutations *eya^1* and *eya^2* enhanced eye fusion, suggesting an

opposite role for Eya in cell proliferation. Similarly, a two-fold reduction in Eya

suppressed notch formation at the wing margin, supporting the positive role for Eya in

cell proliferation. However, overexpression of type I and type II *eya* transcripts also

suppressed wing notches despite the decline in *neu^{YC}* expression by diluting the available

GAL4 proteins over two UAS targets. This supports inhibition of cell cycle entry by Eya.

How Eya regulates the cell cycle to coordinate tissue development remains unclear. What

is clear from this data is the requirement for Eya in Neu^{YC}-specific signalling. Further

characterization of *eya* will clarify the regulatory mechanisms that synergize to

coordinate cell proliferation and organ development.

In conclusion, despite an inefficient screen design, we have been successful in

identifying modifiers of the YC pTyr output of Neu. Identification of *eya* as a modifier of

neu^{YC} supports the validity of our screen in identifying modifiers downstream of

RTK/Ras1 signalling. The mapping and molecular characterization of the remaining modifiers, such as $E(neu^{YC})69$ and $E(neu^{YC})32J$, may lead to the identification of novel proteins involved in YC-specific signalling that link the YC pTyr to Ras1-dependent or Ras1-independent pathways. Together, this will provide new insight into the mechanism of Neu^{YC} signalling and its regulation.

CHAPTER 4 : CONCLUSIONS

This thesis describes studies aimed at genetically elucidating signalling partners from the specific pTyr 1201 of the Neu RTK. The objective of this thesis was to determine if signalling from Neu^{YC} involved both Ras-dependent and Ras-independent mechanisms. Two genetic modifier screens were conducted to accomplish this: 1) a dosage-sensitive modifier screen was conducted to identify roles for known modifiers of the Ras/MAPK pathway; and 2) novel, Ras-dependent and independent modifiers, were sought in a dominant F1 enhancer-suppressor screen.

Neu is the rat ortholog of the human RTK HER-2/ErbB2 that is overexpressed in 18-20% of breast cancers. Overexpression results in increased mitogenicity and migration that correlates with the highly aggressive and metastatic nature of tumours common to HER-2-positive patients.

Molecular analysis of signalling from Neu^{YC} identified *in vitro* binding partners Nck and Crk (Dankort *et al.*, 2001a; Marone *et al.*, 2004). Two studies using different Ras competition assays came to different conclusions when attempting to identify Ras-dependency in Neu^{YC} signalling. The first inhibited Ras by introducing an α-Ras antibody. Transformation in *neu*^{YC}-expressing lines was inhibited by the α-Ras antibody (Dankort *et al.*, 1997; Dankort *et al.*, 2001b). The second study took advantage of the natural inhibition of Ras by Rap1A. Transformation was not greatly affected by Rap1A in YC-expressing lines (Dankort *et al.*, 2001b). Together, these lines of evidence suggest that Neu^{YC} has both Ras-dependent and Ras-independent signalling mechanisms. Preliminary dosage-sensitive studies in *Drosophila* also suggested Ras-dependency in

Neu^{YC} signalling, however, failure of the loss of one copy of Ras to completely suppress *neu*^{YC} suggests that it is not the primary mediator of Neu^{YC} signalling (Settle *et al.*, 2003).

4.1 The Role of Known *Drosophila* Ras GTPases in Neu^{YC} Signalling

Extensive assessment of the role of Ras in Neu^{YC} in *Drosophila* required a quantitative approach due to the expressivity of *neu*^{YC} in the wing margin and developing eye. Therefore, wing notches generated by ectopic *neu*^{YC} expression were measured. Suppression and enhancement were successfully identified using loss- and gain-of-function alleles of known modulators of the Ras/MAPK pathway, such as Grb-2 and Raf, which were identified as suppressors of *neu*^{YC}. We further established a level of greater sensitivity in detecting modification of phenotype, as the suppression of notches by Grb-2 was more pronounced than previously reported by qualitative analysis (Settle *et al.*, 2003).

Exploiting this approach, the behavior of Neu^{YC}-dependent notch formation was studied in the presence of loss- and gain-of-function alleles of the *Drosophila* Ras GTPase orthologue, Ras1. Neu^{YC} is strongly sensitive to reduced and hyperactivated Ras1. Strength of sensitivity appears to be a combination of signalling from Neu^{YC} and the kinase domain of Neu. In Rat1 fibroblast cells, the kinase domain retains limited transforming potential (Dankort *et al.*, 1997). Phosphotyrosine residues within the kinase domain are hypothesized to couple to distinct effector pathways, such as PLC-γ and Src kinase, which physically interact with the kinase domain of Neu to mediate signalling (Peles *et al.*, 1991; Muthuswamy *et al.*, 1994; Kim *et al.*, 2005).

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The potent suppression of Neu^{YC} by Grb-2 mutants suggests that Grb-2 mediates Ras1 activation by Neu^{YC}. This is supported by the moderate sensitivity of the *neu^{YC}* phenotype to haplosufficiency of the Ras1-specific GEF, *Sos*, and the *Ras1^{V12S35}* allele of Ras1, which specifically activate the MAPK effector pathway. Other adaptors we identified as modulators of Neu^{YC} include Dab, Nck and Crk. In mammalian studies, although Grb-2 does not mediate Neu^{YC}-dependent transformation, Nck and Crk both bind Neu^{YC} *in vitro* (Dankort *et al.*, 1997; Dankort *et al.*, 2001b; Marone *et al.*, 2004). Nck is an SH2-SH3 adaptor protein that links cell surface receptors to the actin cytoskeleton by activating the serine/threonine kinase Pak (Li *et al.*, 2001). The Crk adaptor protein functions downstream of RTKs and interacts with GEFs Sos and C3G to activate members of the Ras superfamily of small GTPases (Mochizuki *et al.*, 2000). In particular, Crk activation of C3G results in the activation of the Ras superfamily members Rap1A and R-Ras (Gotoh *et al.*, 1995; Mochizuki *et al.*, 2000). A model for Ras-independent activation of Neu^{YC} was proposed to occur through Rap1A by recruitment and activation of the Crk adaptor. We observed mild Nck and Crk-dependent suppression of *neu^{YC}* in only one tissue, indicating that these adaptors are not likely major throughputs for Neu^{YC} activated signalling.

Drosophila has a single R-Ras ortholog, Ras2 (Neuman-Silberberg *et al.*, 1984). Ras2 is roughly 50% identical in sequence to Ras1. Ras2 overexpression augments not only Neu^{YC} signalling, but also signalling from the kinase domain and the pTyrs YB and YE. Therefore, it is probable Ras2 acts in parallel to Neu signalling. Ras2 is not involved in canonical RTK signalling in *Drosophila*. Ras2 fails to rescue loss of R7

photoreceptor cells in *sev* mutants, and overexpression of constitutively active Ras2 does not modify the wing margin by itself in our assay (Banerjee *et al.*, 1987a).

Like mammalian H-Ras, R-Ras directly binds the effectors Raf kinase, PI3'K and RalGDS, however, it only potently activates PI3'K (Spaargaren *et al.*, 1994; Herrmann *et al.*, 1996; Marte *et al.*, 1997). Activation of R-Ras mediates cell-cell adhesion by promoting integrin activation and focal adhesion formation (Zhang *et al.*, 1996; Self *et al.*, 2001). R-Ras is activated by the GEF C3G. C3G is recruited and activated by the adaptor Crk (Mochizuki *et al.*, 2000). Crk binds a number of pTyr-containing proteins, including other adaptors like Paxillin and p130Cas, and the non-receptor tyrosine kinases Abl (Birge *et al.*, 1993; Feller *et al.*, 1994a; Feller *et al.*, 1994b; Ren *et al.*, 1994; Sakai *et al.*, 1994; de Jong *et al.*, 1995; Schaller and Parsons, 1995).

R-Ras is well characterized for its role in semaphorin-plexin B1 signalling in axon guidance (Negishi *et al.*, 2005; reviewed by Reuther and Der, 2000). The cell surface receptor plexin B1 inactivates R-Ras through the activity of the GAP Rnd. This inhibits integrin activation, leading to actin collapse in non-neuronal cells and growth cones.

R-Ras encourages integrin activation by antagonizing H-Ras-mediated integrin suppression. Additionally, targeting of R-Ras to focal adhesions is critical for the ability to regulate integrin function. FAK, which acts downstream of integrin signalling, and modulates the turnover of integrin based focal adhesions, cooperates with Neu/ErbB2 to promote metastasis (Benlimame *et al.*, 2005). R-Ras activation may also enhance Neu-dependent metastasis. Study of loss-of-function alleles or dominant-negative alleles of R-

Ras and R-Ras signalling partners are required to clarify the possible involvement of R-Ras in Neu signalling.

4.2 Identification of Novel Genetic Modifiers of Neu^{YC} Signalling

To identify novel genetic modifiers of Neu^{YC}, a dominant F1 mutagenesis screen was conducted using the chemical mutagen EMS. After screening over 19 000 flies, a total of 17 modifiers have been isolated. Of these, ten modifiers are dependent to some degree on Ras1 activation, as they are sensitive to either constitutive active Ras1, dominant negative Ras1 or *neu* add-back alleles NYPD, YB or YD. Two modifiers appear to function in a Ras1-independent manner. Only *E(neu^{YC})32J* is an YC-specific modifier that functions in a Ras1-independent manner. Identification of the gene mutated in the *E(neu^{YC})32J* mutant may lead to the identification of a novel Ras1-independent pathway activated by Neu^{YC}.

Of the Ras1-specific modifiers, *E(neu^{YC})69* is the most promising and has been partially mapped (Appendix G). *E(neu^{YC})69* is likely a mutation in a novel gene that connects Neu^{YC} to Ras1, as *E(neu^{YC})69* modifies both constitutively active and dominant negative Ras1 alleles. Moreover, YB and YD, which activate the canonical Ras/MAPK pathway, fail to be modified by *E(neu^{YC})69*. This may lead to the identification of a non-canonical Ras1-activating pathway stimulated by Neu^{YC}.

