PHOSPHOTYROSINE SPECIFIC OUTPUTS OF Neu/ErbB2

A GENETIC APPROACH TO IDENTIFY THE REQUIREMENTS FOR PHOSPHOTYROSINE SPECIFIC OUTPUTS OF Neu/ErbB2

By

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ABSTRACT

DER, the *Drosophila* Epidermal Growth Factor Receptor (DEgfr) is the only known fly orthologue of vertebrate Neu/ErbB2 receptor tyrosine kinase family. Receptor Tyrosine Kinases (RTKs) like DER and ErbB2 play an important role in regulating cell differentiation, cell proliferation and cell survival in metazoan animals. Neu/ErbB2 is over-expressed in 20-30% human breast cancers, which correlates with poor clinical prognosis in cancer patients.

Our previous studies showed that rat-Neu/ErbB2 could successfully signal *in vivo* using *Drosophila* adaptor and second messenger molecules. Here we regenerated the transgenic fly lines with various neu add-back alleles. We further re-established mis-expression phenotypes in various adult structures such as wings and eyes, the tissues known to require DEgfr signaling. By using genetic approach, we have demonstrated that the tyrosine residue at the 1028 site (Neu^{YA}), might have an inhibitory role in RTK signaling. In addition we have already generated a number of double add-back *neu* alleles where tyrosine site at the 1028 site (neu^{YA}) was added back to another *Neu* allele and made neu^{YAB}, neu^{YAC} neu^{YAD} and neu^{YAE}. Transgenic flies with these alleles will be generated to further study the inhibitory role of Neu^{YA}.

Finally, our on going large-scale genetic screening is likely to reveal the component(s) of *Neu*^{YE} (Y1253) pathway that does not utilize the function of Ras.

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
DEgfr	Drosophila EGFR
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal Growth Factor Receptor
EMS	Ethyl Methanesulfonate
ESEM	Environmental scanning Electron Microscopy
GAL4	Galactosidase transgene with 4 binding sites
KD	Kinase dead
KDa	Kilodalton
MG	Midline Glia
PBD	Phosphotyrosine Binding Domain
PI3K	Phosphotidyl inositol 3 kinase
PTyr	Phosphotyrosine
RTK	Receptor Tyrosine Kinase
Sev	Sevenless
SH2	Src homology 2
SH3	Src homology 3
UAS	Upstream Activating sequence

CONTRIBUTIONS

Zhong Xiaoli of the Dr. Ana Campos Lab, McMaster University performed microinjection of most of the UAS-neu alleles.

CHAPTER 1:

INTRODUCTION:

The organism that originates from a fertilized egg into a multicellular individual must coordinate the growth and differentiation of many different types of cells during the course of development. In doing this, each individual cell must be able to recognize environmental cues, process multiple signals and generate appropriate developmental response (Huang and Rubin, 2000). Extra-cellular peptides act as ligands, which ultimately initiate the intra and inter cellular signaling. A great number of ligands bind to cell surface receptors from where the signals are transmitted to the nucleus through a cascade of signaling molecules. Therefore, the transduction of extra-cellular signals requires: (1) transmembrane receptors that recognize extracellular cues, (2) intracellular proteins that relay and amplify these signals, and finally, (3) effector molecules that convert the signals into the developmental output(s). The signaling molecules play an important role in fulfilling the requirements during every developmental process.

In eukaryotes, a large group of genes encode for proteins that function as cell surface receptors. One large family of cell surface receptors, such as Receptor Tyrosine Kinases (RTKs), has an intrinsic kinase activity by which they catalyze the transfer of gamma phosphate of ATP to hydroxyl group of tyrosine on target proteins (reviewed by Schlessinger, 1998). These RTKs are comprised of a ligand binding extra-cellular domain, a trans-membrane single

helix, and a cytoplasmic domain containing both a conserved kinase core and an additional regulatory sequence where autophosphorylation and phosphorylation occurs (Hynes and Stern, 1994).

The signaling pathways vary from prokaryotic to eukaryotic organism. However, the highly conserved nature of eukaryotic signaling pathways has also been revealed and defects in signaling has been found as an underlying mechanism of cancer and various types of human diseases (Hunter, 2000). For example, structural changes in the transmembrane receptors lead to increased kinase activity, and result in oncogenic potential. In some cases, major structural changes cause receptor activation while in different instances even a single amino acid substitution induces ligand-independent constitutive activity (Wides et al., 1990). Therefore, understanding the mechanism of kinase deregulation of these receptor molecules, due to the structural changes, is not only important for understanding the mechanism of normal signal transduction but also for understanding the mechanism of various cancers from therapeutic point of view.

1.1. CELL SIGNALING BY TRANS-MEMBRANE RECEPTOR TYROSINE KINASE:

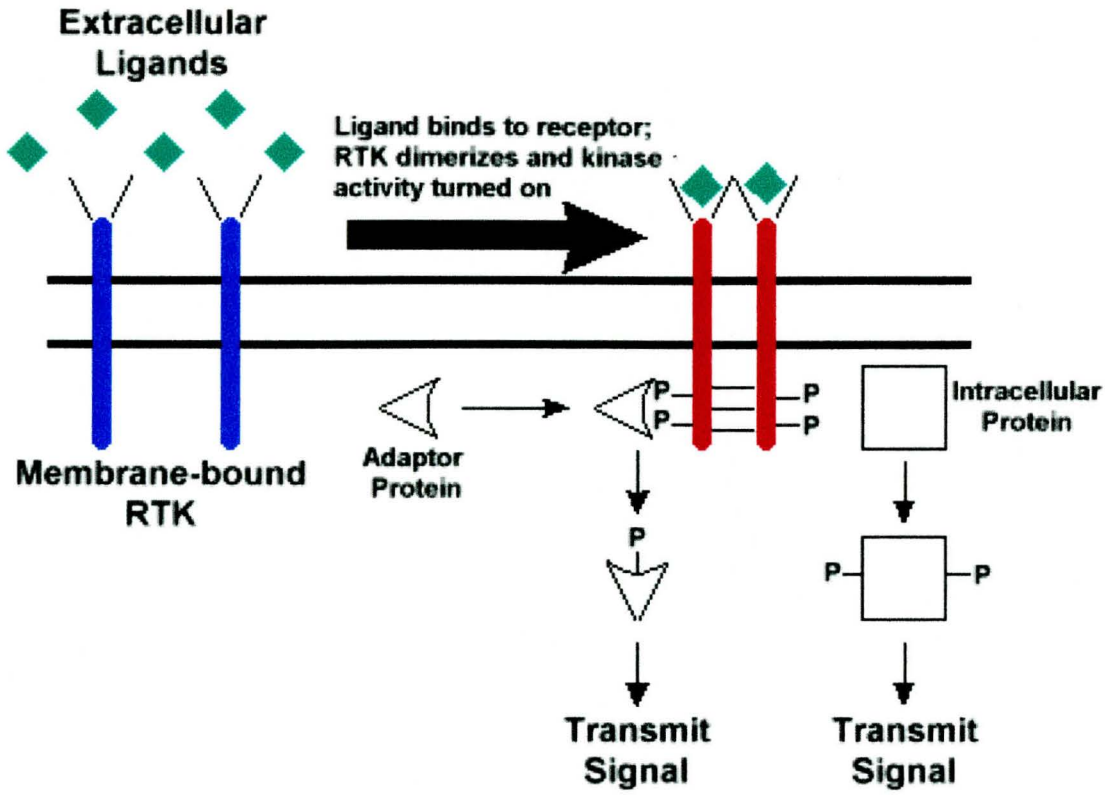
In all eukaryotes, a large group of genes encode for proteins that function as membrane spanning cell surface receptors (reviewed by Schlessinger, 2000). These receptors can be classified on the basis of their ligand recognition, biological response induction and the primary structure of the receptor itself. Ligands can be small organic molecules, lipids, carbohydrates, peptides and proteins (Schlessinger, 2000). They bind to and regulate the activity of cell surface receptors. One large family of cell surface receptors, such as RTKs, play an important role in almost all fundamental cellular processes, including cell cycle, cell migration, cell metabolism, cell survival, cell proliferation and cell differentiation as well (Schlessinger, 2000).

Ligand binding to the extra-cellular domain of the receptors induces RTKs dimerization, which eventually results in the autophosphorylation or transphosphorylation of the tyrosine residues in the docking or C-terminal domains (Simon, 2000). These phosphotyrosine residues can then engage specific cytoplasmic and/or plasma membrane associated proteins, containing the modular Src homology 2 (SH2) or protein tyrosine binding (PTB) domains. These second messenger proteins induce the signaling 'cascades' and transduce a growth or differentiating signal to the nucleus (Fig. 1). The

FIGURE 1.

Activation of Receptor Tyrosine Kinases (RTKs). The monomeric transmembrane RTKs dimerize upon the binding of the extracellular ligand (green filled rectangle). The dimerized and activated RTKs (red) then phosphorylate the specific tyrosine residue at the cytosolic domain. These phosphorylated residues act as the binding sites for the SH2 domain of the adaptor proteins (arrowhead) and second messengers (white rectangle). The adaptor proteins, mostly membrane linked, activate additional messenger proteins (white rectangle) to propagate the signal to the nucleus (not shown). However, not all adaptors or second messengers are phosphorylated upon activation. (This figure is adapted from J.R. Jacobs).

Activity of Membrane-bound Receptor Tyrosine Kinases



second messenger proteins may be enzymes, including kinases, phosphatases or phospholipases, which further activate intracellular signaling 'cascades'. Adaptor proteins, on the other hand, act as intermediates linking the activated RTK with the second messenger proteins (Schlessinger, 2000). For example, adaptor proteins such as Grb-2 or Shc associate with activated RTKs through their SH2 domains and further recruit the guanine nucleotide exchange factor, Son of Sevenless (Sos), through its SH3 domain. This event leads Ras to the sequential activation of the protein kinase Raf, mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (Erk) (reviewed by Haugh, 2002). However, interaction of RTKs with different substrates is thought to result in activation of distinct signaling pathways, thus producing different cellular responses (Schlessinger and Ullrich, 1992).

1.2. RTK SIGNALING SPECIFICITY:

Upon phosphorylation of Tyr, the activated RTKs reveal a short site (3-6 peptides in the carboxyl terminal) to bind specific proteins via SH2 domains (Pawson and Nash, 2000). These proteins include the adapter GRB-2, which ultimately activates RAS, phospholipase C-γ (PLC-γ), the tyrosine phosphatase (SHP-2) (Adachi et al., 1996), Ras GTPase-activating protein (GAP), and the regulatory subunit of phosphatidylinositol-3OH-kinase (PI-3K) (Ullrich and Schlessinger, 1990; Koch et al., 1991; Hernandez-Sotomayor

and Carpenter, 1992; Cohen et al., 1995; Heldin, 1995; Pawson, 1995). Interaction of these various types of substrates with the activated RTKs is thought to produce distinct signaling pathways resulting in different cellular responses (Schlessinger and Ullrich, 1992). For example, RTK signaling can trigger either activation or repression of gene expression depending on the cellular context (Roch et al, 2002). This functional duality has been added to the complexity in understanding the RTKs signaling output(s).

Moreover, some growth factor RTKs have shown different activities at different SH2 binding sites. For example, a mutation study found that the sites for PLC-γ (Y1021) and phosphatidylinositol-3OH-kinase (Y740/751), in the platelet-derived growth factor receptor-β (PDGFR-β), promote chemotaxis, whereas the site for GAP (Y771) mediates suppression of migration (Kundra et al., 1994). In the fibroblast growth factor receptor, the site binding PLC-γ is required for phosphatidylinositol turnover and Ca^{2+} flux but not for mitogenesis (Mohammadi et al., 1992; Peters et al., 1992). Another study with the *Caenorhabditis elegans* epidermal growth factor receptor homologue, LET-23, has found that six out of eight potential SH2-binding sites function *in vivo* (Lesa and Sternberg, 1997). However, by analyzing the transgenic nematodes for three distinct LET-23 functions (viability, vulval differentiation and fertility), they found that three sites were sufficient for viability and vulval differentiation, one site induced wild-type fertility, one site mediated all LET-23 functions and the other site mediated tissue specific negative

regulation (Lesa and Sternberg, 1997). Therefore, it is clear that the putative SH2 binding sites not only function in non-equivalent manner *in vivo* and but also mediate either positive or negative tissue specific regulation. RTKs tissue specificity *in vivo* is regulated by at least two independent factors: (1) by tissue specific effectors and (2) by tissue specific regulators. Both of these effector and regulator molecules act synergistically to propagate RTK signaling in some cells, while repressing it in others (Simon, 2002).

1.3. VERTEBRATE FAMILY OF EGF RECEPTORS:

The epidermal growth factor receptor (EGFR) is a member of the tyrosine kinase receptor (RTK) family. Although *C. elegans* and *Drosophila* have only one EGF receptor, vertebrate EGFR family consists of four family members: EGFR/ErbB-1, ErbB2/Neu, ErbB3 and ErbB4. In normal tissue, the ErbB receptors are activated by a variety of receptor specific ligands. The ligands, specific to the EGFR, are epidermal growth factor (EGF), transforming growth factor-∝ (TGF-∝), amphiregulin and heparin-binding EGF (reviewed by Ranson, 2004). Upon ligand binding, these receptors form homo or heterodimeric complexes and activate the tyrosine kinase domain thereafter (Yarden, 2001).

The EGFR is involved in cell proliferation, cell differentiation, cell survival, metastasis, and angiogenesis (reviewed by El-Rayes and LoRusso, 2004). The loss of ErbB2, ErbB3 or ErbB4 expression, in 'knock-out' mice, showed deleterious effects on the developing embryo (Britsch et al., 1998; Gassmann et al., 1995; Lee et al., 1995; Riethmacher et al., 1997). For example, ErbB2 and ErbB3-deficient mice showed similar hypoplastic development of the sympathetic nervous system (Britsch et al., 1998). On the other hand, ErbB2 and ErbB4-deficient mice showed defective formation of cardiac ventricular trabecules (Gassmann et al., 1995; Lee et al., 1995).

Apart from the normal functioning in cell growth and cell differentiation and their individual action mode, EGFR signaling, from the medical perspectives, has been reported to be important for tumour cell proliferation, inhibition of apoptosis, angiogenesis, metastasis and sensitivity to chemotherapy and radiotherapy (Ritter and Arteaga, 2003; Arteaga, 2003).

Although EGFR collectively share the functional similarity in development and diseases, but not all of them follow the linear model of signaling 'cascades'. First of all, ErbB-2 has no known ligand and secondly, ErbB-3 is devoid of catalytic activity (Chan et al., 2002). Moreover, it has been demonstrated that ErbB receptors may partake in any combination of homo- and hetero-dimerization complexes with a preference of having ErbB2 in common, as it shows potent intrinsic kinase activity (Chan et al., 2002).

Therefore, it is obvious that more potent signaling results from the hetero-dimerization than the homo-dimerization and the most potent mitogenic signal originates from the ErbB2 and ErbB3 hetero-dimerization (Yarden and Sliwkowski, 2000). ErbB1 and ErbB2 homodimers may be less potent due to the proteosomal and lysosomal degradation by the ubiquitin ligase, c-Cbl. On the other hand, as heterodimers are targeted to cellular recycling, their signaling persists longer and can be more potent (Yarden and Sliwkowski, 2000).

1.4. SIGNALING BY ERBB2/NEU:

ErbB2/Neu is over expressed in 20-30% of primary human breast cancers and frequently altered in lung and kidney carcinomas (Hynes and Stern, 1994). The activation or overexpression of this gene correlates with poor patient prognosis (Ross and Fletcher, 1999). However, the precise mechanism by which ErbB2 activation leads to oncogenic transformation or metastasis of epithelial cells is unknown. Unlike other members of the EGFR family, ErbB2/Neu has no known specific soluble ligand. However, the tyrosine kinase activity of Neu can be stimulated by other EGFR ligands through the formation of heterodimers with other EGFR family members (Goldman et al., 1990; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996a,b; Tzahar et al., 1996; reviewed in Chan et al., 2004). For example, the

transphosphorylation of ErbB2 occurred through the formation of EGFR-ErbB2 heterodimers while the cells were stimulated with EGF (King et al., 1988; Stern and Kamps, 1988). Similarly, ErbB2 transphosphorylation resulted through the formation of specific heterodimers of ErbB2 and ErbB4 or heterodimers of ErbB2 and ErbB3 (reviewed in Chan et al., 2004).

Neu has five phosphorylated tyrosines (pTyr) in the C-terminal/docking domain. In a series of studies, these five major tyrosine autophosphorylation sites (Y1028, Y1144, Y1201, Y1227 and Y1253) were systematically evaluated for their roles in constitutively activated ErbB2-mediated transformation of fibroblasts cells (Dankort et al., 1997). A single amino acid substitution from tyrosine to phenylalanine at the 1028 site (Y1028F) of a constitutively active Neu allele showed consistent increase in transforming ability. However, restoration of the tyrosine residue at the 1028 site (Y1028) of a tyrosine phosphorylation deficient (NYPD) mutant blocked transformation of cultured fibroblasts. Moreover, the other four out of five individual pTyr of activated Neu, the rat homologue of ErbB2, contributed individually to transform cultured fibroblasts indicating that tyrosine the 1028 site negatively modulates ErbB2 activity (Dankort et al., 1997). They also reported that in the case of Neu, single pTyr that coupled to the Ras pathway through Shc (Y1227) or Grb-2 (Y1144) could act to transform cultured fibroblasts (Dankort et al., 1997, 2001). However, *in vivo* experiments with

the living organism showed that signaling through Grb-2 alone result higher rate of metastasis than signaling through Shc alone (Dankort et al., 2001).

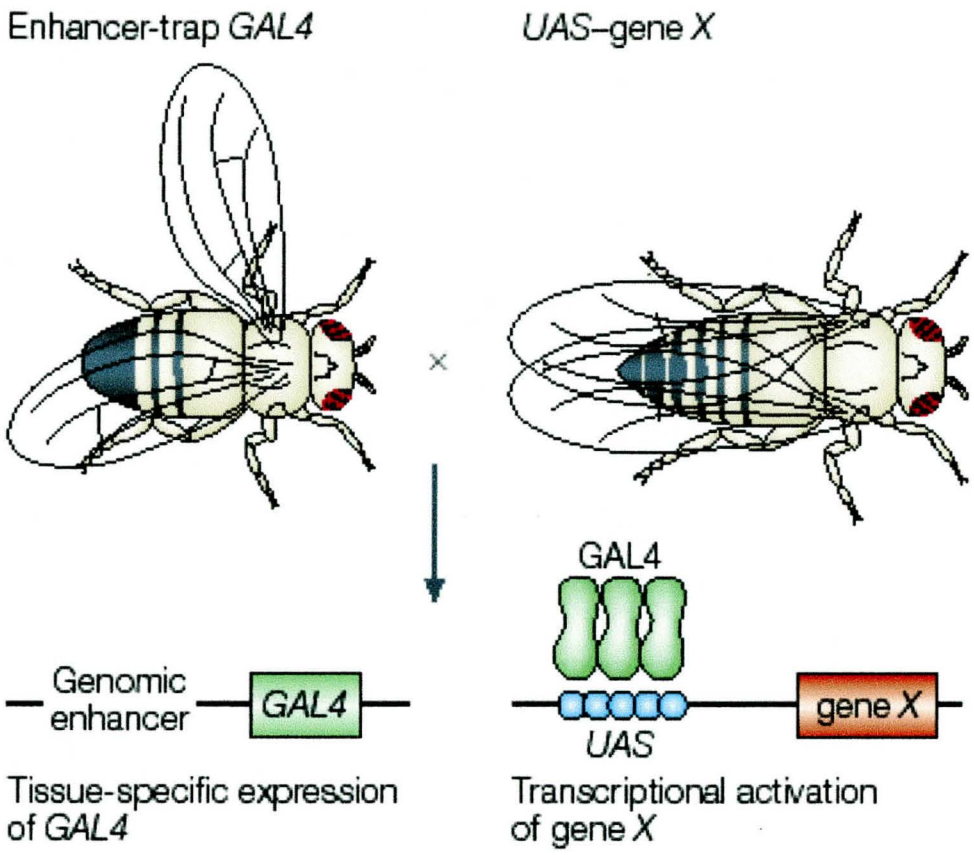
1.5. DROSOPHILA, A GENETICALLY MORE AMENABLE AND POWERFUL MODEL ORGANISM:

The identification of pTyr outputs is largely established by the data from peptide inhibition, phosphotyrosine labeling and protein co-immunoprecipitation experiments *in vitro* (reviewed by Pawson and Nash, 2000; Schlessinger, 2000). The data from pTyr outputs or protein sequence required for receptor binding, from the *in vitro* experiments, therefore, must be validated by functional assessment *in vivo*. *In vivo* experiments reveal more functional distinctions of different pTyr outputs (Lesa and Sternberg, 1997; Dankort et al., 2001). Genetic analysis is an efficient means for identifying signaling pathways *in vivo*. The structure and function of many SH2/PTB proteins in signaling is conserved in model organisms like *Caenorhabditis elegans* and *Drosophila*. For example, *C. elegans* SEM-5 has been shown to associate with human EGFR and share its SH2 and SH3 domains with GRB2 (Stern et al., 1993). On the other hand, human GRB2 and *Drosophila* Drk can rescue *sem-5* function in *C. elegans*. Moreover, the PTB and SH2 binding properties of *Drosophila* Shc (Dshc) and mammalian Shc are highly conserved (Lai et at., 1995).