One modifier has been genetically mapped. *Su(neu^{YC})3*, also a modifier of the pTyrs YB, YD and NYPD, has been identified as a lethal mutation in the *eyes absent* (*eya*) locus. Eya is a dual transcriptional coactivator and phosphatase (Tootle *et al.*, 2003). Eya consists of an N-terminal nuclear localization sequence (NLS), a Pro/Ser/Thr-

rich region that contains the conserved Eya domain 2 (ED2), a second C-terminal NLS

followed by an Eya conserved domain (ECD) (Zimmerman *et al.*, 1997; Bui *et al.*,

2000a). The Pro/Ser/Thr-rich region is required for transactivation activity of Eya (Xu *et*

al., 1997; Silver *et al.*, 2003). The ECD contains a phosphatase domain and is conserved

in Eya proteins across invertebrate, vertebrate and plant species (reviewed by Jemc and

Rebay, 2007).

As a transcription factor, Eya is a member of the retinal determination gene

network, a set of transcription factors that act in cooperation to direct retinal tissue

specification (Bonini *et al.*, 1993; Bonini *et al.*, 1997; Pignoni *et al.*, 1997; Leiserson *et*

al., 1998; Niimi *et al.*, 1999; Bui *et al.*, 2000b; Punzo *et al.*, 2002). Other members

include *twin of eyeless* (*toy*), *eyeless* (*ey*), *sine oculus* (*so*) and *daschund* (*dac*). Briefly,

Toy drives expression of *ey*, which directs expression of *eya* and *so*, which, together,

activate *dac*. Eya, So and Dac function in positive feedback loops to modulate output of

upstream genes. The Six domain of So directly binds the Eya ECD domain (Pignoni *et*

al., 1997). A direct interaction between Eya and Dac has not been reported, although

interaction may be indirect through So (Chen *et al.*, 1997).

The role of Eya as a phosphatase is beginning to be understood. The catalytic

phosphatase motif in ECD belongs to the haloacid dehalogenase (HAD) superfamily of

enzymes (Rebay *et al.*, 2005). HADs are found in all organisms and are best

characterized in prokaryotes; their function in eukaryotes is poorly understood. HADs

are unique, using an aspartate residue as the nucleophile for catalysis instead of the

traditional cysteine residue used in thiol-based protein tyrosine phosphatases (PTPs). The

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majority of evidence points to phospho-tyrosine residues as the target of Eya phosphatase activity, however studies using mouse Eya3 suggest dual phospho-serine and phospho-threonine peptide specificity (Li *et al.*, 2003).

Neu^{YC} is sensitive to the lethal mutation *eya$^{cli-IID}$* in the eye and the wing margin. This is synonymous with our identified lethal *eya* mutation *Su(neu^{YC})3*. Lethal mutations in the *eya* locus generally occur in the Eya conserved domain (ECD) (Bui *et al.*, 2000a). This region is shared in both Eya isoforms. Indeed, the *eya$^{cli-IID}$* allele is the result of a point mutation at nucleotide 1395 (C1395T), resulting in the replacement of the Gln amino acid at position 335 with a premature stop codon (Zimmerman *et al.*, 1997; Bui *et al.*, 2000a). The resulting protein product has a truncated ED2 and is therefore missing the ECD, located C-terminal to ED2. Loss of the ECD results in a functional null. Our EMS-induced *eya* lethal allele, *Su(neu^{YC})3*, genetically behaves as *eya$^{cli-IID}$*, therefore, *Su(neu^{YC})3* is most likely null for Eya activity. Sequence analysis the *Su(neu^{YC})3* mutation will identify the location and nature of the DNA lesion.

Eye-specific mutations *eya^1* and *eya^2* and overexpression of type I and type II *eya* transcripts behave unexpectedly in a tissue-specific manner. Contrary to the expected suppression a loss-of-function allele would produce, *eya^1* and *eya^2* mildly enhance *neu^{YC}* –dependent ommatidial fusion, yet suppress wing notch formation. The *eya^1* and *eya^2* hypomorphs are the result of small deletions in the eye-specific promoter of *eya* (Bonini *et al.*, 1998; Bui *et al.*, 2000b; Zimmerman *et al.*, 2000). Homozygous *eya^1* and *eya^2* mutants have an eyeless phenotype, while heterozygotes have normal eyes (Bonini *et al.*, 1993). The progenitor cells in the eye imaginal discs from homozygotes fail to

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proliferate, resulting in loss of the eye field. The role of Eya is unclear from these interactions, as deletions affecting the promoter region of *eya* can have multiple effects. The behavior of the strong hypomorphic alleles *eya*$^{cli-IID}$ and *Su(neu*^{YC}*)3* clearly identifies its role downstream of Neu^{YC} signalling. This is supported by the identification of Eya as a direct target of MAPK phosphorylation (Hsiao *et al.*, 2001).

Similar to the inconsistent behavior of the homozygous *eya*^{1} and *eya*^{2} alleles, overexpression of type I and II *eya* transcripts suppress wing notch formation, but enhance ommatidial fusion, consistent with mutant data. The role of Eya during wing development remains uncharacterized. As a phosphatase, Eya may have a negative impact on substrate function; however, Eya phosphatase activity has not been associated with inhibition of activity. Phosphatase activity is required for Eya transactivation potential and association with transcriptional cofactors So and Dac occurs through the ECD (Chen *et al.*, 1997; Pignoni *et al.*, 1997; Xu *et al.*, 1997; Li *et al.*, 2003). Additionally, the mammalian Dac ortholog Dach, which functions as a repressor, requires Eya phosphatase activity to convert Dach to an activator. However, phosphatase activity or direct dephosphorylation of Dac or other transcriptional cofactors has not been observed *in vivo*. Currently, there are only two identified substrate candidates for Eya phosphatase activity *in vitro* – the C-terminal domain repeats of RNA polymerase II and Eya itself (reviewed by Jemc and Rebay, 2007). It is postulated that dephosphorylation of RNA polymerase II may facilitate recycling, and therefore, transcriptional activation. However, Eya autophosphatase activity is unclear. An *in vitro* phosphatase assay, using GST-ECD, dephosphorylated full length tyrosine-phosphorylated Eya purified from

Drosophila S2 cells. However, direct self association of Eya has not been reported. The nature of Eya autocatalytic function and identification of the specific phospho-targets needs further investigation (Rebay *et al.*, 2005).

The identification of *eya* as a modifier of *neu^{YC}* in the mutagenesis screen supports our efforts of finding *bona fide* modifiers downstream of Neu^{YC}/Ras signalling. Further mapping and characterization of *E(neu^{YC})32J* and *E(neu^{YC})69* will lead to the identification of novel Ras1-dependent and Ras1-independent modifiers that are specific to the YC pTyr.

In conclusion, we have successfully identified both Ras1-dependent and Ras1-independent mechanisms of signalling linked to the YC pTyr. Neu^{YC} most likely couples to Ras1 by first recruiting and activating the Grb-2-Sos complex. Additionally, Ras2 is also involved in Neu signalling, most likely in parallel to Neu. The identification of *eya* validates the mutagenesis screen, supporting the authenticity of other modifiers isolated from the same screen. Mapping of *E(neu^{YC})69* will identify a novel YC-specific Ras1-dependent modifier that negatively regulates YC-specific signalling. Ras1-independent modifiers have been isolated, with *E(neu^{YC})32J* being the most promising. Together, these data support the hypothesis that signalling through the Neu pTyr YC occurs with and without Ras1.

4.3 Analysis of Enhancer-Suppressor Screens

Enhancer-suppressor screens are powerful tools with which to identify novel components of signalling pathways. Most loss-of-function mutations are recessive, indicating that 50% gene function is sufficient for normal activity. Taking into account

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that mutations in many genes have no detectable phenotype, a 50% reduction in gene function may not be sufficient for normal function when the pathway is already disturbed. Enhancer-suppressor screens take advantage of this by searching for mutations in genes that modify a sensitized mutant background with a detectable phenotype. Since only one copy of the gene is mutated, these modifiers are termed dominant enhancers or suppressors. Phenotypes can be induced using hypomorphic alleles or gain-of-function alleles of the gene in question. This approach has been most successful in identifying canonical signalling pathway components downstream of the sev RTK, the small GTPase Ras and its substrate Raf (Rogge et al., 1991; Simon et al., 1992; Dickson et al., 1996; Karim et al., 1996). The success of these screens can be attributed to several factors that include the efficiency of the screen design and the strength or suitability of the phenotype being scored.

There are several disadvantages to this approach. Many modifiers identified by this approach affect pathways through unrelated processes, such as pathways that regulate expression of the gene of interest or that direct the development of the tissue being assayed (reviewed by St. Johnston, 2002). Secondly, such a screen will not identify modifiers that do not function in a dosage-dependent manner. That is, modifiers that retain function when reduced by half will not affect signalling through the already perturbed pathway. Lastly, the majority of homozygous viable mutations identified do not have a phenotype and, therefore, may not have an essential role in the signalling pathway.

The two enhancer-suppressor screens presented in this thesis were confronted with several different problems. The failure to identify multiple strong enhancers and suppressors of *neu^{YC}* in the dosage-sensitive modifier screen and mutagenesis screen suggests 2 possibilities: 1) that not all avenues have been exhausted while searching for modifiers of *neu^{YC}* in the genome; and 2) that modifiers of *neu^{YC}* do not function in a dose-dependent manner. Others problems relate to the design of the screens. One major setback was a result of driving *UAS-neu^{YC}* expression using *sev-GAL4*. *sev* is located on the X chromosome, and is therefore subject to dosage compensation in males. Dosage compensation leads to the upregulation of many X-linked genes to almost two-times the levels observed in females (Straub *et al.*, 2005). Ectopic expression of *neu^{YC}* using *sev-GAL4* resulted in a more severe phenotype in males than females (Appendix C.3). Furthermore, the broad phenotypic range of *neu^{YC}* expression in both genders confounded the ability to identify suppressors and enhancers. Future screens seeking to use the *sev-GAL4* driver should use the *sev* enhancer/promoter fused to the target gene instead. A *sev::ras^{V12}* fusion construct was successfully used to identify downstream components of the Ras signalling pathway (Karim *et al.*, 1996). Similar to the expressivity seen with *sev-GAL4*, ectopic expression using *bbg^{C96}* generated a mild to moderate phenotype difficult to screen suppressors with. Promoters, not affected by dosage compensation that may be suited to driving *neu^{YC}* expression include GMR, shv/dpp or tkv.

Major difficulties also arose pertaining to the strength of the GAL4 driver. Over successive generations, the expressivity of *neu^{YC}* decreases when driven with *sev-GAL4* (Appendix C.4). Repetitive removal of accumulated background mutations by

recombination was conducted every 3 to 4 months, stalling the enhancer-suppressor screens. A similar decrease in expressivity with bbg^{C96} was observed to occur at a much slower rate. Removal of background mutations did not restore expressivity to its original range, but did shift expressivity to an acceptable range for screening. A recent report in *Fly* describes the loss of GAL4-driven reporter gene expression due to physical loss of GAL4 protein. The loss of GAL4 protein was linked to a bacterial agent (Barrett *et al.*, 2008). Treatment with tetracycline restored stability of the GAL4 strain. Therefore, reduced neu^{YC} expressivity may be a result of GAL4 protein instability due to a bacterial contagion. Future testing of *p[sev-GAL4] p[UAS-neu^{YC}]* recombinant lines with tetracycline will identify if a bacterial agent is responsible for the decreased expression observed over generations.

Not only did these difficulties confound the screening process, but they also affected genetic mapping of unknown mutations. The genetic mapping strategy used required verification of the modifying phenotype at each step (Figure 3.18; Appendix G.4). As the expression of neu^{YC} decreased, seen by the shift in expressivity, identification of suppressors and enhancers became increasingly arduous during the mapping process. Other mapping procedures, like SNP mapping, were not employed in this screen. Unlike SNP mapping in other organisms like *C. elegans*, SNP mapping in *Drosophila* is not as well implemented. Several different techniques have been explored in combination with SNP mapping to exploit its power in *Drosophila*; however, a well established library of SNPs is only available for a limited number of strains (Berger *et al.*, 2001; Hoskins *et al.*, 2001; Martin *et al.*, 2001; Nairz *et al.*, 2002; Chen *et al.*, 2008a). As

researchers continue to explore this approach, SNP libraries for various strains continue to expand. In order for future screens involving neu^{YC} to take advantage of this approach, the initial stock used for mutagenesis would require all SNPs to be characterized.

4.4 Future Research

The studies presented here provide an idea of the nature of signalling from the YC pTyr of the Neu RTK. The inability to identify strong modifiers of neu^{YC} in the dosage-sensitive modifier screen and mutagenesis screen suggests that we have not identified the major signalling output activated by YC and that modifiers of neu^{YC} may not function in a dose-dependent manner. Expansion of the dosage-sensitive modifier screen and re-designing the mutagenesis screen to identify modifiers that are not dose-dependent can address these issues. Furthermore, several other lines of evidence require further study. These include continued mapping of unknown modifiers identified from the chemical mutagenesis screen and functional analysis of Eya.

4.4.1 Expansion of dosage-sensitive modifier screen

From the dosage-sensitive modifier screen, we now know that Ras1 and Ras2 are involved in Neu^{YC} signalling. Ras2 involvement may be downstream or parallel to Neu activation. Loss-of-function analysis is required to clarify the position of Ras2 in Neu signalling.

In addition to Ras1 and Ras2, *Drosophila* also has a third member of the Ras superfamily of small GTPases – Ras3 or Rap1A (Pizon *et al.*, 1988). In mammals, Rap1A competes with Ras for Raf binding (Hu *et al.*, 1997). Unlike Ras, Rap1A binding does not activate Raf. Contrary evidence showing Ras-independent Raf activation by

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Rap1A downstream of Torso RTK has been reported in *Drosophila* embryos (Hou *et al.*, 1995; Mishra *et al.*, 2005). Rap1A may provide a Ras-parallel link to Raf activation. Dosage sensitive analysis using loss- and gain-of-function Rap1A alleles with *neu*^{YC} in the wing margin and developing eye is currently underway to address this.

Mammalian evidence continues to point to a role for Neu^{YC}-mediated signalling in cell migration and metastasis. Mammary tumour explants expressing Neu^{YC} are highly motile and have fewer mature focal adhesions that are smaller in size (Northey *et al.*, 2008). Add-back mutant cells expressing Neu^{YC} migrate as efficiently as Neu^{NT}-expressing constitutively active mutant cells (Marone *et al.*, 2004). Competition by a phospho-YC peptide inhibits YC-dependent migration, while the non-phosphorylated YC peptide does not interfere with migration. Neu also cooperates with FAK signalling, thereby contributing to Neu-dependent metastasis (Benlimame *et al.*, 2005). Future studies should involve a dosage-sensitive modifier screen of *Drosophila* orthologues known to be involved in cell migration, such as the GTPases Rac and Rho, integrins and integrin-linked adaptor proteins, scaffolding proteins involved in focal adhesions and actin cytoskeleton rearrangement, and Ets transcription factors.

4.4.2 *Mapping of chemically-induced mutations*

It is important to continue mapping of specific unknown modifiers identified from the chemical mutagenesis. Several mutations have been partially mapped, including *E(neu*^{YC}*)32J*, *E(neu*^{YC}*)69*, *E(neu*^{YC}*)6J* and allelic modifiers *E(neu*^{YC}*)3-1* and *E(neu*^{YC}*)3-3* (Chapter 3 Figure 3.19). Mapping of *E(neu*^{YC}*)32J* and *E(neu*^{YC}*)69* remains most vital to the identification of novel YC-specific modifiers. Since *E(neu*^{YC}*)32J* is a viable mutation,

it will be necessary to conduct further mapping using molecularly mapped P-elements.

Mapping of $E(neu^{YC})69$ can continue using deficiencies that uncover chromosomal

regions that have not yet been tested, such as the heterocentric chromatin of chromosome

2 (Appendix G.3).

Identification of $E(neu^{YC})6J$ will further support the validity of the mutagenesis

screen, since $E(neu^{YC})6J$ is not specific to the YC pTyr (Chapter 3 Table 3.2). Deficiency

mapping has narrowed the location to 31D8-31E3. However, a secondary lethal mutation

appears to have mapped with the modifying mutation (Appendix G.1 and G.2). Further

mapping necessitates the removal of the background mutation.

Preliminary mapping results indicate that mutations $E(neu^{YC})3-1$ and $E(neu^{YC})3-3$

are located at the telomeric tip of chromosome 2R (Appendix G.5). Heterozygotes

possess a scalloped wing phenotype similar to the classical *Drosophila LIM-only*

(dLMO)/Beadex (Bx) mutations, suggesting that mutations $E(neu^{YC})3-1$ and $E(neu^{YC})3-3$

may genetically interact with *dLMO* (Mattox and Davidson, 1984; Shoresh *et al.*, 1998;

Zeng *et al.*, 1998). Consistent with the classical *Bx* mutations, $E(neu^{YC})3-1$ and

$E(neu^{YC})3-3$ are completely suppressed with loss of one copy of *dLMO*, and enhanced

with duplication of the locus (Appendix G.6 and G.7). Continued mapping using the P-

element mapping strategy (Appendix G.4) will identify these mutations and further

validate the outcome of the mutagenesis screen.

4.4.3 Functional analysis of Eya

Finally, with the identification of *eya* as a modifier of *neu*, several questions

remain. What is the nature of the DNA lesion in our EMS-induced *eya* allele? Since

reduced Eya suppressed YC-induced wing margin defects, is *eya* expressed during wing development and what is its role downstream of RTK signalling outside the eye field? Can Eya function to both transmit and inhibit Neu signalling? Are other retinal determination genes involved in Neu-mediated signalling? Additionally, *Su(neu^{YC})3* strongly suppressed signalling from other *neu* add-back alleles that activate the canonical Ras/MAPK cascade. Does Eya function downstream of add-back alleles Neu^{NYPD}, Neu^{YB} or Neu^{YD}? Approaches using DNA sequence and RT-PCR analysis, and heteroallelic combinations or deletion constructs of *eya* and other retinal determination genes could answer these questions.

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APPENDICES

Appendix A: Comprehensive stock list used for genetic screening and mapping

Table A.1: Mutant Alleles

Gene Mutated	Allele (Gene)	Lesion (Mutagen)	Proposed Function	Reference
Ras1 oncogene	Ras1^{05703} Source: D. Montell	P-element insertion; hypomorph	GTPase	(Rorth, 1996;Schnorr and Berg, 1996)
	Ras1^{06677} Source: Bloomington	P-element insertion; hypomorph	GTPase	(Galindo et al., 2006;Spradling et al., 1999)
	Ras1^{A014} Source: Bloomington	P-element insertion; hypomorph	GTPase	Unpublished
	Ras1^{EY07538} Source: Bloomington	P-element insertion; hypomorph	GTPase	Unpublished
SHC	DShc$^{111-40, BG}$ Source: S. Luschnig	EMS mutagen; lethal	SH2-SH3 adaptor protein	(Luschnig et al., 2000)
Disabled	dab^{M54-R1} Source: E. Giniger	X-ray mutagen; amorph	X-ray mutagen; amorph	(Hill et al., 1995)
Daughter of sevenless	dos$^{1.46}$ Source: M. Simon	Lethal; recessive	SH2-SH3 adaptor protein	(Herbst et al., 1996)
Pole hole	phl^1 (phl^{C110}) Source: M.P. Martin	X-ray mutagen; hypomorph	Ser/Thr kinase	(Melnick et al., 1993;Perrimon et al., 1995;White and Jarman, 2000)
	phl^{11} Source: Bloomington	Spontaneous; amorph	Ser/Thr kinase	(Li et al., 1997;Melnick et al., 1993)
Dreadlocks	dock^{P1} Source: Y. Rao	P-element activity; hypomorph	SH2-SH3 adaptor protein	(Garrity et al., 1996)
Crk	Crk^{ED107} Source: J. Locke	P-element activity; hypomorph	SH2-SH3 adaptor protein	Unpublished
Rolled	rl^1 Source: Bloomington	Spontaneous; hypomorph	Ser/Thr kinase	(Biggs et al., 1994;Lorenzen et al., 2001)
Rolled	rl^{10a} Source: Bloomington	Deficiency; amorph	Ser/Thr kinase	(Lim et al., 1999;Peverali et al., 1996)