A number of studies have taken the advantage of well-characterized signal transduction pathways in *Drosophila* to screen for proteins that interact with vertebrate transgenes (Bhandari and Shashindra, 2001; Jackson et al., 2002; Kazantsev et al., 2002; Rubinsztein, 2002). The targeted misexpression system in *Drosophila*, using the GAL4-upstream activating sequence (GAL4-UAS) (Brand and Perrimon, 1993), has made this organism more attractive to the geneticists and developmental biologists (reviewed by Johnston, 2002). In this system, the yeast transcriptional activator, GAL4, is being used to regulate gene expression in *Drosophila* by inserting the Upstream Activating Sequence (UAS), to which it binds next to a gene of interest (Fig.2). Using appropriate 'enhancer-trap' GAL4 lines, the misexpression of any particular gene can be done in cell and tissue specific manner (Brand and Perrimon, 1993). Using this modular misexpression system, with the help of appropriate GAL4 driver(s), Settle et al. (2003) were able to misexpress Neu, the rat homologue of ErbB2, in the midline glia, eye and wing tissues and showed that *Drosophila* adaptors signal from individual phosphotyrosine sites of rat Neu. The study also found that activated Neu expression in the midline glia suppressed apoptosis, a similar phenotype seen with the activated *Drosophila* EGFR expression. The authors also showed that Neu-'add-back' alleles, while expressed in the eye and wing tissues, generated graded phenotypes- suitable for the dosage-sensitive modifier genetics. By suppressing the ErbB2/Neu-induced phenotypes in tissues haplosufficient for genes encoding for proteins

FIGURE 2:

The GAL4-UAS system for directed gene expression. In this system, one fly line contains 'enhancer-trap' GAL4 driver and the other contains the upstream activating sequence (UAS) plus particular gene of interest (UAS-gene X). To activate the target gene, in a cell or tissue specific pattern, flies carrying the target (UAS-Gene X) are crossed to flies expressing GAL4 (Enhancer trap GAL4). In the F1 generation, the yeast transcription factor GAL4 binds with the UAS and thus expresses the adjacent gene (gene X). This figure was adapted from Johnston, 2002.


Enhancer-trap *GAL4*
UAS–gene *X*
×
GAL4
Genomic enhancer
GAL4
UAS
gene *X*
Tissue-specific expression of *GAL4*
Transcriptional activation of gene *X*

and second messengers they showed that pTyr 1227 (Y1227) required Shc, and pTyr 1253 (Y1253) signals through Raf but not Ras. This study was the first to use the 'power of *Drosophila* genetics' to reveal the molecules that signal from the oncogenic ErbB2/Neu.

1.6. STRUCTURE AND SIGNALING CONSERVATION BETWEEN NEU AND THE DEGFR:

The Drosophila epidermal growth factor receptor (DEgfr) shares an overall 56% amino acid similarity with the rat Neu. Sequence comparison of Neu with DEgfr showed that the kinase domains of these two receptors are the highest conserved structure (79% amino acid similarity). The C-terminal or docking domain, on the other hand, has the least 31% amino acid conservation (Settle et al., 2003). Out of the three identified phosphotyrosine residues, in Neu, DEgfr shares an NPEYL sequence with neu^{YC} (Y1201) and neu^{YE} (Y1253) (Settle et al., 2003). On the other hand, neu^{YA} (Y1028) has sequence conservation with the DEgfr-δ (Y1261) (Fig.3). Due to the relative structural and sequence similarity between DEgfr and rat Neu, it is possible that Neu might associate with those adaptor proteins involved in endogenous DEgfr signaling. The targeted expression of *neu* 'add-back' transgenes in *Drosophila*, tissues known to require DEgfr signaling in development, was

done in our lab previously to find out which *Drosophila* adaptor may bind to specific Neu pTyr (Settle et al., 2003).

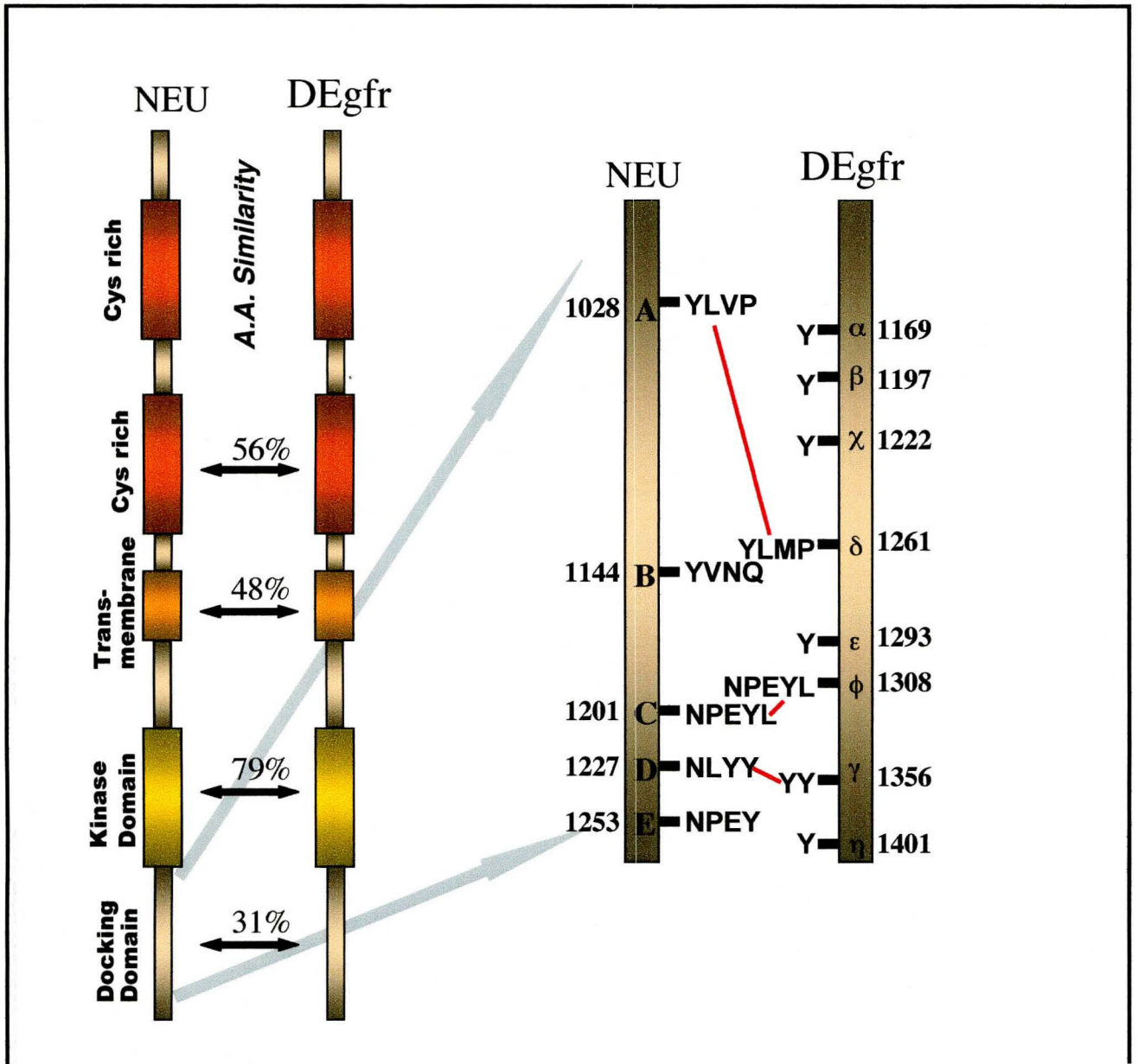
1.7. ROLES OF DEGFR DURING THE DR0SOPHILA DEVELOPMENT:

The *Drosophila* epidermal growth factor receptor (DEgfr) performs multiple functions during development: including apoptosis suppression, cell proliferation, cell differentiation and cell survival (reviewed by Schweitzer and Shilo, 1997). In specific, DEgfr provides polarity information to the ovary by specifying the ventral ectoderm after gastrulation and induces wing vein cell fate during wing development (Raz et al., 1991). DEgfr also suppresses apoptosis of midline glia (MG) cell lineages and provides cell proliferation and cell differentiation during the eye development (Raz et al., 1991). Moreover, it has also been shown that DEgfr, through the Ras/Raf/MAPK pathway, promotes cell survival during the eye development (reviewed by Kurada and White, 1999).

There are four known ligands that activate the DEgfr, such as Spitz, Gurken, Vein and muc4. However, Argos is the only known ligand that negatively regulates this RTK activity. During the eye development, the combinatorial action of Spitz and Argos provide the tight regulation in both

FIGURE 3.

Structural comparison of rat Neu and the DEgfr. Neu and the DEgfr show an overall 56% sequence similarity. However, the kinase domain showed the highest 79% amino acid sequence similarity while the docking domain has the least 31% amino acid sequence conservation. Further sequence analysis showed that DEgfr shares an NPEYL sequence with neu^{YC} (Y1201) and neu^{YE} (Y1253). The other two protein binding sites have a high degree of conservation (compare DEgfr- δ and Neu^{YA}). This figure is adapted from Dr. J.R. Jacobs.


NEU
DEgfr
A.A. Similarity
Cys rich
Cys rich
Trans-membrane
Kinase Domain
Docking Domain
56%
48%
79%
31%
NEU
DEgfr
1028 A YLVP
1144 B YVNQ
1201 C NPEYL
1227 D NLYY
1253 E NPEY
YLMP
NPEYL
YY
Y α 1169
Y β 1197
Y χ 1222
δ 1261
Y ε 1293
ϕ 1308
γ 1356
Y η 1401

time and space manner (reviewed by Freeman, 1997). In case of cell survival, during the eye development, DEgfr functions via the Ras/Raf/MAPK pathway and negatively regulates a pro-apoptotic gene, *head involution defective (hid)*, a cell death regulator in *Drosophila* (Kurada and White, 1998; Bergmann et al., 1998). A loss of function of *hid* promotes cell survival in flies, while ectopic *hid* expression induces massive apoptosis at various stages during *Drosophila* development. During the central nervous system (CNS) development DEgfr, in order to maintain the cyto-architecture of CNS, suppresses apoptosis in the MG cells by phosphorylating and thus inhibiting *head involution defective, hid* (Raz et al., 1991).

1.8. TRANSGENIC NEU ENABLES STUDYING RTK SIGNALING *IN VIVO:*

Due to the structural similarity and conserved signaling 'cascades' among the metazoans, our lab previously investigated whether the adaptor binding sites of a vertebrate RTK (such as Neu) could successfully signal through the adaptor proteins in *Drosophila*. By mis-expressing the Neu 'add-back' alleles in the tissues, known to require DEgfr signaling during pattern formation, Settle et al. (2003) showed that *neu* alleles generate phenotypes similar to increased activation of DEgfr. The mis-expressed Neu alleles showed dosage-sensitive lethal effects as well. By taking the advantage of

these graded phenotypes and the dosage-sensitive genetics, they showed that Neu^{YD} signals require Shc, while Neu^{YE} signal requires Raf but not Ras. Their findings tempted us to further study Neu^{YE}, which could signal through Raf without employing Ras, the primary molecule of the conventional Ras/Raf/MAPK signaling pathway. In order to identify this possible non-conventional pathway, the primary step would be identifying gene(s) responsible for neu^{YE} signaling. A mutation of the gene that positively regulates neu^{YE} signaling would enhance the wing phenotypes in the mis-expressed (neu^{YE}; *C96*) adult wings. On the other hand a mutation on the gene that has negative or inhibitory role in neu^{YE} signaling would suppress the wing phenotypes in the mis-expressed flies. Therefore, a genetic screen with the widely used chemical mutagen, Ethyl Methanesulfonate (EMS), to find either the suppression or enhancement to wing phenotype of neu^{YE};*C96* flies, would reveal the gene required for neu^{YE} signaling in *Drosophila*.

Since our previously established all Neu transgenic fly lines lost their mis-expressed phenotypic characteristics, we reestablished all the transgenic Neu 'add-back' alleles into the *Drosophila* and re-established phenotypes in the wing and eye the tissues know to require DEgfr signaling.

In our previous study, Settle et al. (2003) designated the function of a number of candidates molecules required for mammalian *neu* signaling in *Drosophila* by examining wings from the *UAS-*neu^{YE}*; C96* flies also

heterozygous for the amorphic alleles of *ras, shc, raf, dos, dab, drk and csw*. Using their standards, here we genetically verified our newly generated transgenic *neu*^{YE} lines by scoring the suppression or enhancement of the phenotypes rendered by the amorphic alleles of second messenger and adaptor molecules.

We have already standardized the EMS dosage and verified the X-chromosome linked recessive lethality in order to get one mutational hit per 2000-5000 genes. So far we have screened 307 EMS treated males or roughly 3000 F1 flies. We are optimistic that our ongoing EMS mutagenesis scheme would reveal the gene(s) responsible for Neu^{YE} signaling that possibly signals through other than conventional Ras/Raf/MAPK signaling pathways.

Moreover, while expressed in the eye, Neu^{YA} showed less strong phenotype than that of Neu^{NYPD}, suggesting a negative regulatory role in Neu signaling. Dankort et al. (1997) also suggested that Neu^{YA} might provide inhibitory signals and normally attenuate signaling outputs from other pTyr. A recent study, with 'knock-in mice', shows that YA (Erbb2-Y1028F) mutant could rescue the perinatal lethality in the hemizygous *Erbb2* animals and suggested that YA might provide inhibitory role in ErbB2 signaling (Chan et al., 2004). All these data tempted us to verify whether Neu^{YA} could suppress the phenotypes while heterozygous with the other individual Neu 'add-back' alleles.

Our current data verified that neu^{YA} confers suppression of wing phenotypes while heterozygous with neu^{YB}, neu^{YC}, neu^{YD}, neu^{YE} and neu^{NYPD}. We have also generated the *neu* 'double add-backs' of neu^{YAB}, neu^{YAC}, neu^{YAD} and neu^{YAE} and these molecules are ready for generating the transgenic fly lines. Finally, the mutation(s) that confer a phenotype to neu^{YA} would reveal the gene(s) responsible for the repression of RTK signaling. Therefore, our study will have profound effects in understanding the RTK signaling pathways and may eventually help to develop therapeutic targets for many forms of human cancers caused by the aberrant signaling pathway/molecule.

CHAPTER 2:

MATERIALS AND METHODS:

2.1 DROSOPHILA MELANOGASTER FLY SOCKS:

Unless otherwise indicated, *Drosophila melanogaster* fly strains were obtained from Bloomington Stock Centre. All fly lines were stored at room temperature (22-25°C) in polypropylene shell vials (Fisher Scientific, AS 519) or 16X100 mm glass culture tubes (Fisher Scientific) supplemented with a sucrose-yeast agar food medium and capped with rayon rope (Fisher Scientific, APS205). The wild type Oregon R strain was used in all controls. Microinjection was performed on yellow white$^-$ (yw^-) embryos. *p[UAS-neu]* expression was regulated by *p[GAL4]* strains, GMRGAL4 (Hay et al., 1997) and C96 (Gustafson and Boulianne, 1996; Stewart et al., 2001). Crosses with GMRGAL4 were conducted at 18°C in order to suppress a weak ommatidial defect, which is intrinsic to the stock. On the other hand crosses with C96 were always conducted at 25°C.

2.2 MUTATIONS AND pUAST LINES:

All mutants and pUAST lines used are reported in the following two tables.

TABLE 2.1:

pUAST and DER mutant lines used for genetic interaction experiments.

pUAST lines	Genetics	Reference
GMRGAL4 Source: Rubin	Driver line containing yeast transcription factor, GAL4, under the control of glass multimer reporter (GMR).	Hay et al., 1997.
C96 Source: Boulianne	Enhancer trap P-insert line containing yeast transcription factor, GAL4. C96 expression has been observed in imaginal discs and adult wing margins (Settle et al., 2003)	Kim et al., 1998; Gustafson and Boulianne, 1996; Stewart et al., 2001.
UAS-DER^{wildtype} (11-9) Source: N. Baker	*Drosophila* epidermal growth factor receptor	
DER-Ellipse (DER^{ElpB1}) Source: N. Baker	Gain of function mutant of DEgfr	Baker and Rubin, 1989.
UAS-DER^{A887T} (12-4) Source: N. Baker	Constitutively active DEgfr that contains an alanine to threonine mutation at residue number 887.	Lesokhin et al., 1999.
UAS-DER^{DN} Source: M. Freeman	Dominant negative *Drosophila* Egfr receptor	Freeman, 1996.

TABLE 2.2:

Mutant lines used for Genetic screening (Adapted from Settle et al., 2003)

Gene (Allele)	Cytological location	Genetics	Proposed function	Reported Mutant Phenotype	Reference
Polehole (phl^{C110}) Source: M.P. Martin	3A1-15	X-ray mutagen, hypomorph	Protein kinase involved in signal transduction downstream of Ras; required for normal rate of cell proliferation	Rough eye due to loss of R7 during ommatidial formation; mild wing vein phenotype.	Perrimon et al., 1985; White and Jarman, 2000.
Ras oncogene at 85D ($ras85D^{05703}$) Source: D. Montell	85D21	P-element activity mutagen, hypomorph	Ras small monomeric GTPase involved in perineural glial growth; growth, survival and differentiation in the eye.	Defects in ovarian development and cuticle formation, rough eye phenotype.	Rorth, 1996; Schnorr and Berg, 1996.
Son of Sevenless (sos^{34Ea-6} Adh^{n4}) Source: ?	34D4	EMS mutagen, amorph	Ras guanyl-nucleotide exchange factor involved in Ras protein signal transduction.	Some ommatidial lack receptor cells leading to mild rough eye phenotype.	Rogge et al., 1991.
Corkscrew (csw^{LE120}) Source: L. Perkins	2D1	EMS mutagen, amorph	Protein tyrosine phosphatase involved in multiple receptor signaling pathways and R7 cell fate determination.	Maternal effect lethality, loss of muscle precursor cells.	Perkins et al., 1996.

SHC-adaptor protein ($dshc^{111-40}$) Source: S. Luschnig	67B4	EMS mutagen recessive	Adaptor protein involved in multiple receptor signaling pathways	Semi-lethal; germ band retraction defects; female sterility.	Luschnig et al., 2000.
Downstream of Receptor kinase (drk^{10626}) Source:	50B7	P-element activity mutagen, recessive.	SH3/SH2 adaptor protein involved in Ras protein signal transduction.	Embryos with patterning defects in head and tail regions.	Simon et. al., 1993; Spradling et al., 1999.
Daughter of Sevenless ($Dos^{2.42}$) Source: S. Simon	62E7	EMS mutagen, recessive	SH2/SH3 adaptor protein involved in signal transduction	Cells in the eye can proliferate, but do not differentiate as photoreceptors (Similar to lack of csw function).	Herbst et al., 1996.
Disabled ($dab^{[M54-R1]}$]	73C1	X-ray mutagen, amorph	Adaptor protein involved in axon guidance downstream of RTK signaling	Abnormal photoreceptor development	Hill et al., 1995

2.3 TRANSGENES:

The generation of constitutively active rat *erb-B-2* (neu^{NT}), a mutant form of the *neu* gene, was done by E664V point mutation in the transmembrane domain (Dankort et al., 1997). The generation of neu^{NYPD} (neu phosphorylation deficient or tyrosine to phenylalanine transition at 1028, 1144, 1201, 1226/1227 and 1253 amino acid residues) and add-back alleles: restoring tyrosine to 1028 (YA), 1144 (YB), 1201(YC) and 1226/1227(YD), were also done by Dankort et al. (1997). By using the Site-Directed (SD) mutagenesis technique (Nickoloff and Deng, 1992), we generated the neu^{YE} allele

by restoring the tyrosine at the 1253 position on the *neu*^{NYPD} background. The SD mutagenesis was done according to the manufacturers protocol (Quick-Change XL Site-Directed mutagenesis kit, Stratagene, cat# 200517). The *UAS-neu*^{NYPD} plasmid was amplified by using the mutagenic oligonucleotide pair 5'GCAGAGAACCCTGA GT**AC**CTAGGCCTGGATGTACC3' (forward, ML1977) and 5'GGTACATC CAGGCCTAG**GT**ACTCAGGGTTCTCTGC3' (Backward, ML1978), which will restore tyrosine at the site E (1253). Nucleotides that differ from the neu^{NYPD} sequence are in bold and underlined. The PCR amplification was performed for 14 cycles of 60s at 94°C, 1 min 40s at 60°C and 30 mins at 68°C. The PCR product was then treated with 1μL *Dpn I* endonuclease and incubated at 37°C for 1 hour to digest the parental DNA template and to select for mutation containing synthesized DNA. 2 μL of the digested product was then used to transform 50 μL of XL10-Gold ultracompetent cells provided with the Mutagenesis kit. 100 μL of the transformed cells (in NZY^{+} broth, see Stratagene's instruction manual, cat# 200517) was spread on the LB Ampicillin agar plate and allowed to grow at 37°C for 16-18 hours. Several well distant colonies were picked up and grown overnight in 14 ml falcon tubes (Corning, cat.# 4-2059-3) supplemented with LB Ampicillin broth medium. The plasmid was prepared separately from all the bacterial samples using the Plasmid Minipreps kit (Qiagen, cat# 27106). All the plasmid preparations were then restriction mapped with XhoI (Invitrogen, cat# 15231-012) and EcoRI (Roche, cat# 703737) restriction enzymes and only a few of the chosen samples which have the right band size revealing proper length and orientation as well, were sent to the Mobix Lab (McMaster university) for sequencing.

The sequences were then aligned with Rat mRNA for *neu* oncogene (p185) encoding an epidermal growth factor receptor-related protein (Accession# X03362, Version X03362.1 gi# 56745). The result was then analyzed and only that sample was considered, which does not have any mutation but the expected tyrosine restoration at the site E (1253).