Gene Mutated	Allele (Gene)	Lesion (Mutagen)	Proposed Function	Reference
	Df(2R)M41A10 *Source: Bloomington*	Deficiency; amorph	Ser/Thr kinase	(Zhang *et al.*, 1999)
Downstream of Raf	$Dsor^{G42}$ *Source: Bloomington*	P-element insertion; hypomorph	Ser/Thr kinase	Unpublished
	$Dsor^{G79}$ *Source: Bloomington*	P-element insertion; hypomorph	Ser/Thr kinase	Unpublished
	$Dsor^{S-1221}$ *Source: Bloomington*	P-element insertion; hypomorph	Ser/Thr kinase	(Karim *et al.*, 1996)
	$Dsor^{LH110}$ *Source: Bloomington*	P-element insertion; hypomorph	Ser/Thr kinase	(Hsu and Perrimon, 1994;Lu *et al.*, 1994)
Paxillin	$Pax^{EY11633}$ *Source: Bloomington*	P-element insertion; hypomorph	Scaffolding protein	Unpublished
Corkscrew	csw^{LE120} *Source: L. Perkins*	EMS mutagen; amorph	Phosphatase	(Perkins *et al.*, 1996)
Son of sevenless	Sos^{34Ea-6} *Source: Bloomington*	EMS mutagen; amorph	Guanine nucleotide exchange factor	(Rogge *et al.*, 1991)
Downstream of receptor kinase	drk^{10626} *Source: Bloomington*	P-element activity; hypomorph	SH2-SH3 adaptor protein	(Simon *et al.*, 1993;Spradling *et al.*, 1999)

Table A.2: Transgenic Modifiers

pUAST Line	Gene	Genetics	Reference
Ras1^{V12}	Ras1 oncogene	Amino acid substitution G12V	(Lee *et al.*, 1996;Therrien *et al.*, 1996)
Ras1^{N17}	Ras1 oncogene	Amino acid substitution S17N	(Lee *et al.*, 1996)
Ras1^{V12S35}	Ras1 oncogene	Amino acid substitution G12V and T35S	(White *et al.*, 1996)
Ras1^{V12G37}	Ras1 oncogene	Amino acid substitution G12V and E37G	(White *et al.*, 1996)
Ras1^{V12C40}	Ras1 oncogene	Amino acid substitution G12V and Y40C	(Rodriguez-Viciana *et al.*, 1997)
Ras2^{V14}	Ras2	Amino acid substitution G14V	(Fortini *et al.*, 1992;Walker *et al.*, 2006)
Pax^{EY02020}	Paxillin	P-element mutagenesis	(Read *et al.*, 2005)
DP110	PI3'K	Wild type DP110	(Leevers *et al.*, 1996)

Table A.3: Second chromosome deficiency stocks (Bloomington)

Stock #	Deficiency Strain	Breakpoint 1	Breakpoint2
6608	Df(2L)BSC16	21C3-4	21C6-8
8673	Df(2L)BSC107	21C2	21E2
3084	Df(2L)ast2	21D1-2	22B2-3
3133	Df(2L)dp-79b	22A2-3	22D5-E1
7144	Df(2L)BSC37	22D2-3	22F1-2
6648	Df(2L)dpp^{d14}	22E4-F2	22F3-23A1
90	Df(2L)C144	22F4-23A1	23C2-4
1567	Df(2L)JS17	23C1-2	23E1-2
6875	Df(2L)BSC28	23C5-D1	23E2
6965	Df(2L)BSC31	23E5	23F4-5
6507	Df(2L)drm-P2	23F3-4	24A1-2
5330	Df(2L)ed1	24A2	24D4
693	Df(2L)sc19-8	24C2-8	25C8-9
7497	Df(2L)Exel6011	25C8	25D5
160	Df(2L)cl-h3	25D2-4	26B2-5
490	Df(2L)E110	25F3-26A1	26D3-11
6299	Df(2L)BSC5	26B1-2	26D1-2
6338	Df(2L)BSC6	26D3-E1	26F4-7
24378	Df(2L)BSC354	26D7	26E3
6374	Df(2L)BSC7	26D10-E1	27C1
2414	Df(2L)spd^{j2}	27C1-2	28A
5420	Df(2L)Dwee1-W05	27C2-3	27C4-5
4956	Df(2L)XE-3801	27E2	28D1
7147	Df(2L)BSC41	28A4-B1	28D3-9
9502	Df(2L)BSC142	28C3	28D3
140	Df(2L)Trf-C6R31	28DE	28DE
7807	Df(2L)Exel7034	28E1	28F1
179	Df(2L)TE29Aa-11	28E4-7	29B2-C1
8836	Df(2L)BSC111	28F5	29B1
9298	Df(2L)ED611	29B4	29C3
2892	Df(2L)N22-14	29C1-2	30C8-9
6478	Df(2L)BSC17	30C3-5	30F1
1045	Df(2L)Mdh	30D-30F	31F
8469	Df(2L)BSC50	30F5	31B1
3366	Df(2L)J2	31B	32A
9503	Df(2L)BSC143	31B1	31D9
6121	Df(2L)J77	31C-D	31E7-F2
6504	Df(2L)BSC144	31B1	31E4
6118	Df(2L)J3	31D	31F2-5
6904	Df(2L)J106	31D1	31F2

Stock #	Deficiency Strain	Breakpoint 1	Breakpoint2
7999	Df(2L)Exel7048	31E3	31F5
9638	Df(2L)BSC210	31F4	32A2
7142	Df(2L)BSC32	32A1-2	32C5-D1
9505	Df(2L)BSC145	32C1	32C1
7143	Df(2L)BSC36	32D1	32D4-E1
5869	Df(2L)FCK-20	32D1	32F1-3
3079	Df(2L)Prl	32F1-3	33F1-2
7420	Df(2L)ED778	33E9	34A7
6999	Df(2L)BSC30	34A3	34B7-9
9594	Df(2L)BSC159	34B4	34C4
3138	Df(2L)b87e25	34B12-C1	35B10-C1
9506	Df(2L)BSC147	34C1	34C6
6092	Df(2L)b80e3	34C4	35A4
6056	Df(2L)64j	34D1-2	35B9-C1
3588	Df(2L)TE35BC-24	35B4-6	35F1-7
1491	Df(2L)r10	35D1	36A6-7
2583	Df(2L)cact-255rv64	35F-36A	36D
420	Df(2L)TW137	36C2-4	37B9-C1
567	Df(2L)pr-A16	37B2-12	38D2-5
167	Df(2L)TW161	38A6-B1	40A4-B1
7531	Df(2L)Exel6049	40A5	40D3
9510	Df(2L)BSC151	40A5	40E5
4308	Df(2R)nap14	41BC	42A16-B1
1007	Df(2R)nap9	42A1-2	42E6-F1
1888	Df(2R)ST1	42B3-5	43E15-18
3368	Df(2R)cn9	42E	44C
198	Df(2R)H3C1	43F	44D3-8
201	Df(2R)H3E1	44D1-4	44F12
3591	Df(2R)Np5	44F10	45D9-E1
4966	Df(2R)w45-30n	45A6-7	45E2-3
6917	Df(2R)BSC29	45D3-4	45F2-6
9410	Df(2R)BSC132	45F6	46B4
1743	Df(2R)B5	46A	46C
1702	Df(2R)X1	46C	47A1
596	Df(2R)stan2	46F1-2	47D1-2
520	Df(2R)E3363	47A	47F
190	Df(2R)en-A	47D3	48B2
1145	Df(2R)en30	48A3-4	48C6-8
7145	Df(2R)BSC39	48C5-D1	48D5-E1
4960	Df(2R)CB21	48E	49A
7146	Df(2R)BSC40	48E1-2	48E2-10

Stock #	Deficiency Strain	Breakpoint 1	Breakpoint2
442	Df(2R)CX1	49C1-4	50C23-D2
7543	Df(2R)Exel6061	48F1	49A6
7869	Df(2R)Exel7121	49B5	49B12
7916	Df(2R)Exel8056	49C2	49E1
7872	Df(2R)Exel7124	49D4	50A1
7870	Df(2R)Exel7123	49D5	49E6
7544	Df(2R)Exel6062	49E6	49F1
7871	Df(2R)Exel8057	49F1-4	49F10
7873	Df(2R)Exel7128	50C5	50C9
6516	Df(2R)BSC18	50D1	50D2-7
7875	Df(2R)Exel7130	50D4	50E4
9496	Df(2R)BSC134	50E1	50E6
7876	Df(2R)Exel7131	50E4	50F6
7877	Df(2R)Exel8059	51A4-5	51B1
7749	Df(2R)Exel6284	51B1	51C2
7879	Df(2R)Exel7135	51E2	51E11
3518	Df(2R)Jp1	51D3-8	52F5-9
7273	Df(2R)vir130	59B	59D8-E1
7256	Df(2R)3-70	59B3	59D5-11
7262	Df(2R)bw-WI3128	59C	60AB
2355	Df(2R)bw-S46	59D8-11	60A7
4542	Df(2R)Chig230	60A3-7	60B4-7
9691	Df(2R)BSC155	60B8	60C4
2604	Df(2R)Px2	60C5-6	60D9-10
9068	Df(2R)ED4061	60C8	60D13
24117	Df(2R)ED4071	60C8	60E8
9069	Df(2R)ED4065	60C8	60E8
2471	Df(2R)M60E	60E2-3	60E11-12
1465	Df(2R)Dll-MP	60E3-4	60E5-6
2528	Df(2R)gsb	60E9-10	60F1-2
4961	Df(2R)Kr10	60F1	60F5
24758	Df(2R)ED50004	60F5	60F5

Table A.4: Third chromosome deficiency stocks (Bloomington)

Stock #	Genotype	Breakpoint 1	Breakpoint2
5126	Df(3L)XS533	76B4	77B
2052	Df(3L)rdgC-co2	77A1	77D1
3127	Df(3L)ri-79c	77B-C	77F-78A
5878	Df(3L)ri-XT1	77E2-4	78A2-4
4429	Df(3L)ME107	77F3	78C8-9
4430	Df(3L)Pc-2q	78C5-6	78E3-79A1

Appendix B: Verification of point mutations in *p[UAS-neu^{YC}]* vector

Figure B.1: Sequence alignments of the Neu^{YC} C-terminus

Sequence alignment of the 3' end of *neu*^{YC} in the pUAST vector with the *neu* sequence reported in Genbank (AY116182) to confirm site-directed mutagenesis at the autophosphorylation sites YA (3183-3185 nt; 1053 a.a), YB (3531-3533 nt; 1144 a.a.), YD (3778-3783 nt; 1226/7 a.a.) and YE (3859-3861 nt; 1253 a.a.) using ClustalW sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The Tyr residue at site YC (3703-3705 nt; 1201 a.a.) should not be mutated. [A] DNA sequence alignment following sequencing of nucleotides 3180-3885 at the 3' end of the *neu*^{YC} coding sequence in the pUAST vector. Alignment reveals nucleotide substitution of thymine for adenine at positions 3184 (YA), 3532 (YB), 3779 and 3782 (YD) and 3860 (YE). Tyr codon is identical between the two sequences at position 3703-3705, indicating the autophosphorylation site YC is intact. Other nucleotide substitutions are most likely silent mutations due to polymorphisms in the genetic code. [B] The sequenced 3' end of *neu*^{YC} translated and aligned to reported *neu* sequence (Genbank AY116182). Tyr to Phe amino acid substitutions are observed at positions 1053 (YA), 1144 (YB), 1226/7 (YD) and 1253 (YE), but not at position 1201 (YC), indicating this autophosphorylation site remains intact. Blue highlighting indicates mutated autophosphorylation sites YA, YB, YD and YE. Green highlights the autophosphorylation site YC and red shows the location of the stop codon.