2.4 SUB CLONING OF CDNA:

The newly made neu^{YE} cDNA was subcloned into a fresh pUAST vector. 2.4 μg of plasmid DNA and 2.4 μg of pUAST vector were digested separately with 1.5μl of EcoRI at 37°C for 1.5 hours. The digested product from the plasmid DNA was electrophoresed on a 1% agarose gel and 4 kb band was excised and purified using Gel extraction kit (Qiagen, cat.#20021). On the other hand, 2.5 μl of Shrimp Alkaline phosphatase, SAP, (Boheringer, Germany) along with 10X buffer was added to the pUAST digestion product for 1 hour at 37°C. Following incubation, SAP was inactivated at 65°C for 15 minutes and the reaction mix was kept in room temperature for additional 30 mins. The concentration of linearized pUAST and purified neu^{YE} cDNA were measured using spectrophotometer at 260 nm.

In order to ligate the insert into pUAST (Brand and Perrimon, 1993), 500 ng of insert DNA and 100 ng of pUAST DNA were mixed along with 3 μl of 5X ligation buffer, 1 μl of T4 ligase (Invitrogen, cat.# 15224-041) to make the final volume of 19 μl. The reaction was kept at 14°C overnight. 4μl

of ligation mix was used to transform 50µl of DH5alpha competent cells according to the manufacturer's specification. Colonies were screened using Minipreps (Qiagen, cat.# 27106) and EcoRI (Roche, cat.# 703737). Clones with positive inserts were further digested with XhoI (Invitrogen, cat.# 15231-012) to reveal the orientation.

In order to make the double mutant we restored the tyrosine at 1028 (A) amino acid residue in neu^{YB}, neu^{YC}, neu^{YD} and neu^{YE} respectively to generate neu^{YAB}, neu^{YAC}, neu^{YAD} and neu^{YAE}. The mutagenic oligonucleotide primer pair was 5'GGTAGACGCTGAAGAGT**A**TCTGGTGCCCCAG3' (Forward, ML 2538) and 5'CTGGGGCACCAGA**T**ACTCTTCAGCGTCTACC3'(Backward, ML 2539). The nucleotide that has been changed to restore tyrosine at the 1028 position (YA) has been bold and underlined. Here we followed exactly the same procedure and experimental parameters, starting from the site directed mutagenesis to the sample storage as described above for the neu^{YE} in section 2.3 and 2.4. The blast results have been added at the appendix.

2.5 PREPARATION OF DNA CONSTRUCTS FOR MICROINJECTION:

DNA extractions were done individually using the endo free plasmid maxi kits (Qiagen, cat# 12362). The prepared plasmid DNA size was compared with the High Mass DNA ladder prior to make a 30 µg of sample

for microinjection. This sample contains the pUAST and helper vector pπ in a ratio of 5:1. The volume of the sample was then made 100 μL with distilled water followed by precipitation by adding additional 10μL of 3M sodium acetate. After precipitation, 250μL of absolute and cold ethanol was added and kept in –80°C for 15 minutes before spinning at 13000 rpm for 15 minutes at 4°C. Following a rewash with 70% ethanol, the pellet was dried for 10-15 minutes in room temperature and resuspended in 50μL of injection buffer (10mM Tris-Hcl [pH7.5], 0.1 mM EDTA, 100 mM NaCl, 30μM spermine and 70μM spermidine).

2.6 DNA MICROINJECTION:

Except for *Neu*^{YB}, Zhong Xiaoli of Dr. Campos Lab, McMaster University, did all other microinjections according to the standard protocol as described by (Spradling, 1986). Microinjection of pUAST constructs was performed on *yw*$^{-}$ embryos. The embryos were collected on an apple juice agar plate attached to a fly house made of 100 ml plastic beaker (Nalgene) with tiny holes to allow airflow. In order to encourage the flies to lay eggs, a bit of yeast paste was added onto the solidified apple juice agar plates. Fly houses were set up and maintained at 25°C and the plates were changed at every thirty minutes so that early stages embryos can be collected and injected before the pole cell formation. After 5 minutes dechorionated with 50% bleach solution, embryos were collected in a nitex sieve chamber. Then

approximately 30-35 embryos were lined up on a double-sided adhesive tape attached on a standard glass slide within a 20 minutes time limit. The embryos were then placed on Anhydrous Calcium Sulfate bed (W.A. Hammond Company product # 23001) in a dessicator. After appropriate dessication, embryos were covered with a thick layer of halocarbon oil (Halocarbon® cat.# 9002-83-9). Injection was performed under a Leica inverted microscope (Leica, Germany). Needles were pulled from 100X1 mm Borosil glass capillary tubes (FHC, cat.# 30-30-0) and were broken by gently touching the side of the glass slide. Approximately 2-3 μL of the desired DNA construct was loaded into the glass needle using a Hamilton 26 gauge needle (Fisher Scientific, cat.# 14-813-1). A tiny amount (a barely visible bubble) of DNA construct was injected into the posterior of the embryos. Injected embryos were kept in a Petri dish containing a wet paper towel to provide necessary moisture to the embryos. The embryos were then kept at 18°C for 48 hours and the surviving embryos (usually crawled out from the oil) were transferred to yeast agar food vials at 25°C until they eclosed. Individual flies were then crossed with *yw*⁻ adults and the F1 generation was screened for eye pigmentation. Flies with the eye pigmentation were crossed again with *yw*⁻ adults and the purified stocks were balanced and p-element insertion was mapped by crossing (individual stock) with various marked balancers.

2.7 LARGE SCALE WING SCREENING:

Selected flies were crossed in 16X100 mm glass culture tubes (Durex™ Borosilicate glass, cat # 60825-471) supplemented with 4 ml of sucrose-yeast agar food and 2-3 drops of dry active yeast to stimulate egg laying. All the experiments (crossings) were, at least, in triplicate to see the duplication of the results. All crosses were maintained at 25°C until majority of the F1 flies had eclosed. Wings were examined and scored as either 'no interaction', 'enhancement' or 'suppression' depending upon the severity of the wing notches, the amount of ectopic vein tissue and the number of wing deltas. A 1-9 quantitative scale was designed to rank the severity of the wing phenotype where 5 indicate 'no interaction', 1 as 'extreme suppression' and 9 as 'severe enhancement' (See appendix. 1). For blind scoring Dr. Jacobs mounted 2 wings on each slide and at least a total of 40 wings from a particular genotype. He then assigned an arbitrary number (without letting me know the genotype) on the slide and asked me to score the phenotype on the basis of 1-9 scale. The blind test score from each slide was then assigned to the representing genotype that was unknown to the scorer (myself). Finally, a particular value was assigned if only when 75%-100% observed wings of the same genotype represented that score.

2.8 WING MOUNTING AND LIGHT MICROSCOPY:

Flies were anesthetized by keeping them at -20°C for 5-10 minutes. The anesthetized flies were then dehydrated in ethanol gradient (50%, 70%,

90%, 95% and 100%). Wings were clipped by using a wing clippers scissor (F.S.T 91500-09, Germany) and were kept in methyl salisilate prior to mounting. Wings were then mounted in D.P.X neutral mounting medium (Aldrich, cat# 31761-6) on frosted glass slides (Corning) using 24X24 mm coverslips. The slides were then stored or photographed through a Nikon SMZ1500 microscope and a Nikon digital camera (Nikon Coolpix990). The photographs were then stored directly on a Mac OS X (version 10.2.8) computer and prepared for figures by Adobe Photoshop® version7.0.

2.9 ENVIRONMENTAL ELECTRON MICROSCOPY:

Adult flies, from the appropriate crosses, were anesthetized by keeping them at the freezer chamber for at least 5 minutes. Each time one fly head was dissected by using the clippers scissor (F.S.T 91500-09, Germany) and mounted on the centre of the stand covered with homogenously mixed white glue and charcoal. The fly eyes were viewed, photographed at 100X, 150X and 300X at 3.0 Torr of a Philips Environmental Scanning Electron Microscope and images were saved in the Hard disk of a Philips computer. The images were processed with Adobe Photoshop® Version 7.5.

2.9 EMS MUTAGENESIS:

For EMS mutagenesis experiments we used the isogenized wildtype Oregon R fly strain. During the experiment, approximately 80 males of 3-4

days old were collected at 12 pm and kept in an empty polystyrene vial (Fisher Scientific, AS 519) for 5 hours to make them thirsty. At 4:30 pm of the same day, a neutralizing solution was made in a plastic container by adding 0.5 gm of thioglycolic acid (Sigma, cat.# 367-51-1) in a 100 ml of 4% NaOH solution. In addition a 25 ml of 1% sucrose solution was made in a 50 ml centrifuge tube (Corning, cat.# 430290). In the mean time, 2 pieces of 7X1.5 cm of no.1 whatman filter paper (Whatman®, cat.# 1001-125), were attached separately to the inside wall of the vial with adhesive tape. The vial was then properly capped with rayon rope (Fisher Scientific, cat.# APS205). From this point all the preparations were done into the fume hood assigned for EMS uses. At 5 pm 66 µL of Ethyl methane sulfonate (EMS) (Sigma, EC # 200-536-7) was gently dispensed into the sucrose solution by using a 1 c.c syringe (Becton Dickinson & C0. cat. # 309604) with 22 gauge needle (Becton Dickinson & Co., cat. #. 305156) The needle was then immediately filled with neutralizing solution and discarded into that plastic container. By using another 10 c.c syringe with 22-gauge needle, EMS and sucrose solution was mixed gently for 6-7 times. This syringe was also neutralized and discarded into the plastic container containing neutralizing solution. Afterwards 0.5 ml of EMS mix was added onto the two filter paper stripes attached to the inside walls of the capped polystyrene vial by using another fresh 1 cc syringe with 22 g needle. Then the thirsty flies were transferred to the vials treated with EMS and kept in the fume hood for overnight. At 11 am

next morning the EMS fed flies were transferred to vials, containing yeast sucrose agar fly food, for 2 times at 1 hour interval so that no EMS was left on the vials that would be carried to the fly pushing room. All the vials including the EMS treated and the one with fly food were neutralized and discarded as mentioned earlier. The EMS treated males (after all these procedure approximately 50 out of 80 are available) were then crossed pairwise with the virgins of *uas-neu*^{YE}*;C96* flies and kept at 25°C (See Appendix 15, and 16).

CHAPTER 3:

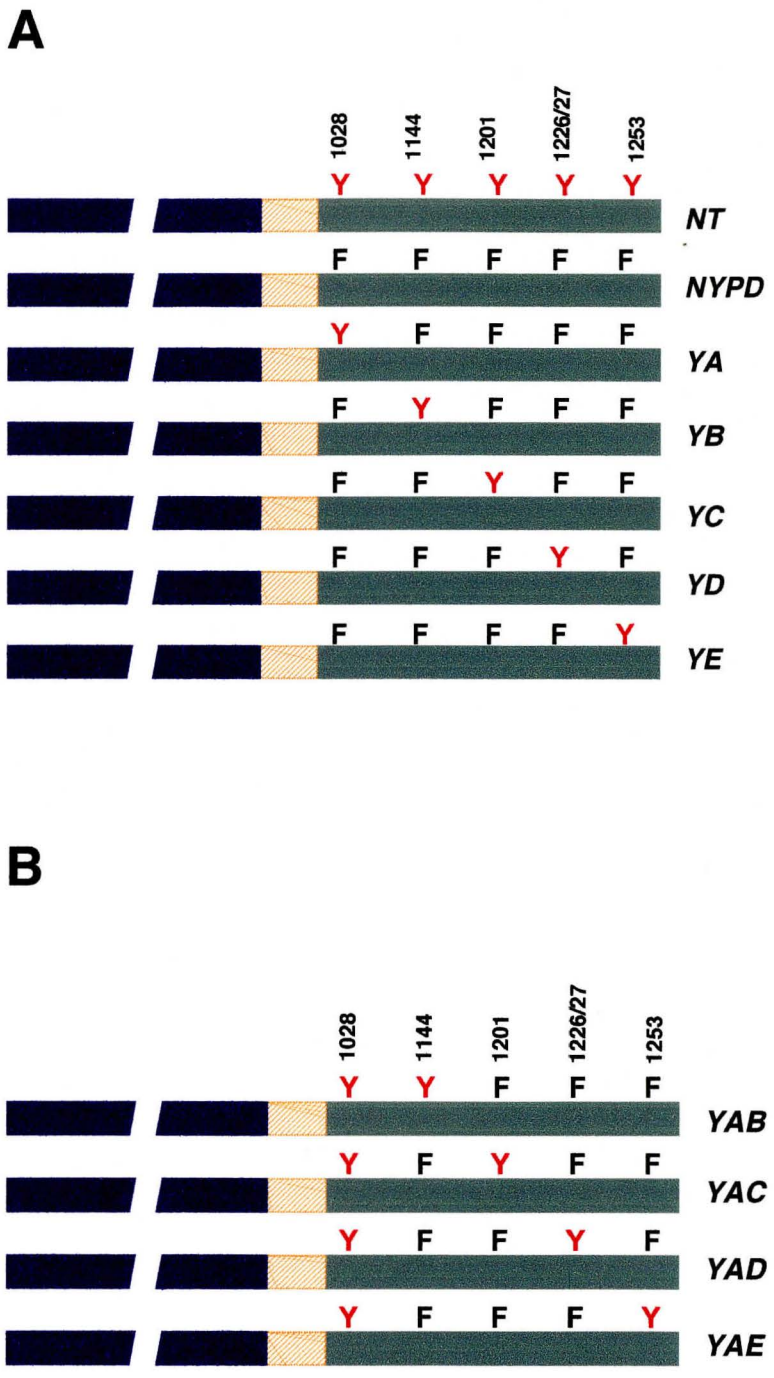
RESULTS

3.1 GENERATION OF NEU ADD-BACK ALLELES:

In order to assess the individual Neu phosphotyrosine (pTyr) output, Dankort et al. (1997) generated a constitutively active Neu allele (neu^{NT}), by substituting a single amino acid (V664E) in the transmembrane region of the wild type Neu. Then they generated a Neu Tyrosine Phosphorylation Deficient (neu^{NYPD}) allele, by simultaneous point mutations that changed all of the five known pTyr to phenylalanine. Afterwards, by adding back (on the NYPD background) a single pTyr to a particular position at a time, they created the following five 'add-back' alleles: neu^{YA} (Y1028), neu^{YB} (Y1144), neu^{YC} (Y1201), neu^{YD} (Y1227), and neu^{YE} (1253) (Fig 4A). Dankort et al. (1997) had generously provided us all these alleles. However, I had to regenerate the neu^{YE} allele as our stock had been lost. In order to do this, I had to add-back a Tyrosine molecule on the neu^{NYPD} allele by using the site directed mutagenesis (Fig 4A and also see Materials and Methods). Since all our previously established Neu transgenic fly lines lost their mis-expressed phenotypic characteristics, we reestablished all the transgenic Neu 'add-back' alleles into the *Drosophila* and re-established phenotypes in the wing and eye that were suitable for the dosage-sensitive

FIGURE 4.

Schematic representation of Neu receptor kinase alleles. (A) The structures of Neu single 'add-back' alleles where two cysteine rich domains are blue, transmembrane domain is striped (orange) and C-terminus is grey. Phosphorylation sites are indicated as 1028 (site A), 1144 (site B), 1201 (site C), 1226/1227 (site D) and 1253 (site E). The constitutively active neu^{NT} allele was made by a point mutation (V664E) at the transmembrane domain to the wild type Neu cDNA. For Neu phosphorylation deficient allele- neu^{NYPD}, all the five known tyrosine residues were converted to phenylalanine. Only one particular tyrosine residue, at a particular site, was restored to make neu^{YA}, neu^{YB}, neu^{YC}, neu^{YD} and neu^{YE}. (B) To make double add-back alleles, the tyrosine residue at the 1028 position was restored to neu^{YB}, neu^{YC}, neu^{YD} and neu^{YE} to make neu^{YAB}, neu^{YAC}, neu^{YAD} and neu^{YAE} respectively. All mutants were derived from the neu^{NT}.


A
1028
1144
1201
1226/27
1253
NT
NYPD
YA
YB
YC
YD
YE
B
YAB
YAC
YAD
YAE

modifier genetics. The reduction of mis-expression phenotypes could be due to accumulation of spontaneous mutations over time.

Moreover, a recent study, with 'knock-in mice', shows that YA (Erbb2-Y1028F) mutant could rescue the perinatal lethality in the hemizygous *Erbb2* animals and suggested that YA might provide inhibitory role in ErbB2 signaling (Chan et. al., 2004). Our trans-allelic data also suggests an inhibitory signaling by neu^{YA}, while it is heterozygous with the other *neu* alleles (section 3.4). Therefore, in order to study the functional outputs of homozygous neuYA with other neu alleles, we generated the Neu 'double add-backs' of neu^{YAB}, neu^{YAC}, neu^{YAD}, and neu^{YAE} alleles (See Fig 4B). To generate these neu alleles, I added back a Tyrosine molecule at the 1028 site (YA) on the neu^{YB}, neu^{YC}, neu^{YD} and neu^{YE} alleles by using the site directed mutagenesis (See Materials and Methods). All of these molecules will be used for generating the transgenic fly lines for further future studies.

3.2 NEU ALLELES CAN BE EXPRESSED IN ADULT STRUCTURES:

Drosophila Epidermal Growth Factor Receptor (DEgfr) is the only known vertebrate orthologue of ErbB receptors. In order to assess the consequences of misexpression of *neu* alleles, we have selected the tissues that require signaling from the *DEgfr*. Both wing and eye require the *DEgfr* signaling for the cell proliferation and patterning of the imaginal disc tissues

(Sturtevant and Bier, 1995). *DEgfr* signaling in the wing alters the structure of the wings and the patterns of the wing veins. On the other hand, changes of cell identity and the patterning of the ommatidia have been associated with *DEgfr* signaling in the eye (Baker and Rubin, 1989; Shilo and Raz, 1991). Therefore, by using the GAL4 activation system (Brand and Perrimon, 1993) with *P[GAL4]C96* (Gustafson and Boulianne, 1996; Stewart et al., 2001) and *p[GMR-GAL4]* driver (Hay et al., 1997), we mis-express *neu* alleles in the wing and eye, the two known tissues not required for the viability of the organism.

3.3 MIS-EXPRESSION OF NEU ALLELES PRODUCES DISTINCT WING AND EYE PHENOTYPES:

Neu signaling in the wing margin

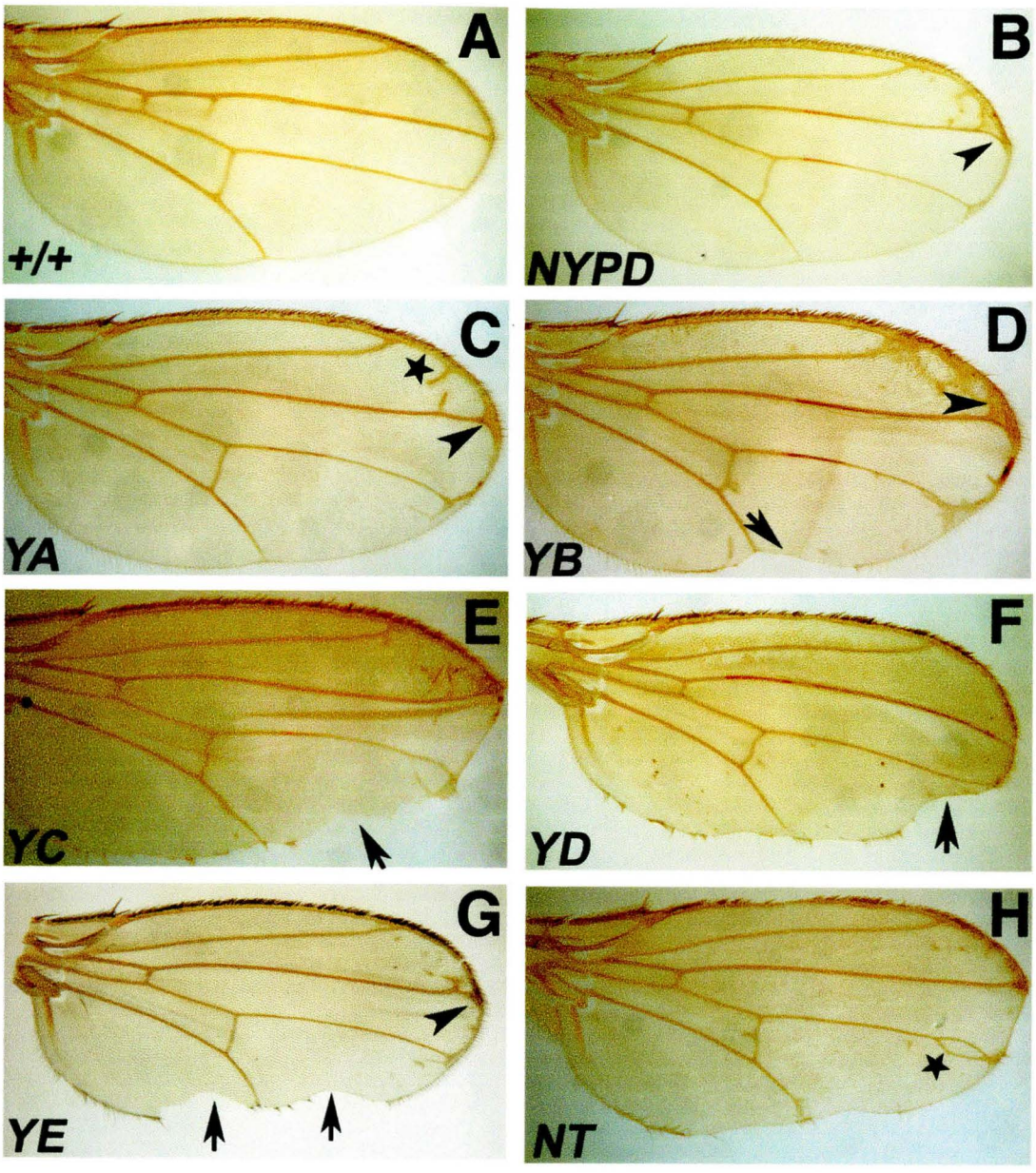
Our lab previously investigated that the adaptor binding sites of a vertebrate RTK (such as Neu) could successfully signal through the adaptor proteins in *Drosophila,* an invertebrate (Settle et al., 2003). Dankort et al. (1997) generated a Neu Tyrosine Phosphorylation Deficient (neu^{NYPD}) allele, by simultaneous point mutations that changed all of the five known pTyr to phenylalanine. Then, by adding back (on the NYPD background) a single pTyr to a particular position at a time, they created the following five 'add-

back' alleles: *neu*^{YA} (Y1028), *neu*^{YB} (Y1144), *neu*^{YC} (Y1201), *neu*^{YD} (Y1227), and *neu*^{YE} (1253). Furthermore, they generated a constitutively active Neu allele (*neu*^{NT}), by substituting a single amino acid (V664E) in the transmembrane region of the wild type Neu. This activating mutation maintains constitutively active Neu signaling through the increased homodimerization of mutant receptors (Bargmann and Weinberg, 1998; Weiner et al., 1989).

In order to mis-express the Neu in the adult wing, we selected C96 GAL4 enhancer trap, which expresses GAL4 at the dorsal to ventral boundary of the wing (Gustafson and Boulianne, 1996; Stewart et al., 2001). Neu mis-expression in the wings of *C96; p[UAS-neu]* flies resulted in ectopic veins, loss of wing margin and formation of wing delta (Fig. 5A-H). Unlike our previous studies (Settle et al., 2003), *neu*^{YA} showed ectopic veins or vein branching (Fig. 5C) although it (*neu*^{YA}) lacks transforming potentials in mammalian cells (Dankort et al., 1997). The severity of the wing phenotypes was dependent on the particular add-back allele. The phenotype penetrance was 100% in all add-back alleles however every wing was unique in the fine details of the phenotype (see complete wing photographs' archive in Appendix. 2-8). The most severe phenotypes were observed in the constitutively active allele, *neu*^{NT}, which include unique vein branching, vein

FIGURE 5.

Mis-expression of Neu add-back alleles produces distinct wing phenotypes. Neu alleles were mis-expressed at the wing margin with *p[C96] GAL4*. The activated neu^{NT} showed the most severe wing phenotype that includes vein branching, vein deltas and mild wing margin loss (H). neu^{NYPD} showed a nearly wild type wing margin along with a very small vein delta (B). neu^{YA} (C) showed ectopic vein formation in the anterior half of the wing blade. A gradual increasing severity of wing notch phenotypes were observed in neu^{YC} (E), neu^{YD} (F), and neu^{YE} (G) respectively (neu transgenes indicated at the bottom left of each panel). An asterisk marks show the wing branching, while arrowheads and arrows indicate the wing deltas and wing margin loss respectively.


A
+/+
B
NYPD
C
YA
D
YB
E
YC
F
YD
G
YE
H
NT

deltas and mild wing margin loss (Fig. 5H). Apart from neu^{NT}, wing margin loss was common in neu^{YB}, neu^{YC}, neu^{YD} and neu^{YE} (Fig. 5D, E, F and G). However, neu^{YD} showed the highest loss of wing margin (Fig. 5G). On the other hand the most severe phenotypes of ectopic veins and deltas were found in neu^{YB} followed by neu^{NYPD} (Fig. 5B and D). Although it lacks any phosphorylation site at the docking domain, the phenotype shown by the neu^{NYPD} was not unexpected as this allele showed a weak transforming potential in mammalian cells (Dankort et al., 1997).

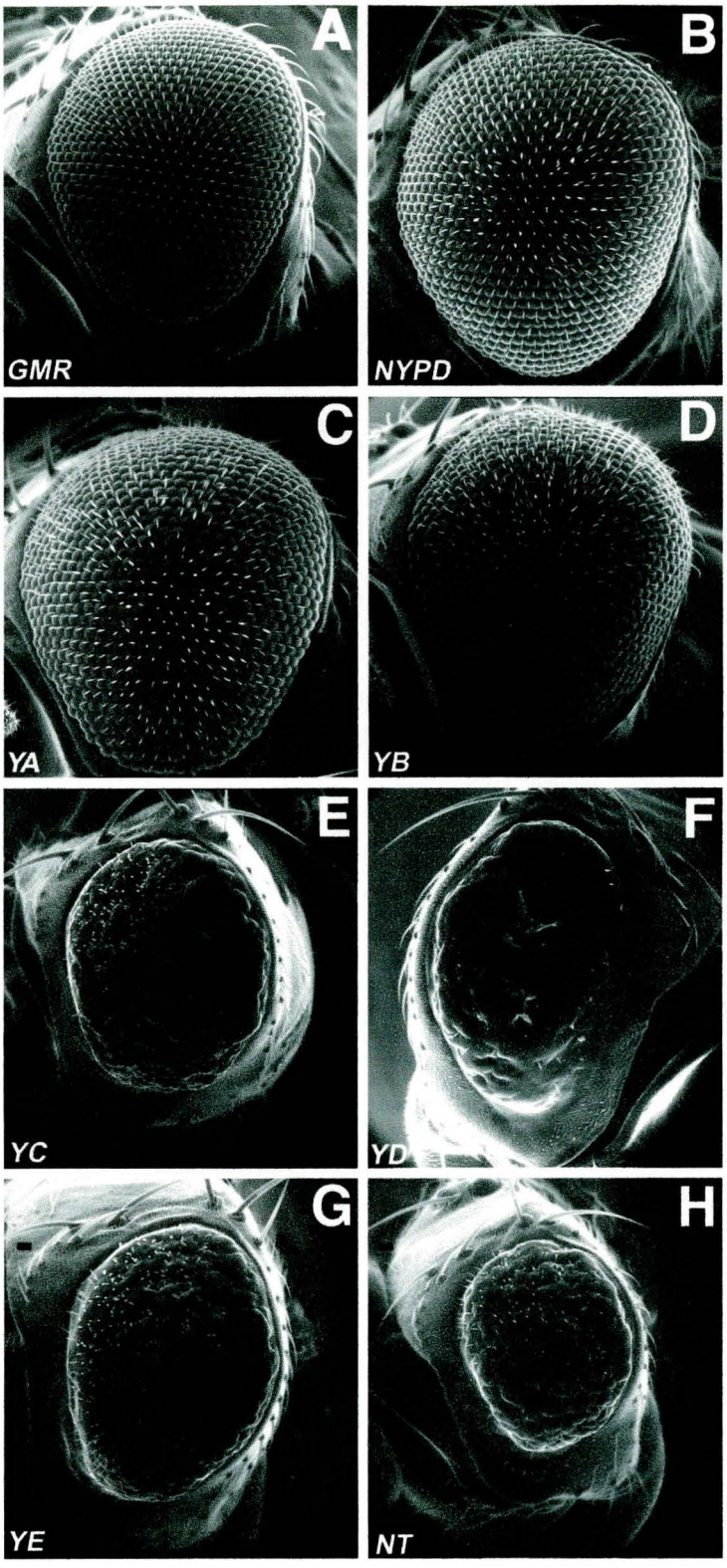
Neu signaling in the eye

For the mis-expression of neu add-back alleles in the adult eye, we had chosen Glass Multimer Reporter (GMR) directed expression system, as this driver directed expression revealed a sensitivity to much lower levels of Neu signaling (Settle at al., 2003). GMR is usually expressed in the eye imaginal disc. The ommatidia and the adjacent bristles are regularly spaced in the adult eye of GMR flies raised at 18°C (Fig. 6A and 7A, B).

The mis-expression of *neu* 'add-back alleles' resulted in rough eye surface, multiple adjacent bristles and in most cases reduced eye size (Fig. 6 and Fig. 7). Activated neu^{NT} showed the strongest phenotypes including severely reduced eye size along with complete loss of ommatidial shape and

FIGURE 6.

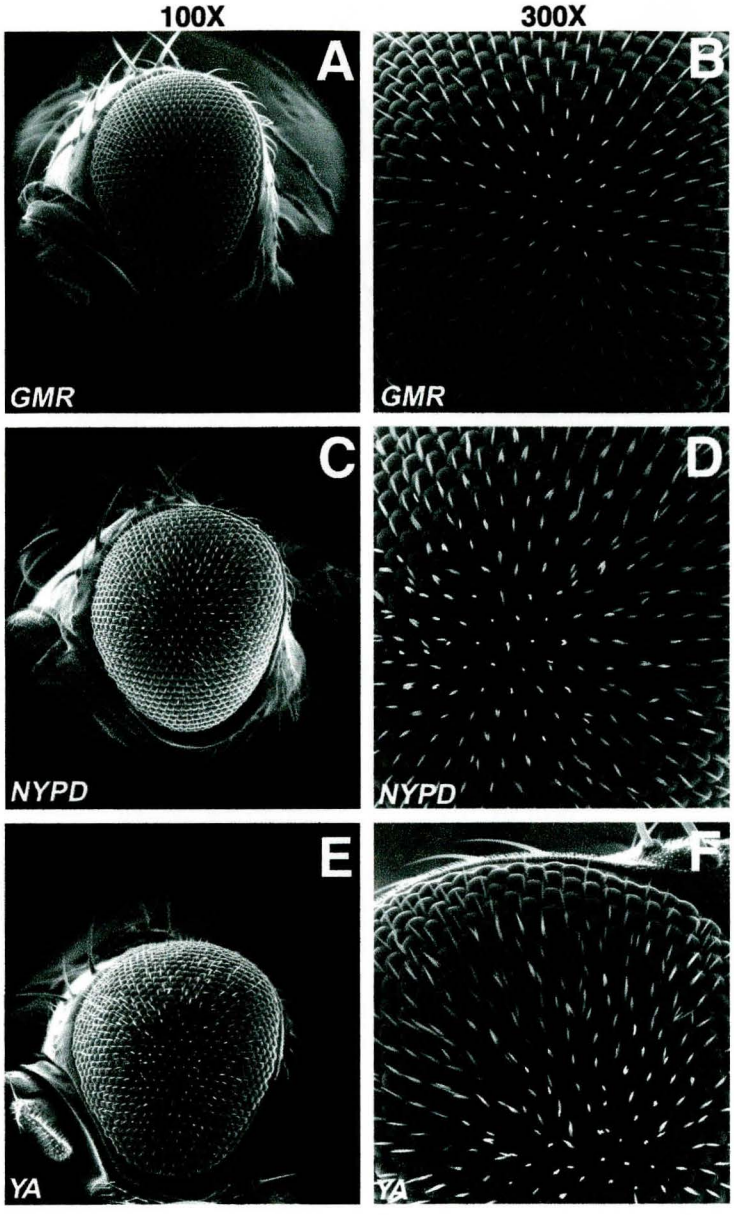
Mis-expression of Neu add-back alleles produces distinct eye phenotype. The mis-expression of eye phenotype was induced with the GMR GAL4 driver. The ommatidia and the adjacent bristles are regularly spaced in the adult eye of *p[GMR-GAL4]* flies raised at18°C (A). Activated neu^{NT} produces the severe eye phenotypes that include greatly reduced eye size and complete loss of ommatidial shape and the eye bristles (H). An irregular bristles arrangement along with noticeably flattened ommatidial discs were found in the neu^{YB} flies (D). A near wild type ommatidial shape and arrangement were observed in both neu^{YA} (C) and neu^{NYPD} (B) flies. However, in neu^{NYPD}, paired bristles were distributed throughout the whole eye surface (see also Fig. 3). On the other hand, a group of three bristles at a time was observed in the case of neu^{YA}. The eye phenotypes of neu^{YC} (E), neu^{YD} (F) and neu^{YE} (G) were also regarded severe. Transgenes are indicated at the bottom left of each panel.


A
GMR
B
NYPD
C
YA
D
YB
E
YC
F
YD
G
YE
H
NT

eye bristles (Fig. 6H). This allele also showed the strongest phenotype while mis-expressed in the adult wing (Fig.5H). Reduced eye along with flattened eye surface were also observed in the neu^{YC}, neu^{YD} and neu^{YE} (Fig. 6E, F and G). However, neu^{YC} had a dramatically reduced successful eclosion rate of 17%, while the others (neu^{YD} and neu^{YE}) had an average of 83% surviving eclosion. The eclosion rate was determined as the numbers of adult fly originate from the total number of pupae in F1 generation. neu^{YB} showed mild eye phenotype with irregular bristle arrangement and noticeably flattened ommatidial discs (Fig. 6D). Fewer but more irregular ommatidia than wild type were observed in neu^{YB} (Fig. 6B). Moreover, paired bristles were randomly placed throughout the whole neu^{YB} eyes (Fig. 7C and D). On the other hand, neu^{YA} had nearly wild type ommatidial shape and arrangement except several triplets of bristles were randomly distributed in the whole eye surface (Fig. 7F). This less severe eye phenotype of neu^{YA} relative to neu^{NYPD} was not unexpected as neu^{YA} showed an inhibitory role in a 'knock-in' mouse model (Chan et al., 2004). Perhaps because C96 driven wing mis-expression was insensitive to this much lower level of GMR driven *neu* expression in the eye, wing phenotypes both in neu^{YA} and neu^{NYPD} were hardly distinguishable (Fig. 5B and C). This result was not inconsistent, as it had been reported earlier that there were tissue-specific differences in the response to signals from individual phosphotyrosine (pTyr) (Settle et al., 2003).

FIGURE 7.

Bristle arrangement and ommatidial patterning were disrupted by expression of *neu*. The bristles and the facet of each ommatidia were regularly spaced and arranged in the eyes of *p[GMR-GAL4]* flies (A and B). neu^{NYPD} showed random distribution of a pair of bristles at different places of the eye surface (D). This allele also showed a relatively flattened ommatidial outer surface. The ommatidial outer surface was nearly wild type in the neu^{YA} flies (F). However, groups of three bristles were randomly distributed throughout the whole eye surface. Transgenes are indicated at the bottom left of each panel.

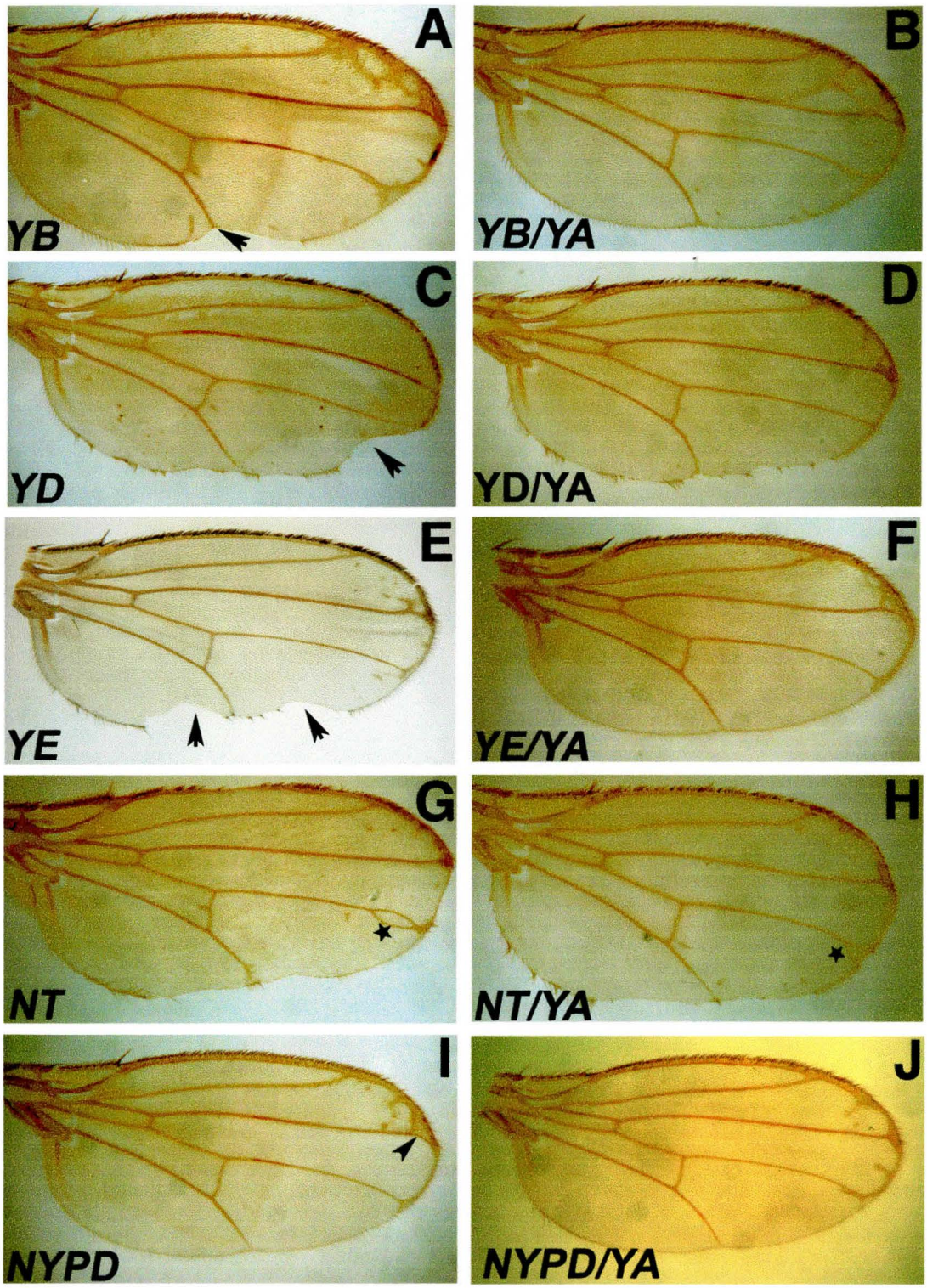

100X
300X
A
B
GMR
GMR
C
D
NYPD
NYPD
E
F
YA
YA

3.4 NEU^{YA} SUPPRESSED WING PHENOTYPES RENDERED BY THE OTHER ADD-BACK ALLELES.

Dankort et al. (1997) reported that the phosphorylation deficient allele, *neu*^{NYPD}, showed a weak transforming potential in mammalian cells while *neu*^{YA} completely lacked the potential. Consistent with Settle et al. (2003), our data showed that *neu*^{NYPD} had more irregular ommatidial patterns than those of *neu*^{YA}, while these are mis-expressed in the adult eye (Fig. 7). This result indicated that *neu*^{YA} might have an inhibitory role in RTK signaling. Therefore, we asked whether *neu*^{YA} could suppress the wing phenotypes induced by the other add-back alleles. While mis-expressed in the wing, *neu*^{YA} significantly rescued the wild type wing phenotypes in heterozygotes with *neu*^{YE} and *neu*^{YB} (Fig.8A and B; E and F; also see Appendix 9 and 11 for complete archives). Moderate wing notch phenotype suppression was observed in *neu*^{YD}/*neu*^{YA} heterozygote (Fig. 8C, D; also see Appendix10). The unique vein branching, observed in *neu*^{NT} (Fig. 8G), was noticeably suppressed in the trans-*neu*^{YA} allele (Fig. 8G and H). On the other hand, partial suppression of vein deltas were seen in *neu*^{NYPD} and *neu*^{YB} while trans allelic with *neu*^{YA} (Fig. 8A, B; I, J). Although there was a level of variation, a noticeable suppression was observed in every individual wing of the trans *neu*^{YA} alleles tested in this experiment (See Appendix 9-12 for complete archive). Here we did not expect the complete suppression of the vein deltas

FIGURE 8.

Neu^{YA} suppresses the wing phenotypes induced by other neu alleles. The left panels show the wings of representing Neu alleles while mis-expressed with the *p[C96] GAL4* driver. In right panel, all the wings used herein are heterozygote of the *neu^{YA}* and another allele (either *neu^{YB}*, *neu^{YD}*, *neu^{YE}*, *neu^{NT} and neu^{NYPD}*). *neu^{YA}* completely suppressed the wing notch phenotypes of *neu^{YE}* (compare E and F) and *neu^{YB}* (compare A and B). Moderate wing notch phenotype suppression was observed in the *neu^{YD}/neu^{YA}* heterozygotes (C and D). Partial suppression of the vein defects (branching) and vein deltas (compare G and H; I and J; A and B) was observed in the *neu^{NT}/neu^{YA}* and *neu^{NYPD}/neu^{YA}* heterozygotes. Transgenes are indicated in italics at the bottom left of each panel. An asterisk marks show the wing branching, while arrowheads and arrows indicate the wing deltas and wing margin loss respectively.


A
YB
B
YB/YA
C
YD
D
YD/YA
E
YE
F
YE/YA
G
NT
H
NT/YA
I
NYPD
J
NYPD/YA

as this phenotype was also rendered by the *neu^{YA}; C96* allele itself (see Fig. 5C and Appendix 3).