A

```
neu-AY116182   GAGTATCTGGTGCCCCAGCAGGGATTCTTCTCCCCGGACCCTACCCCAGGCACTGGGAGC  3240
C-UAS-neuYC    GAGTTTCTAGTGCCCCAGCAGGGATTCTTCTCCCCGGACCCTACCCCAGGCACTGGGAGC  92
               **** ***  *************************************************

neu-AY116182   ACAGCCCATAGAAGGCACCGCAGCTCGTCCACCAGGAGTGGAGGTGGTGAGCTGACACTG  3300
C-UAS-neuYC    ACAGCCCATAGAAGGCACCGCAGCTCGTCCACCAGGAGTGGAGGTGGTGAGCTGACACTG  152
               ************************************************************

neu-AY116182   GGCCTGGAGCCCTCGGAAGAAGGGCCCCCCAGATCTCCACTGGCTCCCTCGGAAGGGGCT  3360
C-UAS-neuYC    GGCCTGGAGCCCTCGGAAGAAGGGCCCCCCAGATCTCCACTGGCTCCCTCGGAAGGGGCT  212
               ************************************************************

neu-AY116182   GGCTCCGATGTGTTTGATGGTGACCTGGCAATGGGGGGTAACCAAAGGGCTGCAGAGCCTC  3420
C-UAS-neuYC    GGCTCCGATGTGTTTGATGGTGACCTGGCAATGGGGGGTAACCAAAGGGCTGCAGAGCCTC  272
               ************************************************************

neu-AY116182   TCTCCACATGACCTCAGCCCTCTACAGCGGTACAGCGAGGACCCCACATTACCTCTGCCC  3480
C-UAS-neuYC    TCTCCACATGACCTCAGCCCTCTACAGCGGTACAGCGAGGACCCCACATTACCTCTGCCC  332
               ************************************************************

neu-AY116182   CCCGAGACTGATGGCTATGTTGCTCCCCTGGCCTGCAGCCCCCAGCCCGAGTATGTGAAC  3540
C-UAS-neuYC    CCCGAGACTGATGGCTATGTTGCTCCCCTGGCCTGCAGCCCCCAGCCCGAGTTTGTTAAC  392
               ***************************************************** ***  ***

neu-AY116182   CAATCAGAGGTTCAGCCTCAGCCTCCTTTAACCCCAGAGGGTCCTCTGCCTCCTGTCCGG  3600
C-UAS-neuYC    CAATCAGAGGTTCAGCCTCAGCCTCCTTTAACCCCAGAGGGTCCTCTGCCTCCTGTCCGG  452
               ************************************************************

neu-AY116182   CCTGCTGGTGCTACTCTAGAAAGACCCAAGACTCTCTCTCCTGGGAAGAATGGGGGTTGTC  3660
C-UAS-neuYC    CCTGCTGGTGCTACTCTAGAAAGACCCAAGACTCTCTCTCCTGGGAAGAATGGGGGTTGTC  512
               ************************************************************

neu-AY116182   AAAGACGTTTTTGCCTTCGGGGGTGCTGTGGAGAACCCTGAATACTTAGTACCGAGAGAA  3720
C-UAS-neuYC    AAAGACGTTTTTGCCTTCGGGGGTGCTGTGGAGAACCCTGAATACTTACTACCGAGAGAA  572
               *********************************************** ****** ****

neu-AY116182   GGCACTGCCTCTCCGCCCCACCCTTCTCCTGCCTTCAGCCCAGCCTTTGACAACCTCTAT  3780
C-UAS-neuYC    GGCACTGCCTCTCCGCCCCACCCTTCTCCTGCCTTCAGCCCAGCCTTTGACAACCTCTTC  632
               ******************************************************** **

neu-AY116182   TACTGGGACCAGAACTCATCGGAGCAGGGGCCTCCACCAAGTAACTTTGAAGGGACCCCC  3840
C-UAS-neuYC    TTCTGGGACCAGAACTCATCGGAGCAGGGGCCTCCACCAAGTAACTTTGAAGGGACCCCC  692
               *  *********************************************************

neu-AY116182   ACTGCAGAGAACCCTGAGTACCTAGGCCTGGATGTACCTGTATGA              3885
C-UAS-neuYC    ACTGCAGAGAACCCTGAGTTTCTAGGCCTGGATGTACCTGTATGA              737
               ******************** *  ***********************
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B

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neu-AY116182   LVDAEEYLVPQQGFFSPDPTPGTGSTAHRRHRSSSTRSGGGELTLGLEPSEEGPPRSPLA  1081
C-UAS-neuYC    LVDAEEFLVPQQGFFSPDPTPGTGSTAHRRHRSSSTRSGGGELTLGLEPSEEGPPRSPLA  65
               ******:****************************************************

neu-AY116182   PSEGAGSDVFDGDLAMGVTKGLQSLSPHDLSPLQRYSEDPTLPLPPETDGYVAPLACSPQ  1141
C-UAS-neuYC    PSEGAGSDVFDGDLAMGVTKGLQSLSPHDLSPLQRYSEDPTLPLPPETDGYVAPLACSPQ  125
               ***********************************************************

neu-AY116182   PEYVNQSEVQPQPPLTPEGPLPPVRPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEY  1201
C-UAS-neuYC    PEFVNQSEVQPQPPLTPEGPLPPVRPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEY  185
               **:********************************************************

neu-AY116182   LVPREGTASPPHPSPAFSPAFDNLYYWDQNSSEQGPPPSNFEGTPTAENPEYLGLDVPV-  1260
C-UAS-neuYC    LVPREGTASPPHPSPAFSPAFDNLFFWDQNSSEQGPPPSNFEGTPTAENPEFLGLDVPV-  244
               ************************::*************************:********
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Appendix C: Ectopic expression of *neu*^{YC} in the developing compound eye

Figure C.1: Eye disc morphogenesis is disrupted in *neu^{YC}*-expressing animals

Visualization of developing ommatidial clusters 3rd instar larval eye imaginal discs using α–dlg antibody at a magnification of 600x. Anterior is the left. [A] *CS-P* wandering 3rd instar eye imaginal discs show ordered 4- and 5-cell ommatidial pre-clusters posterior to the morphogenetic furrow. [B] Wandering 3rd instar larvae eye discs expressing *p[sev-GAL4] p[UAS-neu^{YC}]*. Following the passing of the morphogenetic furrow, differentiating photoreceptor cells appear greater in number, smaller in size and lack cluster organization.

Figure C.2: Comparison of *neu^YC*-dependent rough eye phenotypes as viewed under light and environmental scanning electron microscopes.

Light microscopy images of *neu^YC*-dependent eye fusion [A, C, E] with corresponding scanning electron micrographs of the same eyes [B, D, F]. [A-B] Suppression of ommatidial fusion by *Su(neu^YC)3*. Individual ommatidial units are visible and eye pigment is consistent in anterior and posterior regions of the adult eye. Larger fused areas are visible in both images, indicated by the arrowhead. [C-D] Mild rough eye phenotype in *p[sev-GAL4] p[UAS-neu^YC]*-expressing flies. Increased fusion results in loss of pigment in ommatidial, producing a yellow colour, seen in the anterior of the eye [C]. Corresponding electron micrograph in panel D shows ommatidial fusion more clearly. [E-F] Enhancement of *neu^YC* by *E(neu^YC)32J*. Increased fusion results in a flattened eye surface void of individual facets. Loss of pigment spreads further posterior in the eye. Darkened tissue surrounded by pigment-free tissue is also observed in this region [E]. In electron micrographs [F] the topography of the eye is seen with increased clarity. Additionally, reduced number of bristles is also observed.

Figure C.3: Gender-specific expressivity of *p[sev-GAL4] p[UAS-neu^{YC}]*.

Expressivity, ranging from mild to strong (left to right), of ectopically expressed *neu^{YC}* under the control of *sev-GAL4* in females [A-D] and males [E-H]. Females exhibit lower strength of expression, or ommatidial fusion, while, in males, eyes are observed to have a greater degree of facet fusion and loss of interommatidial bristles.

Figure C.4: Temporal changes in *neu^{YC}* expression in the adult eye.

neu^{YC} phenotype in the developing eye undergoes changes in expressivity over

generations. Date stamp of electron micrograph is indicated at the bottom of each panel

in the format YY/MM/DD. [A] Degree of ommatidial fusion in female eyes expressing

neu^{YC} when first created, dated December 2005. Fusion extends to the posterior edge of

the eye. Fused areas are large and flatten the surface of eye. Number of bristles is greatly

decreased. [B] *neu^{YC}* expression four months later, in March 2006. Fusion of

ommatidial is moderate, as it does not extend to the posterior edge of the eye field.

Furthermore, the surface of the eye is not smooth and bristles are greater in number,

compared to [A]. [C] *neu^{YC}* expression after removal of accumulated mutations in the

background, taken January 2008. Expression regains its strength, indicated by a return in

fusion severity and loss of bristles. [D] Severe reduction in expressivity nine months

after chromosome clean-up. Individual facets are visible, however, are larger than

wildtype, indicating a mild degree of fusion, and interommatidial bristles are greater in

number.

Appendix D: Normality tests for *neu*^{YC}-dependent wing phenotypes

Figure D.1: Normal probability plot of wing notches produced in *p[GAL4]bbg*^{C96} *p[UAS-neu*^{YC}*]* heterozygous flies.

To identify whether the wing notch phenotype in *neu*^{YC}-expressing heterozygotes follows a normal distribution, the Kolmogorov-Smirnov Normality Test was performed. The following scatterplot graphs normal probabilities versus wing margin loss in *p[GAL4]bbg*^{C96} *p[UAS-neu*^{YC}*]* heterozygotes. The P-value, which is greater than α = 0.15, indicates that the data follows a normal distribution.

Normal Probability Plot

YCC96/+

Probability: .999 .99 .95 .80 .50 .20 .05 .01 .001

Kolmogorov-Smirnov Normality Test
D+: 0.079 D-: 0.038 D : 0.079
Approximate P-Value > 0.15

Average: 10.9464
StDev: 7.12848
N: 56

Figure D.2: Normal probability plot of ectopic wing veins produced in

p[GAL4]bbg^{C96} p[UAS-neu^{YC}] **heterozygous flies.**

To identify if ectopic wing veins in *neu^{YC}*-expressing heterozygotes follows a normal

distribution, the Kolmogorov-Smirnov Normality Test was performed. The following

scatterplot graphs normal probabilities versus ectopic vein occurrences in *p[GAL4]bbg^{C96}*

p[UAS-neu^{YC}] heterozygotes. The P-value, which is greater than $\alpha = 0.15$, indicates that

the data follows a normal distribution.

Normal Probability Plot

Probability

.999
.99
.95
.80
.50
.20
.05
.01
.001

0   1   2   3   4   5   6

YCC96/+

Average: 1.91071
StDev: 1.40488
N: 56

Kolmogorov-Smirnov Normality Test
D+: 0.063 D-: 0.031 D : 0.063
Approximate P-Value > 0.15

Figure D.3: Normal probability plot of wing notches produced in *p[UAS-mCD8::GFP] ; p[GAL4]bbg^{C96} p[UAS-neu^{YC}]* heterozygous flies.

The Kolmogorov-Smirnov Normality Test was performed to determine if the wing notch phenotype in *p[UAS-mCD8::GFP] ; p[GAL4]bbg^{C96} p[UAS-neu^{YC}]*-expressing heterozygotes deviates from a normal distribution,. The following scatterplot graphs normal probabilities versus wing margin loss. The P-value, which is less than $\alpha = 0.15$, indicates that the data does not follow a normal distribution. Therefore, statistical analysis using this data set will use the Mann-Whitney U Test, a non-parametric equivalent of the independent T-Test.

Normal Probability Plot

Average: 6.56452
StDev: 5.73920
N: 62

Kolmogorov-Smirnov Normality Test
D+: 0.142 D-: 0.076 D : 0.142
Approximate P-Value < 0.01

Figure D.4: Normal probability plot of wing notches produced in *yw⁻* ;

$p[GAL4]bbg^{C96}$ $p[UAS\text{-}neu^{YC}]$ heterozygous flies.

The Kolmogorov-Smirnov Normality Test was performed to determine if the wing notch

phenotype produced in *yw⁻* ; $p[GAL4]bbg^{C96}$ $p[UAS\text{-}neu^{YC}]$-expressing heterozygotes

deviates from a normal distribution,. The following scatterplot graphs normal

probabilities versus wing margin loss. A P-value greater than $\alpha = 0.15$ indicates that the

data follows a normal distribution. Therefore, the use standard parametric statistical

analysis is appropriate for this data set.

Normal Probability Plot

Average: 12.5928
StDev: 8.94008
N: 61

Kolmogorov-Smirnov Normality Test
D+: 0.090 D-: 0.051 D : 0.090
Approximate P-Value > 0.15

Appendix E: Modification of *neu* add-back alleles by known modifiers of the

Ras1/MAPK signalling pathway

Figure E.1: Ras1 adaptors modify *neu*^{YC} facet fusion.

[A] Heterozygous female expressing *neu*^{YC} in the eye produces a rough eye phenotype, as described earlier. Enhancement of ommatidial fusion is seen in eyes with reduced [B] *DShc* and [D] *Dab* expression. Return of individual ommatidia is seen in flies that have reduced [C] *drk*, [F] *Crk*, [G] *dos* and [H] *Sos* expression. No change in *neu*^{YC} phenotype is observed with reduction of *dock* [E].

Figure E.2: Loss-of-function *Ras1* alleles modify *neu*^{YC}.

[A] Heterozygous *sev-GAL4* expression over a wildtype chromosome produces no eye defects. Flies heterozygous for loss-of-function *Ras1* alleles [B] *Ras1*^{05703}, [C] *Ras1*^{A014}, [D] *Ras1*^{06677} and [E] *Ras1*^{EY07538} show mild eye defects consisting of missing bristles. No ommatidia fusion is evident. [F] *p[sev-GAL4] p[UAS-neu*^{YC}*]* / + female eyes. [G] *Ras1*^{05703} enhances eye fusion phenotype of *neu*^{YC}. In contrast, [H] *Ras1*^{A014}, [I] *Ras1*^{06677} and [J] *Ras1*^{EY07538} suppress *neu*^{YC}, suggesting that *Ras1*^{05703} is not a hypomorphic allele of *Ras1*.

Figure E.3: Loss of *phl*, *Dsor* and *rl* modulate *neu*^{YC}.

[A] Ectopic expression of *neu*^{YC} in the developing eye results in a rough eye phenotype. Loss of *phl* in [B] *phl^1/yw⁻ ; p[sev-GAL4] p[UAS-neu^{YC}]* and [C] *phl^{11}/yw⁻ ; p[sev-GAL4] p[UAS-neu^{YC}]* flies suppresses the rough eye phenotype. [D] *Dsor*^{G42} hypomorphs show suppression of *neu*^{YC}, as well. [E] *Dsor*^{G79} heterozygotes enhanced *neu*^{YC} rough eye phenotype. Alleles [F] *Dsor*$^{S-1221}$ and [G] *Dsor*^{LH110} have no effect on *neu*^{YC} expression. [H] Genetic null *rl*^{10A} mildly suppresses *neu*^{YC}, however, hypomorphic allele [I] *rl*^{1} greatly enhances *neu*^{YC} ommatidial fusion.

Figure E.4: Other adaptors and second messengers modify *neu*^{YC}.

[A] Control eye expressing *neu*^{YC}. [B] Loss of *Dcbl* enhances *neu*^{YC}-dependent ommatidial fusion and bristle loss. [C] Loss of phosphatase *csw* has no effect on *neu*^{YC} expression. [D] Reduced *abl* tyrosine kinase expression strongly suppresses ommatidial fusion.

Figure E.5: *Paxillin* **and** *PI3'K* **alleles modify** *neu*^{YC}**.**

[A] Ectopic expression of *p[UAS-mCD8::GFP]* by *sev-GAL4* has no effect on eye development. [B] Loss of *Pax* by P-element disruption and [D] overexpression of *DP110* show no defects in the eye when directed by *sev-GAL4*. Although *Pax*^{EY02020} disrupts the *Pax* open-reading frame, it still contains UAS sites that may bind GAL4 protein, thereby reducing expression of *neu*^{YC} by *sev-GAL4*. *Paxillin* (*Pax*) [C] mildly effects eye development when overexpressed using *sev-GAL4*, by way of missing interommatidial bristles. [E] *p[sev-GAL4] p[UAS-neu*^{YC}*] / p[UAS-mCD8::GFP]* heterozygotes possess a mild rough eye phenotype. [F-G] Loss-of-function *Pax* allele enhances *neu*^{YC}, as does gain-of-function *Pax* allele, respectively. The EY element in *Pax*^{EY11633} is inserted in the 5' UTR of both *Pax* and *CG13085*, and is oriented to direct expression of *Pax*. Effects resultant from *Pax*^{EY11633} allele may be due to overexpression of *Paxillin*, disruption of *CG13085* or the combined effects of both. [H] Overexpression of *DP110* enhances *neu*^{YC}-dependent rough eye phenotype.