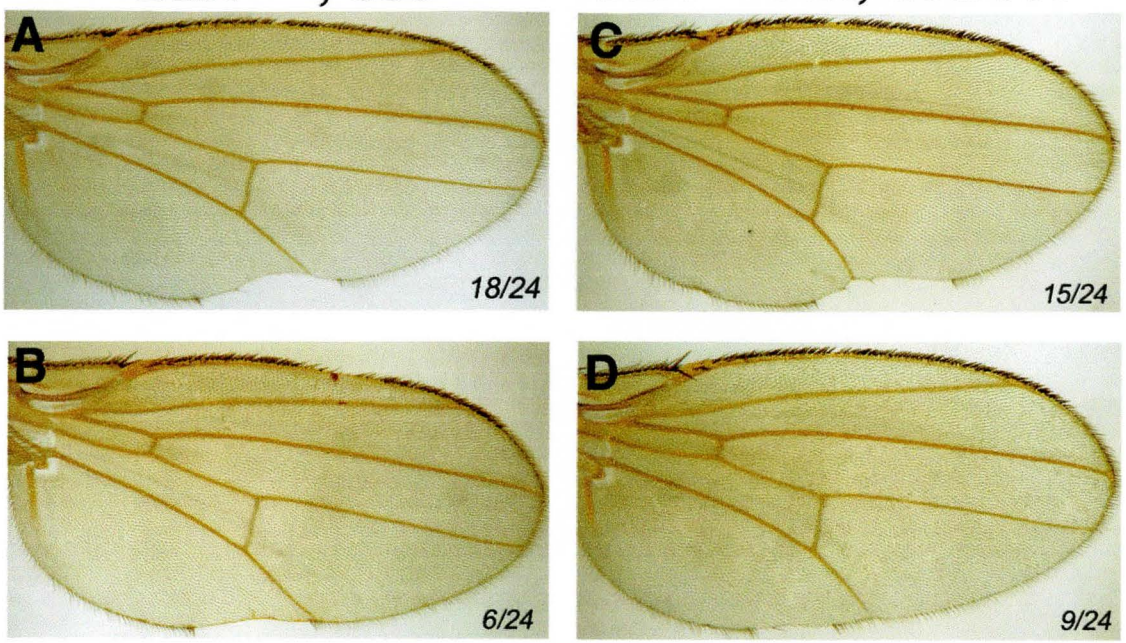
3.5 GAIN OF FUNCION ALLELES OF DER DO NOT GENETICALLY INTERACT WITH NEU^{YA}

Since the GAL4 expression system superimposes neu expression over the intrinsic DEgfr function, it is possible that in *Drosophila*, DEgfr transactivates *neu* through heterodimerization (Settle et al., 2003). Moreover, in knock-in mouse model, it had been shown that Egfr levels were modestly affected depending on the neu/Erbb2 level. In that experiment the authors also showed that *ErbB2 Y1028F* mutant resulted in increased protein level of Erbb2 expression suggesting RKT signaling inhibitory role by the *neu^{YA}* (Chan et al., 2004). Since DER is the only known vertebrate orthologue of ErbB family member in *Drosophila* and *neu^{YA}* has the inhibitory role in RTK signaling pathway, we hypothesize that *neu^{YA}* may suppress the DER phenotype if they (*neu^{YA}* and DEgfr) could heterodimerize.

In order to test this hypothesis we mis-expressed several *DER* alleles both in the wing and eye tissue. The four alleles include a wild type DER (*DER^{WT}*), two gain of function alleles such as *DER^{A887T}* and *DER^{Elp}* (Ellipse) and a dominant negative allele called *DER-DN* (Dominant negative). In case of wing mis-expression, only the gain of function allele of *DER^{A887T}* showed wing phenotype with wing margin loss (Fig. 9). This could be due to the cell

FIGURE 9.

Neu^{YA} did not suppress the wing phenotypes induced by the activated *Drosophila* EGF receptor. The mis-expression of DER, at the wing margin, was induced by *p[C96] GAL4*. The wings in the left panels are from the homozygote of *DER^{A887T}; C96* flies (Fig. 8 A, B). The wings in the right panels are from the heterozygote of *neu^{YA}* and *DER^{A887T}*(Fig. 8 C, D). Mis-expression of DER^{A887T} produced unique phenotype for almost every wing (also see Appendix 13). For each genotype, a total of 24 wings were scored and photographed. 18 out of 24 wings scored had wing margin loss in the *DER^{A887T}; C96* flies (Fig. 8A), while the remaining 6 wings had no significant wing margin loss (Fig. 8B). On the other hand, 15 out of 24 had the same level of margin loss in the *neu^{YA}* heterozygote (Fig. 8C) where as 9 out of 24 wings had no remarkable wing margin loss (Fig. 8D). The number at the bottom right of each panel indicates the particular phenotype scored from the whole data sample.


DER^A887T; C96
DER^A887T/YA; C96/C96
A
18/24
C
15/24
B
6/24
D
9/24

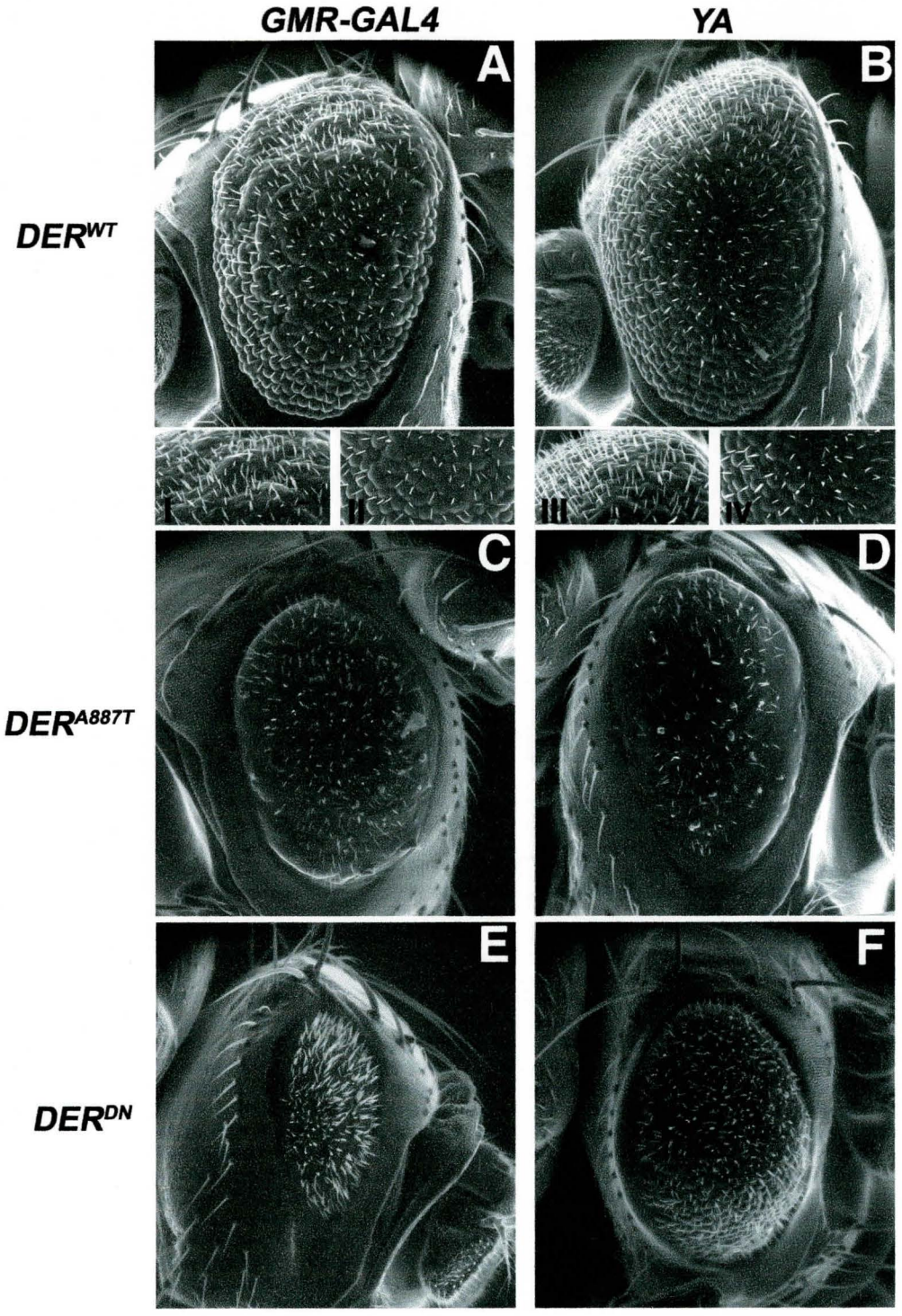
death during the growth of the wing disc (reviewed by Settle et al., 2003). However, the other three DER alleles had no visible wing phenotypes (data not shown). Moreover, while heterozygote with neu^{YA}, DER^{A887T} showed no remarkable wing phenotype suppression, suggesting that it does not heterodimerize with neu alleles, at least with neu^{YA} (Fig. 9). While coexpressed in the Midline Glia cells, in the *Drosophila* Central Nervous System (CNS), a kinase inactive allele of neu^{YD} ($neu^{YD,KD}$) did not suppress an increase in MG cell number caused by DER^{A887T} expression, indicating no interaction between this hypermorph allele and neu (Settle et al., 2003). If these two alleles heterodimerized, $neu^{YD,KD}$ would sequester DER^{A887T} and reduce the level of anti-apoptotic signals.

3.6 NEU^{YA} SELECTIVELY SUPPRESSED DER INDUCED PHENOTYPES.

As GMR driven mis-expression is more sensitive to much lower levels of Neu signaling, we also expressed all the DER alleles in the eye tissues (Fig. 10). The three *DER* alleles- DER^{WT}, DER^{A887T} and DER^{DN} resulted with significant eye phenotypes (Fig. 10). In case of DER^{WT}, a certain anterior portion of the eye was colourless with severely reduced ommatidial facets and bristles (Fig. 10 A and I). In contrast, even though irregularly fused, the coloured area had more ommatidia (II). A complete disruption of ommatidial

FIGURE 10.

Neu^{YA} suppresses the DER (DEgfr)- induced eye phenotypes. Expression of wild type *Drosophila* EGF receptor (*DER^{WT}*) in the eye with GMR GAL4 driver resulted in rough eye (A) with severely reduced ommatidial facet and bristles at the colourless (upper part of the eye) area (I) and irregularly fused facet in the rest of the eye (II). The rough eye phenotypes were greatly reduced in *p[uas-DER^{WT}]/p[GMR-GAL4];p[uas-neu^{YA}]/+* (B) adults. Comparatively reduced colourless region of the eye was seen with increased number of bristles and ommatidial facets (III) and the rest of the eye surface had almost regularly shaped facet (IV). The complete disruption of ommatidial facet structures with irregularly spaced bristles (C) was seen in the gain of function allele (*DER^{A887T}*) (Genotype: *p[uas-DER^{WT}]/p[GMR-GAL4]*). A similar phenotype, with even more reduced bristles numbers, was seen in the *p[uas-DER^{A887T}]/p[GMR-GAL4];p[uas-neu^{YA}]/+* adults (D). However, the dominant negative eye phenotype (E), due to a dominant negative allele of *DER (DER^{DN})*, was noticeably suppressed in the *p[uas-DER^{DN}]/p[GMR-GAL4];p[uas-neu^{YA}]/+* adults (F). The top left caption indicates the heterozygote of GMR-GAL4 and the particular allele, while top left caption indicates the heterozygote of *neu^{YA}* and the particular DER allele while expressed with *GMR-GAL4*.


GMR-GAL4
YA
DER^WT
DER^A887T
DER^DN
A
B
C
D
E
F
I
II
III
IV

facets with irregularly spaced and reduced in number of bristles were observed in the gain of function allele, DER^{A887T} (Fig. 10C). On the other hand, DER^{DN}, the dominant negative allele, had severely reduced eye size with densely packed bristles (Fig. 10E). While heterozygote with neu^{YA}, DER^{A887T} allele resulted with no phenotype suppression (Fig. 10D). This similar result was also seen in the wing misexpression (Fig. 9). However, the other two alleles showed remarkable eye phenotype suppression by the neu^{YA} trans-allele. A reduction of eye roughness with more bristles was observed in the DER^{WT} heterozygote with neu^{YA} resulting moderate suppression by this *neu* allele (Fig. 10B). DER^{DN}, the dominant negative allele, also resulted with partial rescue of eye size (Fig. 10E). Although expected, the DER^{Elp} allele did not show any rough eye phenotype (Data not shown).

3.7 NEU^{YE} TRANSGENE CAN BE GENETICALLY VERIFIED:

Settle et al. (2003) had already reported a number of second messengers (*ras, raf, dab, sos and PLCγ*) and adaptors (*shc, Grb-2, Nck and shp-2*) that participate in signaling of individual Neu pTyr. From that study, they also showed that neu^{YE} signals through Raf without employing the Ras pathway. Therefore, we have been interested in a large scale of genetic screen that might reveal the gene(s) responsible for neu^{YE} signaling in *Drosophila*. In their study, Settle et al. (2003) examined the wings from the *UAS-neu/+;*

C96/+ flies also heterozygous for the amorphic alleles of various second messengers and adaptor proteins. By scoring the suppression or enhancement of the phenotypes rendered by the amorphic alleles, they designated the function of a number of candidates molecules required for mammalian neu signaling in *Drosophila*. Therefore, before starting a large-scale genetic screening, we sought to genetically verify our newly generated neu^{YE} transgenic fly lines. In order to do that we examined wings from the *UAS-*neu^{YE}*; C96* flies also heterozygous for the amorphic alleles of *ras, shc, raf, dos, dab, drk and csw*. The results are summarized in the Table 3.1, where complete suppression of phenotype was scored as 1 and no interaction was scored as 5. Two replicates of each cross were done to examine at least 25 wings for putative interactions. The enhancement or suppression of wing phenotypes were numerically scored on the basis of the severity of wing morphology, vein formation, vein deltas and wing margin loss (See Materials and method and Appendix 1).

Wings examined from flies heterozygous for *raf* (phl^{C110}) showed complete suppression of wing phenotypes suggesting its (Raf) requirement for the neu^{YE} signaling. On the other hand, a reduction in *sos* function (a guanine nucleotide releasing factor for Ras) did not show any significant suppression indicating that neu^{YE} signals through Ras independent manner. As Ras is activated by Sos, this result also suggested that neu^{YE} signals without employing Ras. A reduction in Dab ($dab^{IM54-R1}$) showed a negligible

TABLE 3.1.

Neu^{YE} signaling requires Raf function. A number of amorphic adaptors and second messenger modifiers were screened against *neu^{YE}* for genetic verification of this allele. The results were summarized and scored numerically, with complete suppression of the wing phenotype as one and no interaction as five. The hypomorphic allele of *raf* (*phl^{C110}*) showed the complete suppression of the wing phenotype while little or no interaction was observed with the *ras* mutant. However, wings examined from flies heterozygous for *Dshc$^{111-40}$*, which encodes a SH2 binding adaptor protein, and Shp-2 (*csw^{LE120}*) resulted a noticeable suppression of wing phenotypes.

Second messenger modifiers of *neu*^{YE} wing phenotypes

	Sos	SHC	Raf	Gab-1	Dab	Grb-2	Shp-2
	sos	**Dshc**	*phl*	*dos*	*dab*	*drk*	*csw*
Mean	3.5	2.3	2	3.5	3.8	2.5	2.7
Median	4	2	1	3	4	2.5	2
Mode	4	2	1	2	4	N/A	2
No. of wings scored	28	41	27	20	11	-	23

suppression of *neu*^{YE} phenotype. These results were consistent with the data presented by Settle et al. (2003). However, wings examined from flies heterozygous for *Dshc*$^{111\text{-}40}$, which encodes a SH2 binding adaptor protein, and Shp-2 (*csw*^{LE120}) resulted a noticeable suppression of wing phenotypes. These results were inconsistent with the previous finding (Settle et al., 2003). Regardless of few exceptions with previous study, *neu*^{YE} showed identical interactions with second messengers and adaptor proteins.

3.8 LARGE SCALE GENETIC SCREENING:

Our genetic data confirmed (data from Settle et al., 2003) that *neu*^{YE} signals through Raf pathway without employing Ras. It is possible that *neu*^{YE} signals through a distinct or non-conventional Ras/Raf/MAPK signaling pathway. In order to identify this possible non-conventional pathway, the primary step would be identifying gene(s) responsible for *neu*^{YE} signaling. Therefore, we have undertaken a large-scale genetic screening project to find out the *neu*^{YE} signaling gene(s).

For our screening study, we have been following the chemical (Ethyl Methanesulfonate, EMS) mutagenesis scheme (Methods Book, 1990, 2nd edition, Rubin Lab, Berkeley, California). As EMS dosage is controlled by the concentration, we ran a pilot scheme and found that 25 mM of EMS resulted with 32% induced X-linked lethality (Table 3.2). Approximately, 30% of X-

linked lethality indicates one hit per autosome on average or one hit in 2000 to 5000 for most loci (Roberts, DB, 2003, Drosophila, 2nd edition, Oxford University press, pp56-57). The wild type males (Oregon R) treated with this 25 mM EMS were crossed with *neu*^{YE}*;C96* pair wisely. The F1 flies were observed to score the wing phenotype enhancement or suppression. A complete data summary from this ongoing study has been shown in Table 3.3. Although we expect to run a screening of at least 5000 chromosomal hits (5000 loci roughly), our first 307 chromosomal hits did not show any expected suppression or enhancement. The mutant that would render either suppression or enhancement to wing phenotype of *neu*^{YE}*;C96* flies, would reveal the gene required for *neu*^{YE} signaling in *Drosophila*. Once the desired phenotypic expression is found, the mutant would be mapped out accordingly (See Appendix. 16 for mapping protocol).

TABLE 3.2.

Determination of EMS induced X-linked lethality. EMS treated males were crossed en masse to FM7j/FM7j virgins. The F1 females (X^*/FM7j) were allowed to cross with their brothers (Y/FM7j) for 3/4 days. Then the females were individually placed in tubes and allowed to lay eggs for another few days and finally were discarded. The F2 sons must inherit the X-chromosome from their F1 mother (mutagenized and '*' marks possible mutation). Therefore, F2 sons would be either X^*/Y or Y/FM7j. The X-linked lethal flies would die and eventually be only Y/FM7j (Bar^- eye). The percentage of these lines will be the percentage of induced X-linked lethals.

TABLE:

Determination of X-linked lethality.

EMS Concentration (mM)	No. of lines of Bar^- eye males only (FM7j/Y) or no. of X-linked lethal lines	No. of lines of Bar^+ eye males (X^*/Y)	Total no. of lines tested in F2 generation	X-linked lethality (%)
25	18	39	57	31.57

TABLE 3.3:

The table shows the summary of genetic screening studies. EMS treated wild type males (Oregon R) were crossed individually with homozygous *neu*^{YE}*;C96* virgin. The flies from the F1 generation were observed and scored for either enhancement or suppression of the wing phenotypes. In our study the total 307 mutational hits showed neither enhancement nor suppression of the phenotype.

TABLE:

Summary table of genetic screening.

Batch no.	EMS treated Date	Observation done by	No. of EMS treated males crossed to *ney*^{YE}; *C96*	No. of lines with mutant phenotypes	Total lines observed (EMS treated males)	Gene(S) identified
1	9/28/04	10/21/04	88	None	307	None
2	9/29/04	10/23/04	78	None		
3	10/01/04	10/25/04	68	None		
4	10/05/04	11/01/04	73	None		

CHAPTER 4:

DISCUSSION

It has been well documented that the vertebrate family of Epidermal Growth Factor RTKs are mutated or/and overexpressed in many human cancers. In particular, ErbB2 is overexpressed in 20-30% of human breast cancers (reviewed in Hynes and Stern, 1994). The ErbB2 gene amplication correlates with aggressive tumour formation along with poor clinical prognosis in breast cancer patients (Margolis et al., 1999; Ingham, PW and Hidalgo, A., 1993). A part from these, mouse models well support the notion that ErbB2 signaling participates in oncogenesis (Guy et al., 1992; 1996; 1992). Therefore, the future identification and elucidation of the signaling pathways of RTKs is very important from medical point of view.

RTKs transmit their signals through the individual phosphotyrosine residues that reside in the C-terminal domain. The identification of pTyr outputs of RTKs are largely established by the data from peptide inhibition, phosphotyrosine labeling and protein co-immunoprecipitation experiments *in vitro* (reviewed by Pawson and Nash, 2000; Schlessinger, 2000). In contrast, *in vivo* experiments reveal more functional distinctions of different pTyr outputs (Lesa and Sternberg, 1997; Dankort et al., 2001) and genetic analysis is, therefore, an efficient means of identifying signaling pathways *in vivo*. *Drosophila*, a genetically more amenable organism, is a suitable model system for studying these RTK signaling pathways.

Actually, a number of studies have taken the advantage of well-characterized signal transduction pathways in *Drosophila* to screen for proteins that interact with vertebrate transgenes (Bhandari and Shashindra, 2001; Jackson et al., 2002; Kazantsev et al., 2002; Rubinsztein, 2002). Moreover, the structure and function of many SH2/PTB proteins in signaling is conserved in model organisms like *Caenorhabditis elegans* and *Drosophila*. For example, human adaptor protein GRB2 and *Drosophila* Drk can rescue *sem-5* function in *C. elegans* during vulval induction (Stern et al., 1993). And the PTB/SH2 binding properties of *Drosophila* Shc (Dshc) and mammalian Shc are highly conserved (Lai et at., 1995). Due to the conserved structural and functional nature of RTK signaling in metazoans, it is possible that the mammalian rat Neu (ErbB2) would successfully signal through the adaptor proteins in *Drosophila* (Settle et al., 2003). Therefore, a transgenic Neu fly model is a means to study this mammalian protein *in vivo* in such a genetically more amenable organism like *Drosophila melanogaster*.

4.1 NEU SIGNALING IN THE ADULT STRUCTURES:

In order to understand the Neu signaling in *Drosophila*, we mis-expressed *neu* alleles in both wing and eye tissues. This mis-expression generated a range of phenotypes both in wing and eye tissues. Both wing and eye mis-expressed phenotypes proved to be the most responsive to signaling from the activated neu^{NT}. neu^{NT} also resulted with the highest transforming

ability in Rat1 fibroblasts (Dankort et al., 1997). Our previous study found both neu^{YD} and neu^{NT} to be the most penetrant in both MG and wing tissues (Settle et al., 2003). In our current study, a similar severity of phenotypes was seen with neu^{YC}, neu^{YD} and neu^{YE} while mis-expressed both in the eye and wing tissues (Fig 5 and 6). A smaller eye with reduced ommatidia was characteristic in all of these three neu alleles. However, only 17% of pupae successfully eclosed while the eclosion rate was more than 80% in the other two neu alleles (neu^{YD} and neu^{YE}). But 100% of phenotypic penetration was seen in the successfully eclosed flies in both eye and wing mis-expression.

Unlike our previous study, neu^{YA} resulted with both wing and eye phenotypes. However, mis-expression in both eye and wing tissues showed that neu^{YA} to be less penetrant than that of neu^{NYPD}. Between these two *neu* alleles, a reduced penetrance, in the eye tissues, by neu^{YA} was also demonstrated by Settle et al. (2003). This reduced severity of phenotypes in neu^{YA} signaling may reflect the inhibitory feedback of YA upon the NYPD signal as also discussed by Settle et al. (2003). Neu^{YA} also lacked transforming potential in the mammalian cell culture, suggesting an inhibitory role in RTK signaling (Dankort et al., 1997; Dankort et al., 2001).

Although neu^{NYPD} lacks any phosphorylation site, it showed both wing and eye phenotypes (Fig. 5, 6 and 7) and retained transforming potentials in mammals (Dankort et al., 2001). It is possible that neu^{NYPD} signals through other ErbB receptors by forming a dimer with them. If this is true, it is

possible that *neu*^{NYPD} may activate additional signaling pathways. Further studies and screening may reveal these messenger molecules.

4.2 NEU^{YA} RENDERS INHIBITORY ROLE IN RTK SIGNALING:

Dankort et al. (1997) reported that the phosphorylation deficient allele, *neu*^{NYPD}, showed a weak transforming potential in mammalian cells while *neu*^{YA} completely lacked the potential. Consistent with Settle et al. (2003), our data showed that *neu*^{NYPD} had more irregular ommatidial patterns than those of *neu*^{YA}, while mis-expressed in the adult eye (Fig. 8). Moreover, *neu*^{NYPD} mis-expression in the wings resulted in a thicker mass of wing vein deltas than that of *neu*^{YA}. The reduced level of phenotypes in *neu*^{YA} signaling may reflect the inhibitory feedback of YA upon the NYPD signal. Using mouse as a model, Chan et al. (2004) found that a single amino acid substitution at the 1028 site (ErbB2-Y1028F) of neu/ErbB2 allele resulted in increased ErbB2 protein expression level when compared to the level observed in knock-in embryos expressing the ErbB2 cDNA, the Y1144F cDNA or the Y1227F cDNA alleles. This elevated ErbB2 protein expression level by Y1028F mutant suggests that this amino acid residue (Y1028) may have inhibitory role of RTK signaling. Therefore, a suppression of phenotypes by *neu*^{YA} while heterozygous with other neu alleles would confer this inhibitory role by *neu*^{YA}.

While mis-expressed in the wing, *neu*^{YA} significantly suppressed the wing phenotypes when heterozygous (in *trans*) with *neu*^{YE} and *neu*^{YB} as evidenced by complete suppression of wing notch phenotypes (Fig. 9A and B; E and F). Dankort et al., (1997) showed that addition of site A in *cis* to the NT-YB mutant (NT-YAB) virtually abolished the transforming activity in Rat1 fibroblasts. However, they also found that NT-YAC and NT-YAE less severely impaired transforming activity. Here we couldn't verify the suppression of wing phenotypes of *neu*^{YC} while heterozygote with *neu*^{YA} as we didn't have any line with *neu*^{YC} inserted in the 2nd chromosome. In order to overcome the genetic crossing problem due to particular transgenic neu inserts in specific chromosome, we have already generated the neu double add-back alleles such as *neu*^{YAB}, *neu*^{YAC}, *neu*^{YAD} and *neu*^{YAE} and these are ready for germ-line transformation. This double add-back (A site in *cis*) allele will also allow us to the study the suppression of *neu* phenotypes in homozygotes, where the number of genes is doubled over that of a heterozygote.

In heterozygotes of *neu*^{YA} and the other *neu* allele, complete wing notch phenotype suppression was seen in some cases. However, the complete suppression of wing vein delta phenotypes was not seen in any of the heterozygote. This result was not unexpected as *neu*^{YA}*;C96* renders these vein delta phenotypes by itself. This trans-allelic neu suppression was only studied in wing tissues (*neu*^{YA}*/other neu*). However, as GMR driven mis-expression is more sensitive to much lower levels of Neu signaling, it would be better to

study the *neu*^{YA} suppression in the eye tissues as well. But due to the time constraint, it was quite impossible to study the trans allelic suppression of *neu*^{YA} as most of the neu alleles and p[GMR-GAL4] driver are inserted into the 2nd chromosome and flies raised at 18°C take much longer time than that of 25°C. We, however, studied the *neu*^{YA} suppression of several DER alleles (Fig. 10).

In a mouse model, it had been shown that *ErbB2 Y1028F* mutant resulted with increased level of ErbB2 protein expression (Chan et al., 2004). This study also found that among all other ErbB family members (Egfr, ErbB3 and ErbB4), only Egfr levels were modestly affected depending on the level of ErbB2. Since DER is the only known ErbB family member in *Drosophila*, we hypothesized that DER expression level might be affected by the rat-Neu/ErbB2. Moreover, if it had an inhibitory role in RTK signaling, *neu*^{YA} would reduce the DER protein expression level in *Drosophila* and eventually suppress the phenotypes while mis-expressed in heterozygous (*DER/neu*^{YA}). Therefore, the genetic suppression of DER phenotypes by *neu*^{YA} would confer its (YA) inhibitory role in RTK signaling.

In the developing eye, DER (DEgfr) activity could both promote photoreceptor (R1-R7) differentiation and suppress ommatidium formation depending on the level of activation (Lesokhin et al., 1999). Depending on the alleles, DER mis-expression resulted with an eye phenotypes ranging from rough eye surface to severely reduced eye size. A moderate suppression of

rough eye phenotypes in DER^{WT} and a partial rescue of wild type eye size in DER^{DN} flies while heterozygous with neu^{YA}, indicated an inhibitory role of YA in RTK signaling.

In order to better understand the Neu signaling pathway further exploration of the inhibitory role of neu^{YA} and identification of the gene(s) required for its (YA) signaling would be of immense importance. A mutation of the gene that positively regulates neu^{YA} signaling would enhance the wing phenotypes in the mis-expressed (neu^{YA}; *C96*) adult wings. On the other hand a mutation on the gene that has negative or inhibitory role in neu^{YA} signaling would suppress the wing phenotypes in the mis-expressed flies. Therefore, a genetic screen to find either the suppression or enhancement to wing phenotype of neu^{YA};*C96* flies, would reveal the gene required for neu^{YA} signaling in *Drosophila*. Our long-term goal is to identify such genes.

In our experiments, all *UAS-DER* alleles, except the gain of function mutant DER^{Elp}, showed eye phenotype while mis-expressed with GMR-GAL4 driver. DER^{Elp} was expected to show rough eye phenotype (Lesokhin et al., 1999). This unexpected result might be due to our pretty old DER^{Elp} stock that might have spontaneous mutation over time.

4.3 NON-GENETIC INTERACTION OF HYPERMORPHIC DER ALLELE, DER^{A887T} AND NEU^{YA} :

The GAL4 expression system superimposes *neu* expression over the intrinsic DEgfr function. It is possible that in *Drosophila*, DEgfr (DER) trans-activates *neu* through heterodimerization as also suggested by Settle et al. (2003). Since neu^{YA} is believed to have an inhibitory role in RTK signaling pathway, the heterodimerization of *DER* and neu^{YA} would result in reduced level of phenotypes in DER/neu^{YA} heterozygote than that of DER alone. While mis-expressed, neu^{YA} moderately suppressed rough eye phenotype and partially rescued eye sizes in DER^{WT} and DER^{DN} respectively. However, while mis-expressed in the eye tissues, neu^{YA} did not suppress the rough eye phenotypes in DER^{A887T} when both are heterozygous (eg. $Uas\text{-}neu^{YA}/DER^{A887T}$). A similar non-suppression result was found in heterozygote of neu^{YA} and DER^{A887T} while mis-expressed in the wings (Fig 10). A genetic non-interaction of neu^{YA} with the hypermorph DER^{A887T} allele does not support the evidence for heterodimerize capabilities of these two alleles.

This hypermorphic DER allele (DER^{A887T}) is characterized with a single substitution of Thr for Ala887 in comparison with wild type DER. This A887T mutation showed increased ligand-independent autophosphorylation and MAP kinase activation in *Drosophila* cells (Lesokhin et al., 1999). This mutation might result in increased homodimerization and eventually increased activity.

On the other hand studies of the transmembrane region of Neu receptors have revealed a conserved, site-specific Val, Glu, and Gly tripeptide

(VEG) domain, which is responsible for transformation and signal transduction of the wild type Neu Receptor (Burke et al., 1997). Loss or mis-localization of this domain greatly reduces the tendency for these receptors to dimerize. Since the transmembrane regions in both alleles (*Neu and* DER^{A887T}) play an important role in dimerization it is not surprising that A887T mutation allows DER^{A887T} allele not to dimerize with *neu* allele, at least with neu^{YA}.

4.4 GENETIC VERIFICATION OF NEU ALLELES:

The identification of genetic enhancers or suppressors of a phenotype of a dominant allele by screening would dissect the complexity and intricacy of signal transduction pathway (reviewed in Settle et al., 2003). A study, using wing and eye in a productive screen, first identified numerous second messengers, such as *sos, ksr* and *tws* (Rogge et al., 1991; Simon et al., 1991; Sturtevant and Bier, 1995; Maixner et al., 1998 and Therrien et al., 1995). It has also been demonstrated that dominant phenotypes generated by a mammalian oncogene expressed in *Drosophila* can be used in a modifier screen to reveal novel genes in a well-characterized signal transduction pathway, such as the conventional Ras/Raf/MAP kinase pathway (Bhandari and Shashidra, 2001). In our previous study it was demonstrated that dosage sensitive modifier genetics in *Drosophila* could be used to dissect signal transduction pathways activated by mammalian oncogenic RTK (Settle et. al.,

2003). Using dosage sensitive modifier genetics, they reported a number of second messengers (*ras, raf, dab, sos and PLCγ*) and adaptors (*shc, Grb-2, Nck and shp-2*) that participate in signaling of individual Neu pTyr. They found that neu^{YE} was the only pTyr insensitive to reduced *ras* function, however YE signals did respond to reduced *raf* levels. This result suggested that neu^{YE} signals through Raf without employing the Ras pathway. Their study also found that a reduction in *sos* function did not show any significant wing phenotype suppression while heterozygous with neu^{YE}. Sos is a guanine nucleotide releasing factor that activates Ras. The insensitivity of neu^{YE} to reduced *sos* function indicated a Ras independent pathway for YE signaling. Therefore, on the basis of sensitivity to reduced levels of *ras*, *sos* and *raf* function, neu^{YE} can be genetically verified repeatedly. In consistent to the previous study, we found that neu^{YE} was insensitive to reduced *sos* while sensitive to *raf*. Here we did not include the data for the *ras* function as we tested less than twenty wings. Unlike our previous study (Settle et al., 2003), here we found that Shc ($Dshc^{111\text{-}40}$), encoding a SH2 binding adaptor protein, and Shp-2 (csw^{LE120}) could bind to and signal from Neu^{YE}. This is consistent with the peptide binding studies that showed that Shc could bind to and signal from pTyr YC and YE (Lai et al., 1995).

In summary, our genetic verification study, neu^{YE} showed identical interactions with second messengers and adaptor proteins with a few exceptions with the previous data (Settle el al., 2003). In our study, although

we scored the enhancement or suppression of wing phenotypes in a 1 to 9 scale, every wing was unique in the fine detail of its phenotypic expression. In order to determine the variability of genetic interactions, we scored ample number of wings from each genotype. Moreover, we scored the wing suppression or enhancement with a double blind protocol (see materials and methods).

4.5 LARGE-SCALE GENETIC SCREENING:

As our previous study demonstrate that Neu^{YE} signals through Raf with a Ras independent manner, it is possible that neu^{YE} signals through a distinct or non-conventional Ras/Raf/MAPK signaling pathway. To find this possible non-conventional pathway, the primary step would be identifying gene(s) responsible for neu^{YE} signaling. Therefore, we have undertaken a large-scale genetic screening project to find out mutant(s) that modify a phenotype of neu^{YE}. Here we have chosen Ethyl Methanesulfonate (EMS), the most commonly used chemical mutagen in *Drosophila.*

EMS is a mono-functional alkylating agent, which produces point mutations by attacking (ethylation) the *O*-6 position of guanine and the *O*-4 position of thymine. This mutation allows mispairing with thymine and guanine resulting in G:C to A:T and T:A to C:G transitions respectively. EMS also produces small deletions and occasionally other rearrangements as well. This mutagen works as a concentration dependent manner rather than dose

dependency. The higher the EMS concentration the higher the mutation rate in chromosome. A higher mutation rate in the X-chromosome would obviously result with higher X-linked lethality. It is established that approximately, 30% of X-linked lethality indicates one hit per autosome on average or one hit in 2000 to 5000 for most loci (Methods Book, 1990, 2nd edition, Rubin lab, Berkeley, California). In order to achieve this level of X-linked lethality, we standardized EMS concentration for our wild type OregonR flies. Here we determined that 25 mM of EMS resulted with 32% induced X-linked lethality in Oregon R flies at the available lab environment. In our ongoing genetic screening, we have so far screened a total of 307 EMS treated chromosomes or approximately 3000 F1 flies. Here we screened the F1 flies from the crossings of EMS treated male and homozygous neu^{YE};*C96* virgins for either enhancement or suppression of wing phenotypes as compared to neu^{YE};*C96*.

Although we screened approximately 3000 F1 flies, we did not observe any suppression or enhancement in the wing phenotypes. In a genetic screening with EMS no one can definitely assign any number of flies to be screened before finding a mutant. This is because any mutation in the non-interacting gene of the particular signaling pathway would not result in any suppression or enhancement. *Drosophila* genome harbours approximately 15000 genes. Therefore, to get a few desired mutants one has should screen flies of tens of times, if not hundreds, of the total gene number in *Drosophila*.

We expect to screen at least a total of 5000 chromosomal loci that is roughly one third of the whole *Drosophila* genome. Once a mutant is identified we will employ the mapping protocol as described in the Appendix 14 and 15. During our study, we also found that neu^{YE};*C96* flies showed a tendency to have wing phenotypic suppression by itself in the course of time. This phenomenon could be due to the spontaneous point mutation in this particular fly stock. Therefore, in order to overcome this problem we have been generating homozygous neu^{YE};*C96* flies in every three months.

Finally, as the X chromosome is approximately half the size of the major autosomes, theoretically 32% X-linked lethality would result in 64% of each of the major autosomes carrying a recessive lethal mutation following treatment with EMS (Roberts, DB, 2003, Drosophila, 2nd edition, Oxford University press, pp 56-57). This high frequency of induced mutations would result in more than one mutation in many chromosomes. If this happens in our study, we will consider removing this second site mutation by lowering EMS concentration.

4.6 FUTURE RESEARCH:

It would be worthwhile to screen amorphic alleles of signal transduction genes and transcription factors to identify additional genes implicated in Neu signaling. This might include Elk-1 transcription factor, members of the JAK/STAT pathway, Ets transcription factors and the

Drosophila orthologues of the Wnt pathway members. In addition, the *Drosophila* orthologue of vertebrate ECM proteins such as Erbin, which are involved in the basolateral localization of the Neu receptor in the vertebrate system deserves attention. Finally, Cdk 1, the kinase involved in regulating apoptosis is another potential candidate to explore.

In a recent study with cultured COS-7 cell lines and the transgenic Drosophila, it has been demonstrated that Torso RTK activates the Extracellular signal Regulated kinase (Erk) signaling through a small G protein, Rap1 in a Ras independent pathway (Mirsha et al., 2005). neu^{YE} has been demonstrated to signals through Raf without employing Ras (settle et al., 2003). Therefore, it would be interesting to find out whether neu^{YE} signal also requires *Drosophila* Rap (D-Rap).

Further demonstration of an inhibitory role in RTK signaling would validate our recent findings of the role of Neu^{YA}. The double add-back Neu alleles would allow us to study them in homozygous in various tissues such as wings, eyes and MG cells. Moreover, our proposed genetic screen to identify mutant that confer a phenotype to Neu^{YA} would reveal the gene(s) required for the repression of RTK signaling. Finally, our ongoing neu^{YE} screening study may reveal the candidate genes that allow neu^{YE} to signal in a Ras independent pathway. Hopefully, the null alleles to be identified by the mutagenesis study would allow us to demonstrate the in *vivo* functions of these putative signaling proteins.

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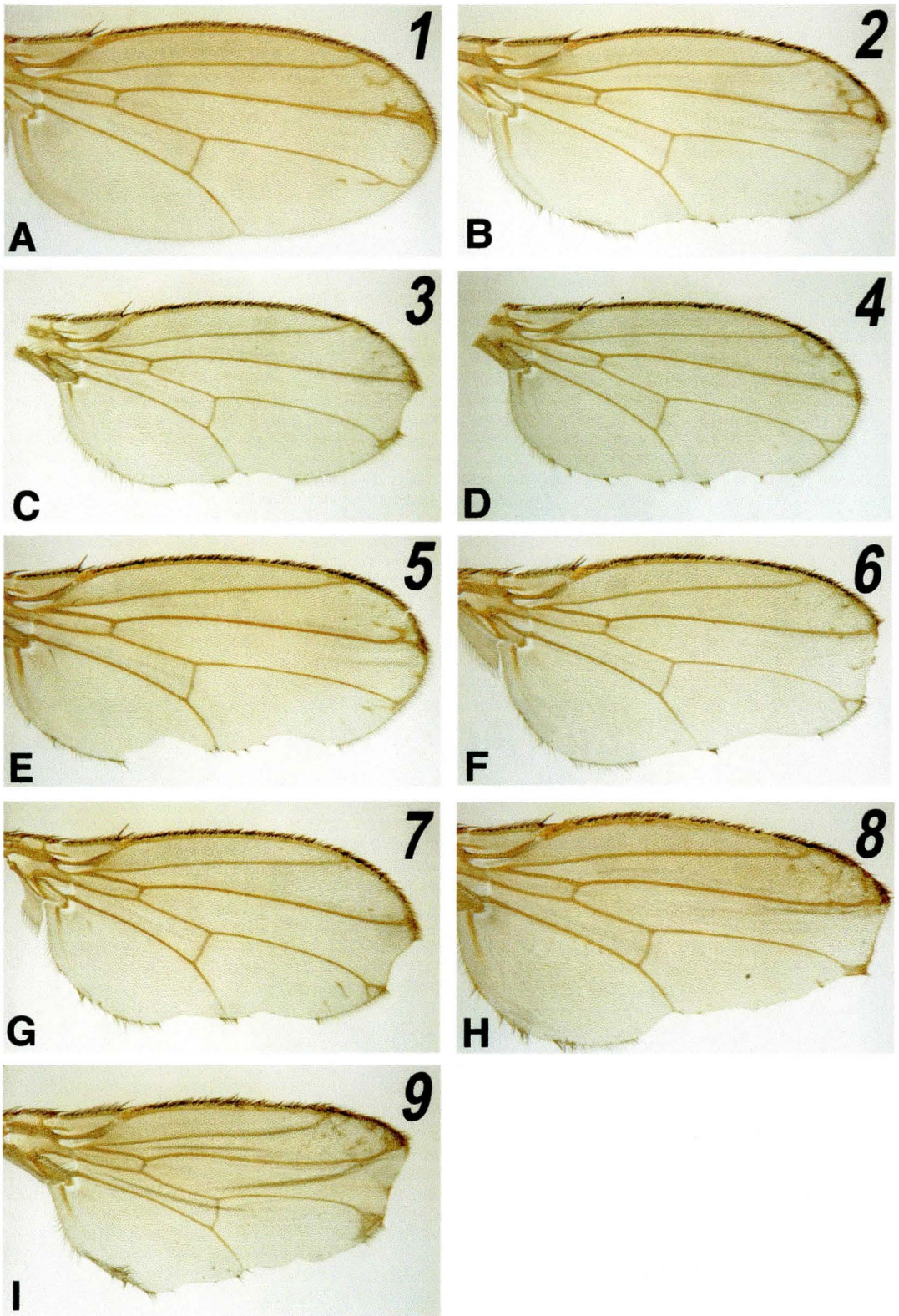
APPENDIX 1.

Range of wing phenotypes and the assigned numerical values to score the enhancement or suppression during genetic interactions with adaptor and second messenger molecules. All genetic interactions herein were assigned numerical values on the basis of the suppression or enhancement of the wing phenotypes. The ectopic wing phenotypes were induced using C96 GAL4 and all wings used here are heterozygote of the neu^{YE} allele and the particular gene deficiency. A complete suppression was assigned with numerical value of one (A). These wings, however, showed one or a few small wing deltas at the tip of one or two horizontal veins. A little loss of wing margin was scored two (B). Loss of wing margins at two different places was scored three (C), while multiple loss of wing region combined with wing deltas was scored as four (D). A typical wing phenotype of neu^{YD}, while mis-expressed with C96 GAL4 alone, was assigned with numerical value of five, indicating no interaction (E). Here the phenotypes include several wing margin loss, vein deltas and in some cases ectopic veins. Further enhancement was scored as six (F), seven (G), eight (H) and the highest assigned was nine (I).