Table E.6: Loss of other 2nd messengers modifies *neu^{YC}* wing phenotype

Modifier Alleles	Mammalian Orthologue	Function	% Average Margin Loss	Overall Wing
neu^{YC}	ErbB2	RTK	10.95; (*n = 56*)	
csw^{LE120}	Shp-2	Phosphatase	6.84; P = 0.00 (*n = 61*)	-
Abl^2	Abl	Non-receptor tyrosine kinase	12.63; P = 0.20 (*n = 67*)	NE
Dcbl^{F0165}	Cbl	E3 ubiquitin ligase	10.47; P = 0.76 (*n = 51*)	NE
neu^{YC}/mCD8::GFP	ErbB2	Control	6.56; (*n = 62*)	
Pax^{EY02020}	Paxillin	Scaffolding protein	13.68; P = 0.00 (*n = 87*)	+

'+' = enhancement; '-' = suppression; 'NE' = no effect

Table E.7: Overexpression of other adaptors and 2nd messengers affects *neu^{YC}*

Modifier Alleles	Mammalian Orthologue	Function	% Average Margin Loss	Overall Wing
neu^{YC}/mCD8::GFP	ErbB2	Control	6.56; (*n = 62*)	
UAS-DP110	PI3K	Kinase	11.38; P = 0.00 (*n = 105*)	+
Pax^{EY11633}	Paxillin	Scaffolding protein	6.03; P = 0.56 (*n = 97*)	NE

'+' = enhancement; 'NE' = no effect

Figure E.8: *neu*^{YB} **and** *neu*^{YE} **add-back alleles are modified by** *Ras1*

[A] Ectopic expression of *mCD8::GFP* has no effect on eye development.

Overexpression of [B] *Ras1*^{V12}, [C] *Ras1*^{N17}, [D] *Ras1*^{V12G37}, [E] *Ras1*^{V12C40} and [F]

Ras1^{V12S35} using *sev-GAL4* generates very mild to mild defects during eye development,

consisting of bristle loss and ommatidial fusion, respectively. [G] *p[sev-GAL4] p[UAS-*

neu^{YB}*] / p[UAS-mCD8::GFP]* and [E] *p[sev-GAL4] p[UAS-neu*^{YE}*] / p[UAS-*

mCD8::GFP] generate a mild eye fusion phenotype. Overexpression of *Ras1*^{V12}

enhances facet fusion in [H] *neu*^{YB} and [N] *neu*^{YE}-expressing flies. Overexpression of

dominant negative *Ras1*^{N17} allele does not modify [I] *neu*^{YB} or [O] *neu*^{YE} rough eye

phenotypes. *Ras1*^{V12G37} and *Ras1*^{V12C40} also fail to modify [J-K] *neu*^{YB} and [P-Q] *neu*^{YE}.

Overexpression of *Ras1*^{V12S35} enhances both [L] *neu*^{YB} and [R] *neu*^{YE}.

Appendix F: EMS mutation phenotypes

Figure F.1: EMS mutations mildly modify eye development.

Scanning electron micrographs of adult eyes from heterozygous EMS mutants. [A] *CS-P* adult eyes show regular size and patterning of ommatidial units in adult compound eyes. Panels B-R are from the following genotypes: [B] *Su(neu^{YC})3*; [C] *Su(neu^{YC})77*; [D] *Su(neu^{YC})93*; [E] *Su(neu^{YC})99*; [F] *Su(neu^{YC})138*; [G] *Su(neu^{YC})162*; [H] *E(neu^{YC})3-1*; [I] *E(neu^{YC})3-3*; [J] *E(neu^{YC})5*; [K] *E(neu^{YC})6J*; [L] *E(neu^{YC})32*; [M] *E(neu^{YC})32J*; [N] *E(neu^{YC})42AJ*; [O] *E(neu^{YC})45J*; [P] *E(neu^{YC})46*; [Q] *E(neu^{YC})50*; and [R] *E(neu^{YC})69*. All display mild eye defects, ranging from missing interommatidial bristles to bristle doublets.

Appendix G: Incomplete mapping and characterization of unknown modifiers

Figure G.1: Genetic mapping of *E(neu^{YC})6J*

[A] Table of meiotic mapping recombinant lines generated that carry *E(neu^{YC})6J* as determined by enhancement of *neu^{YC}*-dependent eye facet fusion. The table shows the visible markers present in each recombinant and whether these recombinants are allelic to the original *E(neu^{YC})6J* mutation, based on lethal complementation. '+' indicates the presence of the wildtype allele and '-' indicates the presence of the mutant allele. Together, this suggests that *E(neu^{YC})6J* is located between visible markers *al^1* and *b^1*. The 'Y' indicates the recombinant complements the original mutation and 'N' indicates that the recombinant fails to complement *E(neu^{YC})6J*. The identification of lines that complement the original mutation suggests that a background mutation may exist in the original *E(neu^{YC})6J* and that *E(neu^{YC})6J* may be homozygous viable.

[B] Schematic representation of deficiency mapping of *E(neu^{YC})6J* by lethal complementation. *Df(2L)J2* uncovers region 31B-32A and fails to complement *E(neu^{YC})6J*. Within this region, 3 deficiencies fail to complement *E(neu^{YC})6J.*, overlapping in the region 31D -31E5. This region is further refined by deficiencies that complement *E(neu^{YC})6J* lethality, therefore outlining 31D8-31E3 as the region containing the *E(neu^{YC})6J*-associated lethality. Deficiencies are listed down the left side of the schematic. Those with red bars represent deficiencies that fail to complement *E(neu^{YC})6J*, whereas green bars represent deficiencies that complement *E(neu^{YC})6J*.

A

$E(neu^{YC})6J$ Recombinant Line	al^1 21C1	b^1 34D1	pr^1 38B3	c^1 52D3-7	px^1 58E4-8	$E(neu^{YC})6J$ Complementation
5	+	-	-	-	-	Y
7	+	-	+	+	-	N
18	-	+	+	+	-	N
24	-	+	+	+	+	N
31	-	+	-	-	+	Y

'-' indicates the presence of the marker allele in the recombinant line with $E(neu^{YC})6J$
'+' indicates the presence of the wildtype gene in the recombinant line with $E(neu^{YC})6J$

B

Figure G.2: Deficiencies that fail to complement $E(neu^{YC})6J$ suppress neu^{YC} eye phenotype.

Environmental scanning electron micrograph taken at 140x of adult compound eyes. [A] Ectopic expression of neu^{YC} by sev-$GAL4$ results in a rough eye phenotype due to fusion of individual ommatidial units. [B] Enhancement of fusion phenotype by $E(neu^{YC})6J$. Suppression of ommatidial fusion is seen with deficiencies [C] $Df(2L)J2$, [D] $Df(2L)BSC144$, [E] $Df(2L)J3$ and [F] $Df(2L)J106$, all of which fail to complement $E(neu^{YC})6J$. With Appendix G.1, this suggests that, although the lethal mutation and $E(neu^{YC})6J$ are located between bands 31B and 32A, that they are separate mutations and that the deficiencies uncover the second lethal mutation, and not $E(neu^{YC})6J$.

Figure G.3: Mapping results of *E(neu^{YC})69*, a Ras1-independent modifier of *neu^{YC}*

E(neu^{YC})69 has been mapped to two regions on the second chromosome: between 38B3 and 52D7, and 58E4 and 60C2. Green bars indicate regions that complement known deficiencies (Appendix A3) and white areas are gaps that have not been tested. The largest gap is the heterocentric chromatin.

Figure G.4: Schematic representation of P-element mapping strategy

Schematic outlining the basis of the mapping strategy using molecularly mapped P-elements. The power of this strategy comes from the use of the mini-white marker in the P-elements as an eye colour marker in a w^- background. Molecularly mapped P-elements are recombined away from the unknown mutation, M, to determine the recombination frequency between M and two flanking P-elements, P1 and P2. Assuming that M is linked to the second chromosome, female virgins, heterozygous for P1 and M on a recombinant chromosome and P2 on the second chromosome, are crossed to the second chromosome balancer stock *Sco/CyO* to recombine the two P-elements away from M. Two recombination events – a and b – are possible. These recombination events generate the following specific recombinant progeny: recombination event a) *yw⁻ ; P1 P2/CyO* and *yw⁻ ; M/CyO*; and recombination event b) *yw⁻ ; P1 M P2/CyO* and *yw⁻ ; +/CyO*. Recombinant progeny are selected by the absence of eye colour. Progeny that contain at least one P-element will have red eyes and those progeny that have recombined out the P-elements will have white eyes. White eyed progeny carrying M are identified by verifying the original modification of neu^{YC}. The percentage of white eyed progeny that carry M is roughly the percent distance between P1 and M. Results are confirmed using recombinants of M and P2.

SCHEMATIC OF P-ELEMENT MAPPING STRATEGY

P1
28C

M
29B

♀   X   X   X   w⁻ ; $\frac{Sco}{CyO}$; +   ♂

a   b

P2
30C

Recombination
Events Generate:

a   yw⁻ ; $\frac{P1\ P2}{CyO}$

    yw⁻ ; $\frac{M}{CyO}$

X   yw⁻ ; $\frac{sev\text{-}GAL4\ UAS\text{-}neu^{YC}}{CyO}$; +   Verifies for M

              Does not verify for M

b   yw⁻ ; $\frac{P1\ M\ P2}{CyO}$

    yw⁻ ; $\frac{+}{CyO}$

Percentage of white-eyed progeny that carry mutation M
is roughly the percent distance M is located from P1

Appendix G.5: Mapping results for $E(neu^{YC})3\text{-}1$

Meiotic recombination mapped $E(neu^{YC})3\text{-}1$ between cytological bands 58E4 and 60C2 (Figure 3.19). Continued mapping of $E(neu^{YC})3\text{-}1$ used the molecularly mapped flanking P-elements $P[w^+]KG06675$ at cytological band 59C1 and $P[w^+]KG06046$ at cytological band 60F5. $E(neu^{YC})3\text{-}1$ was recombined onto the chromosome containing the $P[w^+]KG06675$ insertion and used for mapping. Recombinants of $E(neu^{YC})3\text{-}1$ and $P[w^+]KG06046$ occurred once in every 2000 flies, and stable lines were unable to be generated.

A total of 654 flies were screened; 630 were red-eyed and therefore contained at least one P-element and 24 were white-eyed recombinant flies. Verification of the enhancing phenotype in white-eyed recombinant progeny identified 18 flies where $E(neu^{YC})3\text{-}1$ recombined away from $P[w^+]KG06675$. This indicates that $E(neu^{YC})3\text{-}1$ is located at approximately 75% distance from $P[w^+]KG06675$, within a 640 kb range that corresponds to approximately 10% of the distance between the two P-elements. Since the molecular insertion point of $P[w^+]KG06675$ is at nucleotide 18 886 463, $E(neu^{YC})3\text{-}1$ is located between 20 338 801 nt (60C7) and 20 785 674 nt (60E12).

Figure G.6: *E(neu^{YC})3-1* genetically interacts with *dLMO*

Modification of the *E(neu^{YC})3-1* wing scalloped phenotype. [A] Flies heterozygous for

E(neu^{YC})3-1 have a moderate wing scalloped phenotype. [B] *Bx/+* ; *E(neu^{YC})3-1/+*

heterozygotes have enhanced wing scalloped phenotype. Wings have greater loss of the

posterior wing margin and are narrower. [C] *Df(1)Exel7463* completely suppresses the

E(neu^{YC})3-1 wing scallop phenotype. Together this suggests that *E(neu^{YC})3-1* is involved

in the *dLMO* pathway.

Figure G.7: *E(neu^{YC})3-3* genetically interacts with *dLMO*

Modification of the *E(neu^{YC})3-3* wing scalloped phenotype. [A] Flies heterozygous for

E(neu^{YC})3-3 have a moderate wing scalloped phenotype. [B] The gain-of-function *Bx*

allele enhances wing scalloped phenotype. Wings have greater loss of the posterior wing

margin and are narrower. [C] *Df(1)Exel7463/+* ; *E(neu^{YC})3-3/+* heterozygotes show

complete suppression of the wing scallop phenotype. Together this suggests that

E(neu^{YC})3-3 is involved in the *dLMO* pathway.

Appendix H: pGMR-1::neu^{YC} vector map

Figure H.1: Schematic representation of the pGMR-1::neu^{YC} plasmid

The pGMR-1::neu^{YC} plasmid was constructed to generate transgenic flies expressing

neu^{YC} under the control of the pGMR promoter. *neu^{YC}* was shuttled from a pUAST

construct into the pGMR-1 construct using the *Eco*RI restriction enzyme. Restriction

digest using *Nco*I was performed to identify colonies that contained the *neu^{YC}* insertion in

the pGMR-1 plasmid in the proper orientation. Fragments of 8.3 kb and 4.5 kb were

expected. Proper orientation and sequence was validated by sequencing, with 5' end of

neu^{YC} 3' to the hsp-70 promoter.

HindIII
9808 3' P
hsp-70 3' flanking region
9240 EcoRI
8312 NcoI
pUC
neu^YC
0/12805
pGMR-1::neu^YC
12.8 kb
HindIII 93
5' P
5285 EcoRI
hsp-70 promoter
Glass binding sites (5x)
3181 NcoI
4810 XhoI

Appendix I: Cloning and expression of *CG13848*

During the course of a previous EP element screen in our lab, *EP(3)3303* was identified as an enhancer of the YC-specific wing notch phenotype. *EP(3)3303* was reported as an insertion in the 5' of the transcriptional start site of the novel gene *CG13848*. It was initially my objective to identify the expression pattern of *CG13848* and begin functional characterization through gain-of-function analysis and by attempting to create a loss-of-function mutant by imprecise excision of the P-element *EP(3)3303*. I was later able to determine that *CG13848* overexpression by the P-element *EP(3)3303* did not enhance the YC-specific wing notch phenotype (Appendix I.5), and further study was terminated.

In 2005, the novel CRAL-TRIO domain protein prolonged depolarization afterpotential is not apparent (PINTA) was identified during a genetic screen for mutations that affect biosynthesis of rhodopsin by Wang and Montell. The CRAL-TRIO domain binds to all-*trans*-retinol. PINTA was expressed and required in the retinal pigment cells and its function was demonstrated after the production of vitamin A. It was proposed that PINTA functions to sequester all-*trans*-retinal in pigment cells of the visual system, so that a concentration gradient for the uptake of vitamin A by these cells is generated.

Figure I.1: Inverse PCR verifies location and direction of *EP(3)3303* insertion

Sequence alignment of the sequence flanking *EP(3)3303*, isolated by inverse PCR

(iPCR), as described in the protocol published by the Berkley Drosophila Genome Project

(http://www.fruitfly.org/p_disrupt/inverse_pcr.html; accessed 13/11/2002). Genomic

DNA from *EP(3)3303* transgenic adults was digested using *Hin*PI for 2.5 hours at 37°C.

DNA flanking the inserted P-element was amplified using the forward Pwht1 primer 5'-

GTAACGCTAATCACTCCGAACAGTCACA-3'and the reverse Plac1 primer 5'-

CACCCAAGGCTCTGCTCCCACAAT-3'. Sequencing of PCR products used the Sp1

sequencing primer 5'-ACACAACCTTTCCTCTCAACAA-3'. Sequence alignments

were performed using ClustalW.

[A] The sequence alignment of the 5' iPCR product and genomic region 5' to *CG13848*.

The location of *EP(3)3303* is identified as 8bp from the *CG13848* transcriptional start

site, indicated in green. Sequence strands in the alignment are oriented in the opposite

direction, consistent with the orientation of *CG13848* published on Flybase

(http://flybase.org/reports/FBgn0038966.html; FB2009_01, released January 23, 2009).

[B] Sequence alignment of 5' iPCR product and 5' end of P-element *EP(3)3303*. The

iPCR product sequence continues from the alignment presented in [A] without any gaps,

indicated by the nucleotide count on the right side of both alignments. The 5' end of

CG13848 is adjacent to the 5' end of the *EP(3)3303* insert, indicating that the insert is

oriented in the opposite direction of *CG13848*. This is consistent with the published

orientation of *EP(3)3303* (Bellen *et al.*, 2004; Kraut *et al.*, 2001).

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A