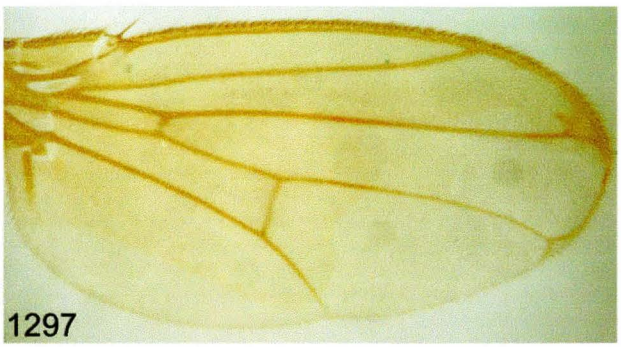

1
A
2
B
3
C
4
D
5
E
6
F
7
G
8
H
9
I

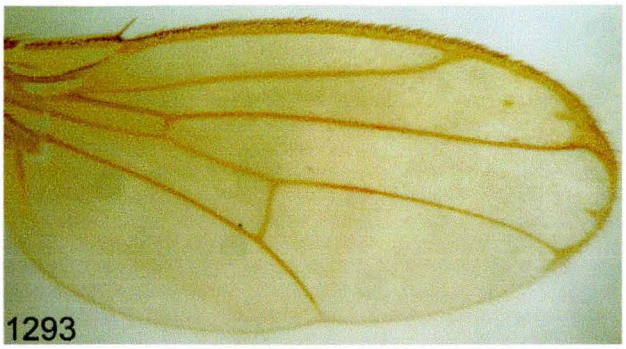
APPENDIX 2:

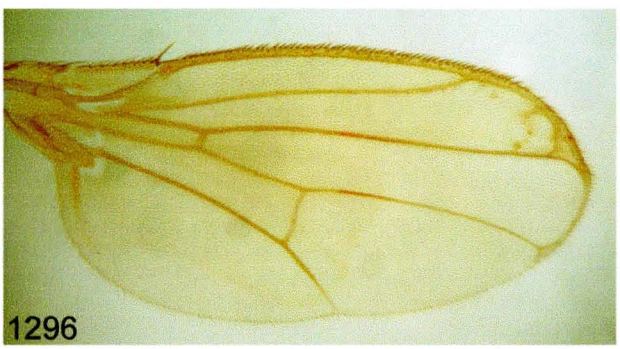
Mis-expression of *neu*^{NYPD} allele produces distinct wing phenotypes.

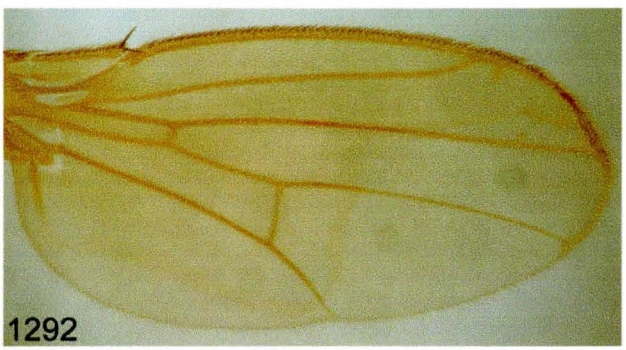
neu^{NYPD} was mis-expressed at the wing margin with *p[C96] GAL4*. *neu*^{NYPD} showed a nearly wild type wing margin along with a very small vein delta. Although there was 100% penetrance of wing phenotype, each wing represents unique amount and pattern of wing phenotype. The bottom left number on each panel indicates the JPEG archive number.

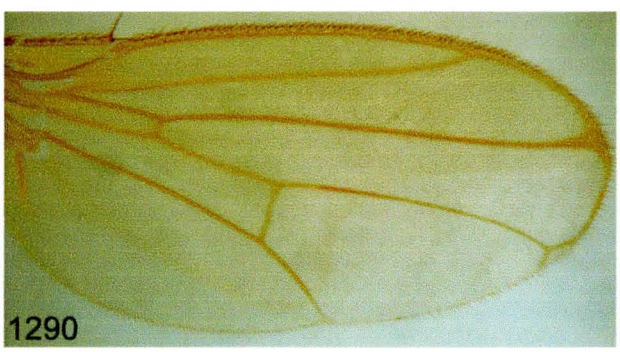
NYPD; +/+; C96

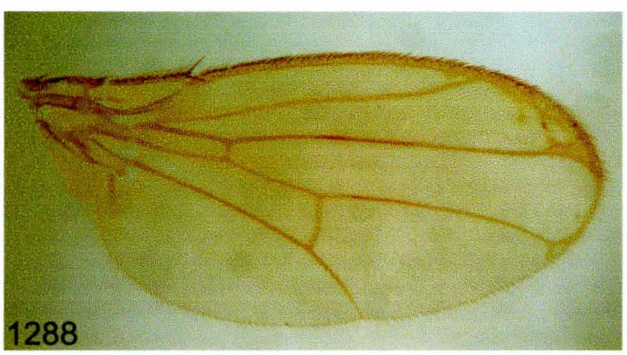








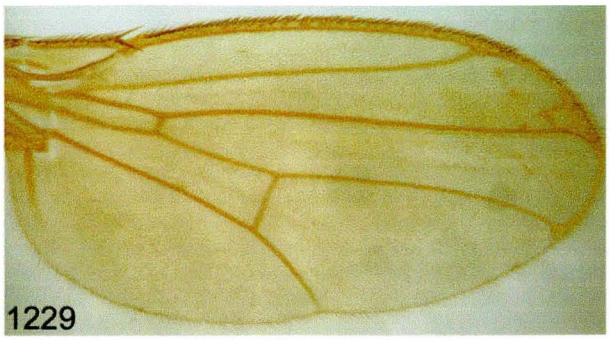


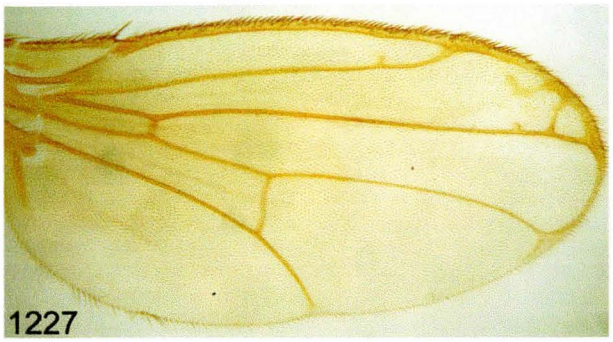


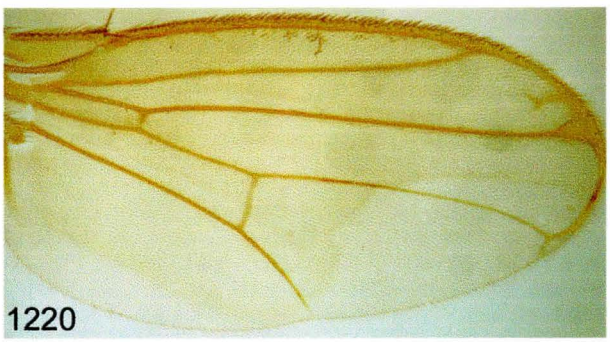
APPENDIX 3:

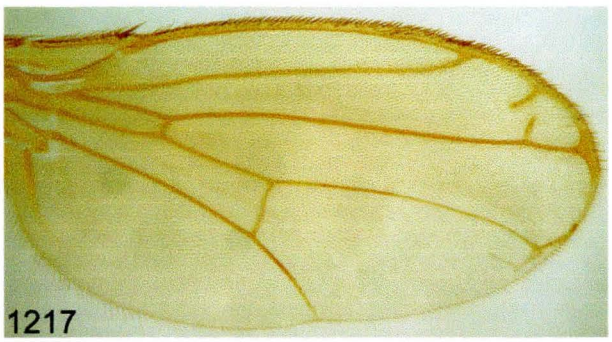
Mis-expression of *neu*^{YA} alleles produces distinct wing phenotypes. Neu alleles were mis-expressed at the wing margin with *p[C96] GAL4*. *neu*^{YA} showed ectopic vein formation in the anterior half of the wing blade. The bottom left number on each panel indicates the JPEG archive number.

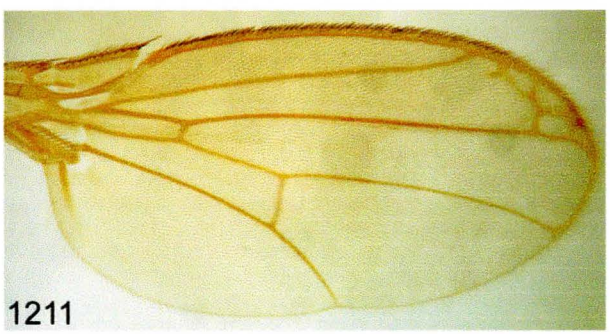
YA;C96

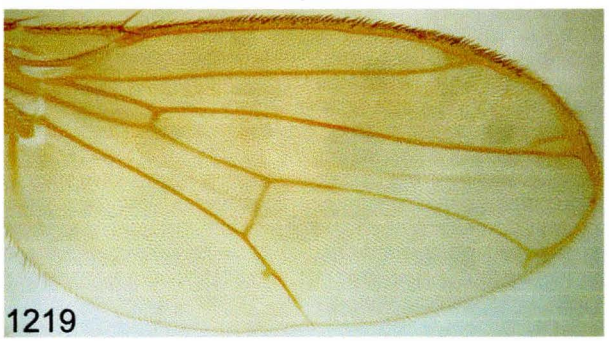


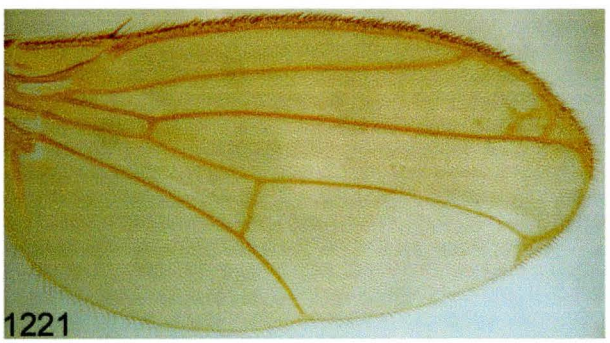


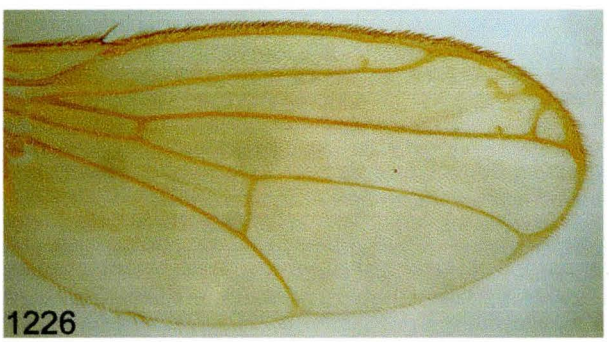








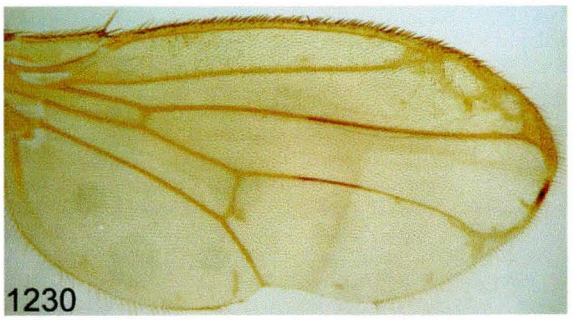


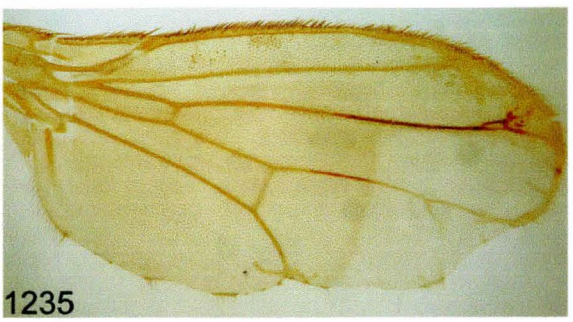


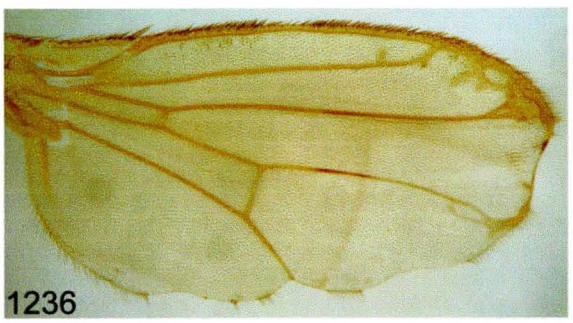
APPENDIX 4:

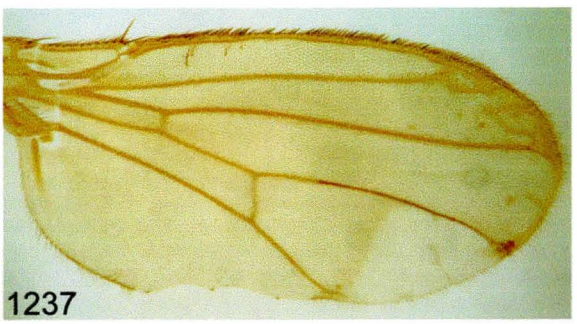
Mis-expression of *neu*^{YB} allele produces distinct wing phenotypes. *neu*^{YB} allele was mis-expressed at the wing margin with *p[C96] GAL4*. The bottom left number on each panel indicates the JPEG archive number.

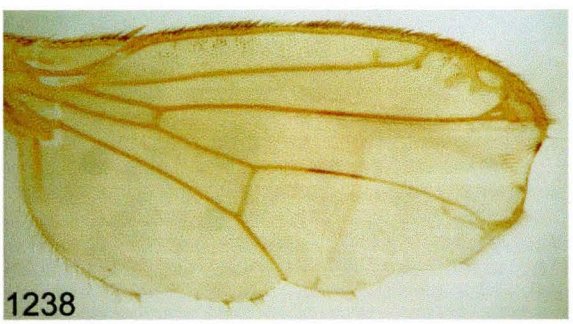
YB; C96

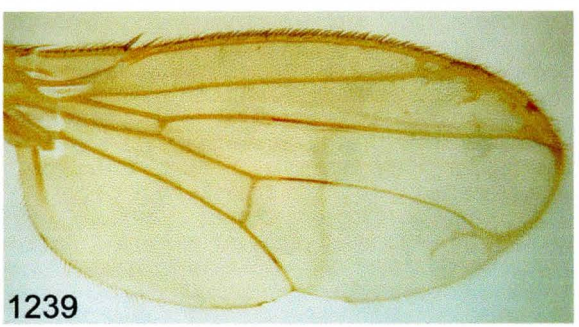


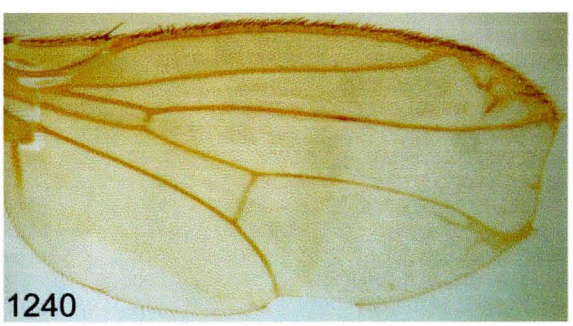


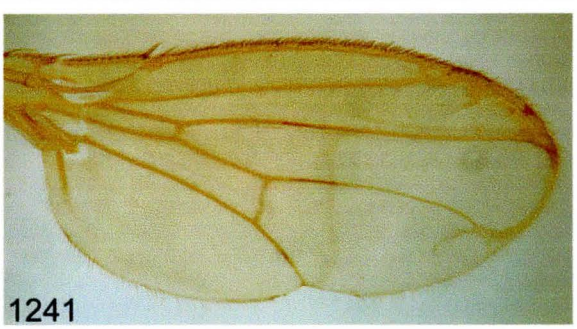


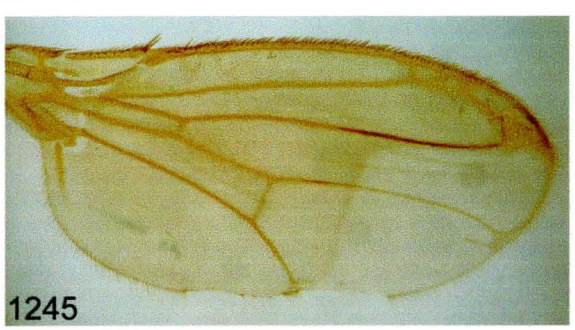


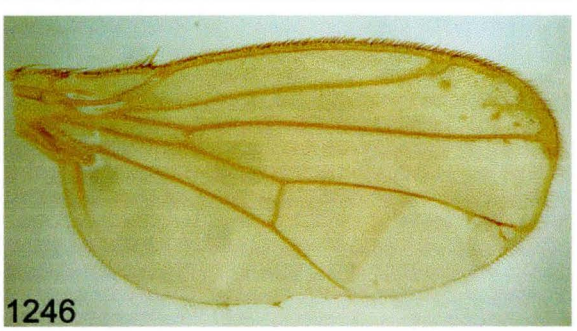








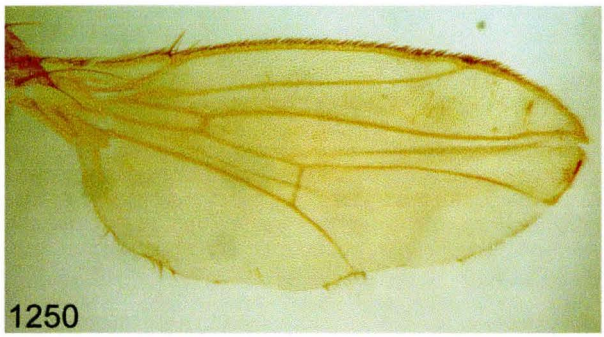


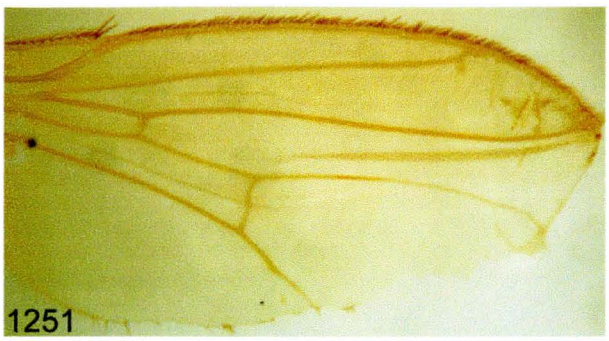


APPENDIX 5:

Mis-expression of *neu*^{YC} allele produces distinct wing phenotypes. *neu*^{YC} allele was mis-expressed at the wing margin with *p[C96] GAL4*. The bottom left number on each panel indicates the JPEG archive number.

YC/C96

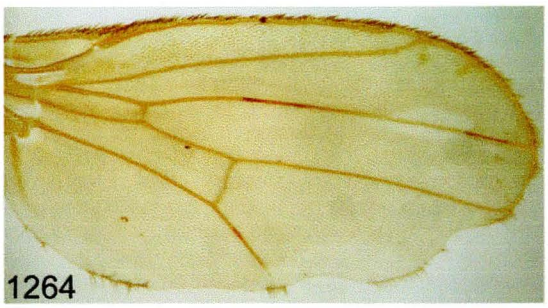


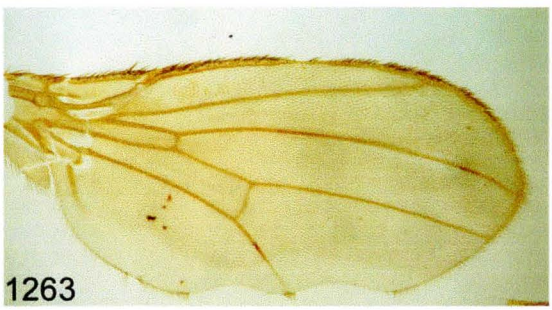


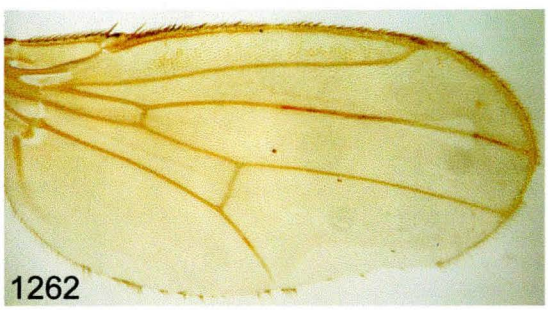
APPENDIX 6:

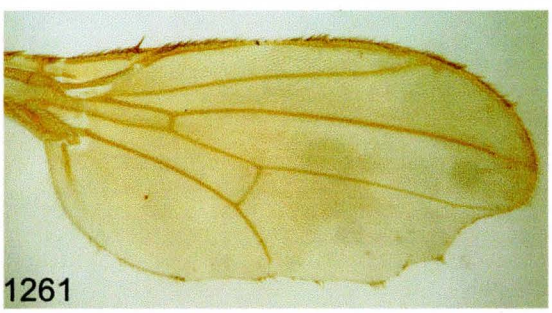
Mis-expression of *neu*^{YD} allele produces distinct wing phenotypes. *neu*^{YD} allele was mis-expressed at the wing margin with *p[C96] GAL4*. The bottom left number on each panel indicates the JPEG archive number.