```
HinP1_5'_iPCR    --------------------------------CTTTGGCGGTAACGACTGTCGGCGAAC 27
CG13848          TTGGAACTGCTGCTGCGCGACCACATGGCGCTCCTTTGGCGGTAACGACTGTCGGCGAAC 2001
                                                 *************************

HinP1_5'_iPCR    TAAAGCAATTGTTTATAAACAAATTGCGACATGCACTCGCC------------------- 68
CG13848          TAAAGCAATTGTTTATAAACAAATTGCGACATTCACTCGCCAAATGAAATAAGCTTTTCA 1941
                 ****************************************
```

B

```
HinP1_5'_iPCR    CATGATGAAATAACATAAGGTGGTCCCGTCGATAGCCGAAGCTTACCGAAGTATACACTT 128
EP3303           CATGATGAAATAACATAAGGTGGTCCCGTCGATAGCCGAAGCTTACCGAAGTATACACTT 60
                 ************************************************************

HinP1_5'_iPCR    AAATTCAGTGCACGTTTGCTTGTTGAGAGGAAAGGTTGTGTGCGGACGAATTTTTTTTTG 188
EP3303           AAATTCAGTGCACGTTTGCTTGTTGAGAGGAAAGGTTGTGTGCGGACGAATTTTTTTTTG 120
                 ************************************************************

HinP1_5'_iPCR    AAAACATTAACCCTTACGTGGAATAAAAAAAAATGAAATATTGCAAATTTTGCTGCAAAG 248
EP3303           AAAACATTAACCCTTACGTGGAATAAAAAAAAATGAAATATTGCAAATTTTGCTGCAAAG 180
                 ************************************************************

HinP1_5'_iPCR    CTGTGACTGGAGTAAAATTAATTCACGTGCCGAAGTGTGCTATTAAGAGAAA-------- 300
EP3303           CTGTGACTGGAGTAAAATTAATTCACGTGCCGAAGTGTGCTATTAAGAGAAAATTGTGGG 240
                 ***************************************************
```

Figure I.2: Schematic representation of the pBSKII::CG13848 construct

The following figure represents the pBSKII::CG13848 construct used to generate RNA probes for *in situ* hybridization of *CG13848*. Full length *CG13848* cDNA was isolated from genomic DNA using RT-PCR. RT-PCR primers were designed with *Bam*HI and *Xba*I restriction sites at the 5' and 3' ends, respectively, to confer directional specificity for cloning into pBSKII. Positive clones were identified using ampicillin resistance and insertion direction was determined by sequencing.

Figure I.3: mRNA expression pattern of *CG13848* during late embryogenesis

The results from an *in situ* hybridization depict mRNA expression of *CG13848* during late embryogenesis. Early embryogenesis showed no expression of *CG13848*. The panels A, C, E and G show controls using the sense probe of *CG13848*, and panels B, D, F and H show results from the α-sense probe of *CG13848*. Low levels of sense probe are detected in the midgut of embryos staged 15 [C], 16 [E] and 17 [G]. Expression of *CG13848* mRNA is detected in the midgut of stage 14 [B], 15[D], 16 [F] and 17 [H] embryos.

CG13848 Sense Probe   CG13848 Anti-Sense Probe

Stage 14

Stage 15

Stage 16

Stage 17

Figure I.4: Schematic representation of the pET29b⁺::CG13848 construct

To generate a His-tagged CG13848 peptide for antibody production, *CG13848* was

ligated into the pET29b⁺ vector. Full length *CG13848* cDNA lacking the stop codon was

isolated using RT-PCR. *CG13848* insert was cloned in-frame into the *Sal*I and *Xho*I

restriction sites in the pET29b⁺ multiple cloning site. Positive clones were identified by

kanamycin resistance and insertional direction and sequence was confirmed by

sequencing.

Figure I.5: Overexpression of *CG13848* does not modify *neu*^{YC} wing notches

EP(3)3303 was previously identified as an enhancer of *neu*^{YC} in the adult wing margin (Settle and McMaster University. Dept. of Biology, 2002). *EP(3)3303* is an EP element that is immediately 5' to *CG13848*. [A] Ectopic expression of *neu*^{YC} in the wing margin produces a moderate wing notch phenotype. [B] Overexpression of *CG13848* using EP(3)3303 does not alter *neu*^{YC} notch formation in the wing.