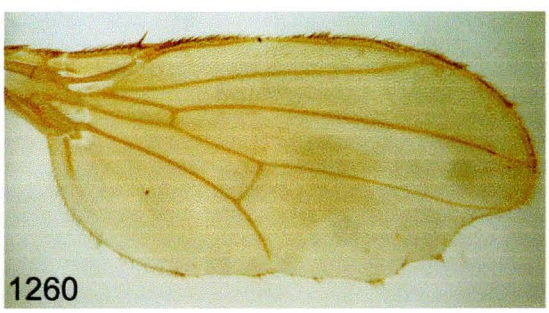
YD; C96

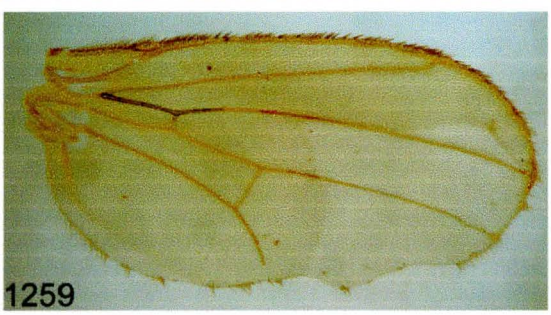


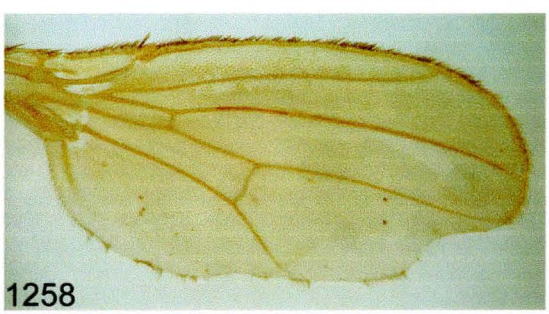








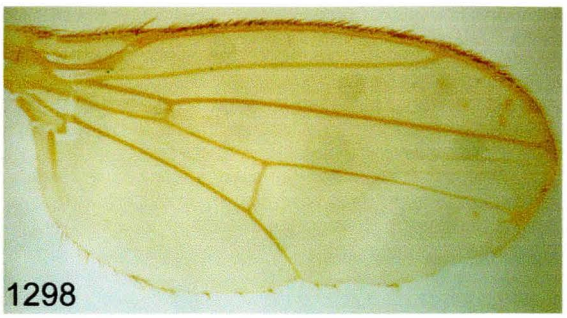


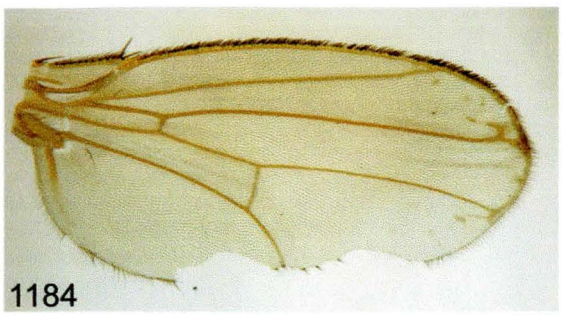


APPENDIX 7:

Mis-expression of *neu*^{YE} allele produces distinct wing phenotypes. *neu*^{YE} allele was mis-expressed at the wing margin with *p[C96] GAL4*. The bottom left number on each panel indicates the JPEG archive number.

YE; C96

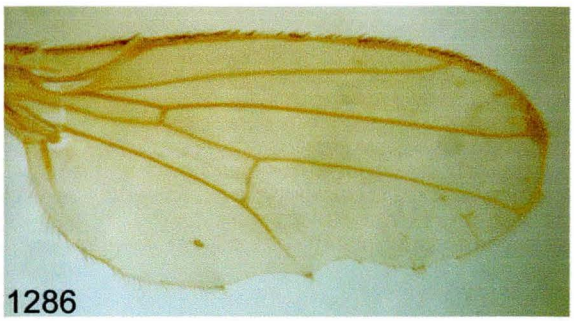


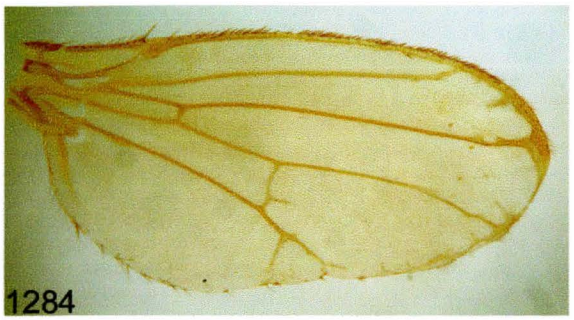


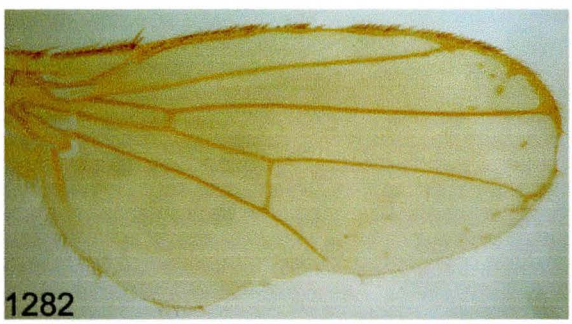
APPENDIX 8:

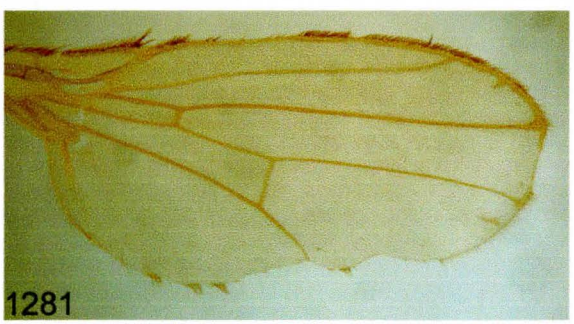
Mis-expression of *neu*^{NT} allele produces distinct wing phenotypes. *Neu*^{NT} allele was mis-expressed at the wing margin with *p[C96] GAL4*. The bottom left number on each panel indicates the JPEG archive number.

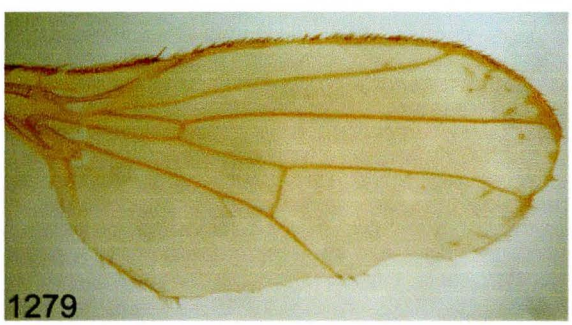
NT; C96

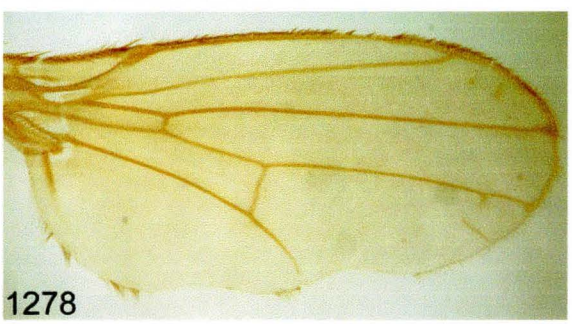


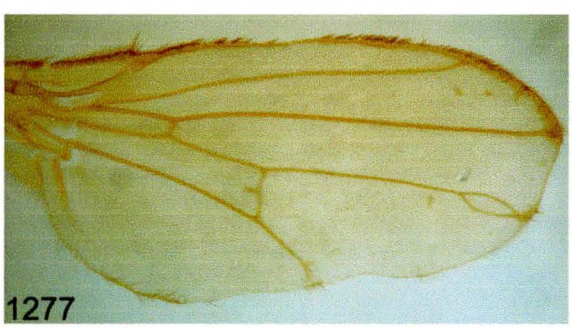


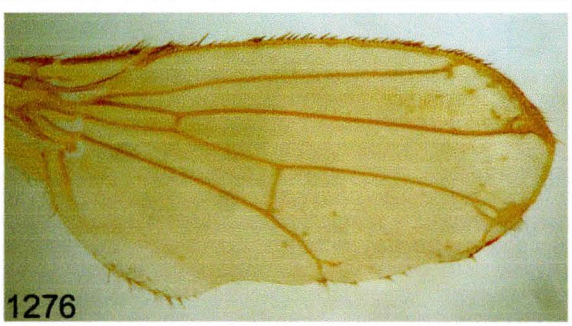


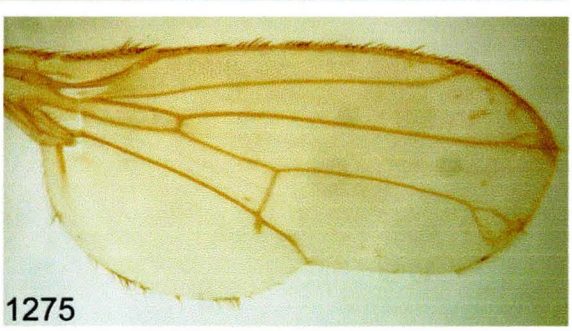


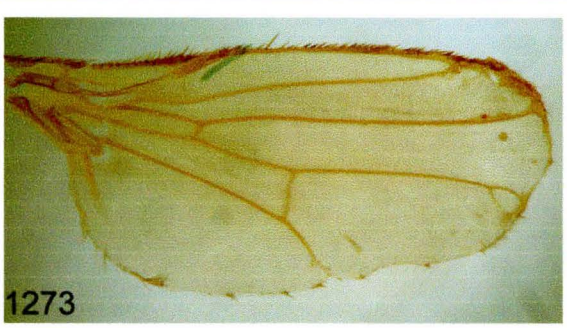








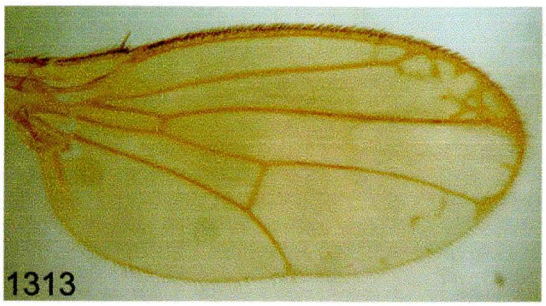


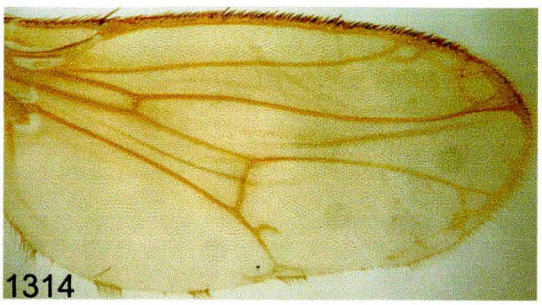


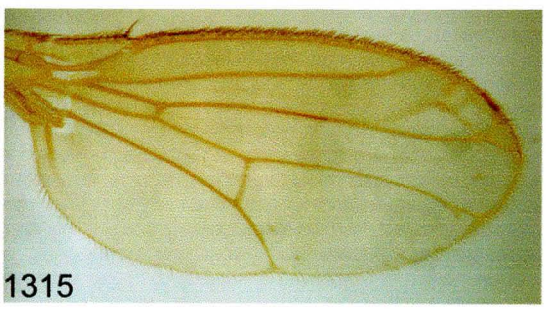
APPENDIX 9:

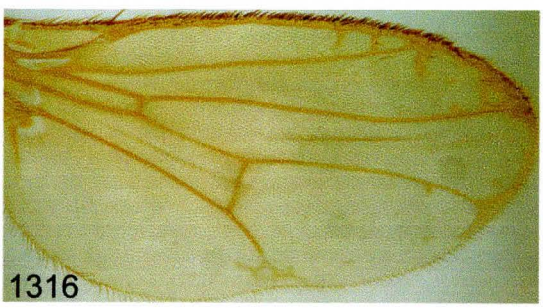
Neu^{YA} suppresses the wing phenotypes induced by *neu*^{YB} allele. The left panels show the wings of representing *neu*^{YB} allele while mis-expressed with the *p[C96] GAL4* driver. In right panel, all the wings used herein are heterozygote of the *neu*^{YA}. The bottom left number on each panel indicates the JPEG archive number.

YB/YA; C96/C96





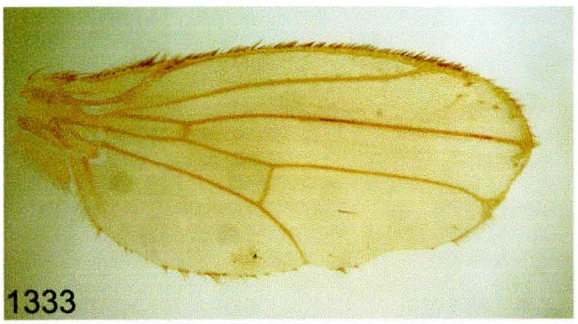


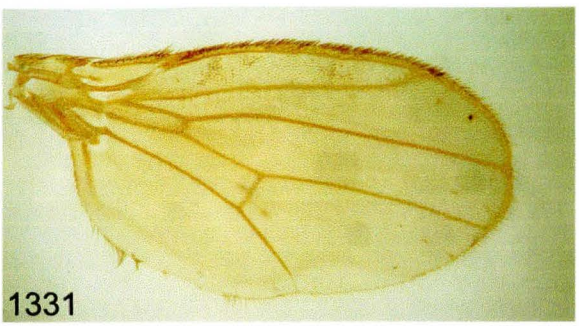


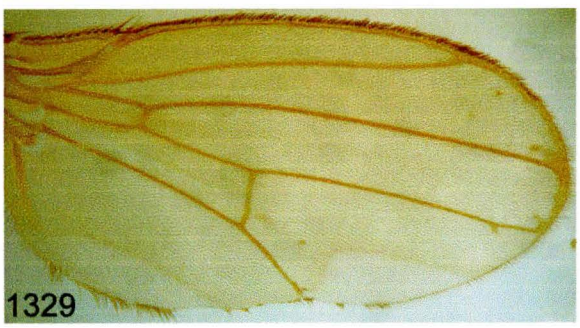
APPENDIX 10:

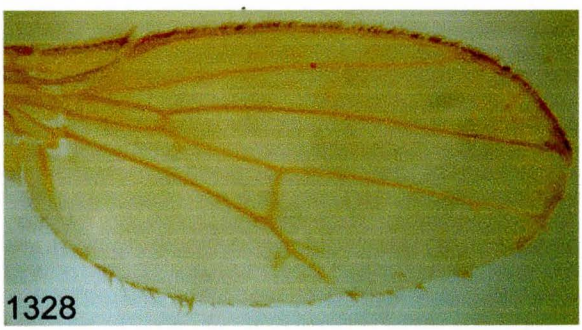
Neu^{YA} suppresses the wing phenotypes induced by *neu^{YD}* allele. The left panels show the wings of representing *neu^{YD}* allele while mis-expressed with the *p[C96] GAL4* driver. In right panel, all the wings used herein are heterozygote of the *neu^{YA}*. The bottom left number on each panel indicates the JPEG archive number.

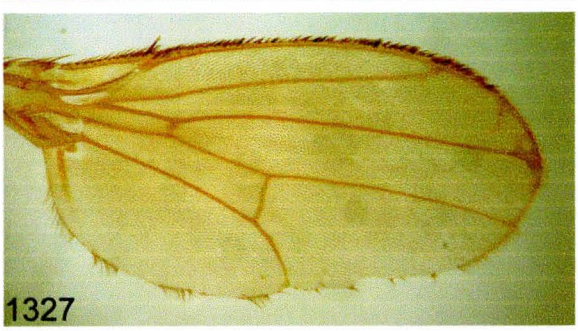
YD/YA; C96/C96

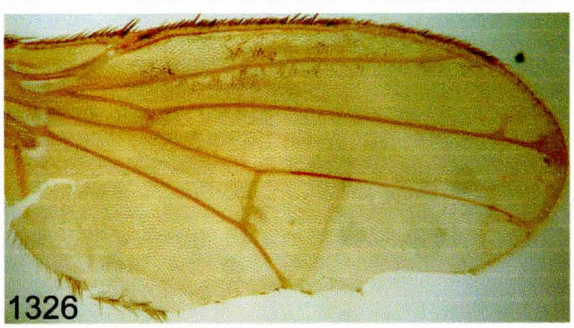


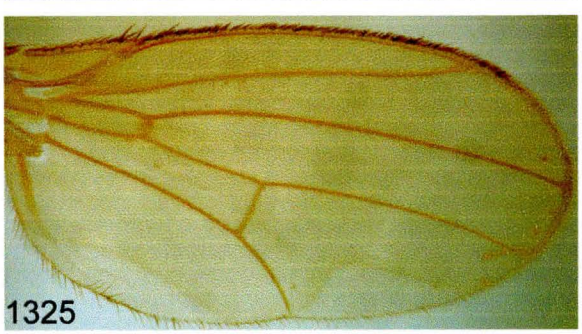


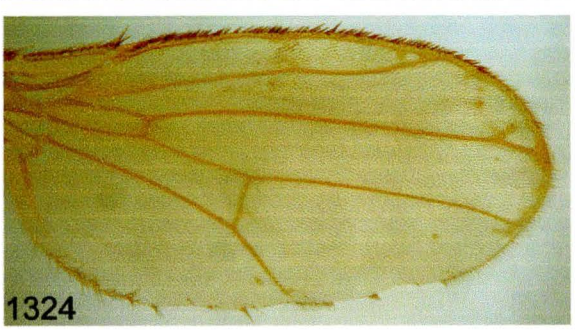


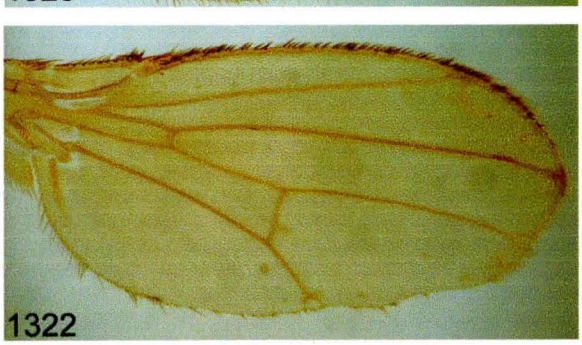


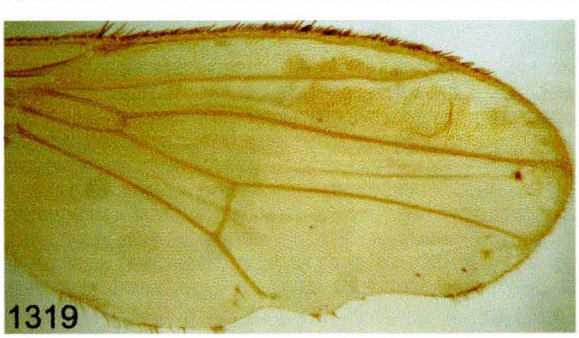








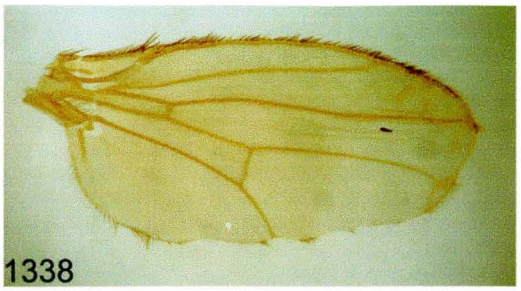


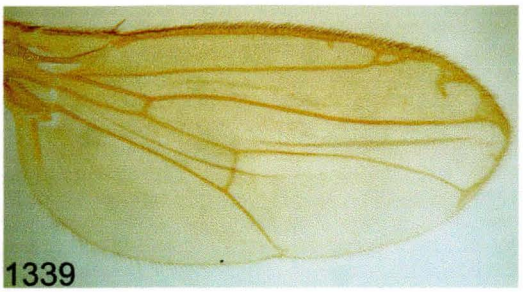


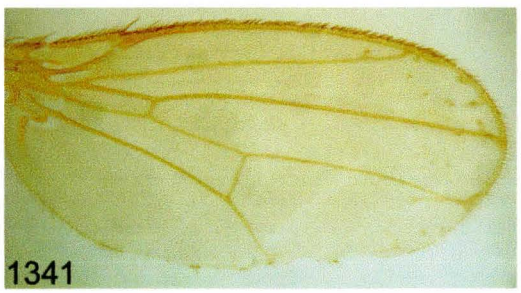
APPENDIX 11:

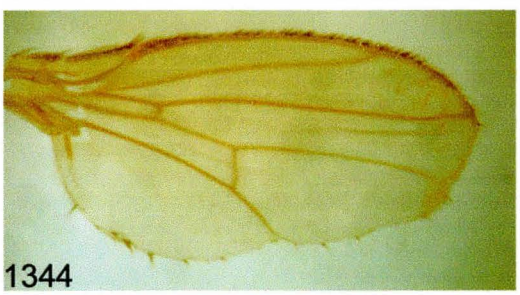
Neu^{YA} suppresses the wing phenotypes induced by *neu^{YE}* allele. The left panels show the wings of representing *neu^{YE}* allele while mis-expressed with the *p[C96] GAL4* driver. In right panel, all the wings used herein are heterozygote of the *neu^{YA}*. The bottom left number on each panel indicates the JPEG archive number.

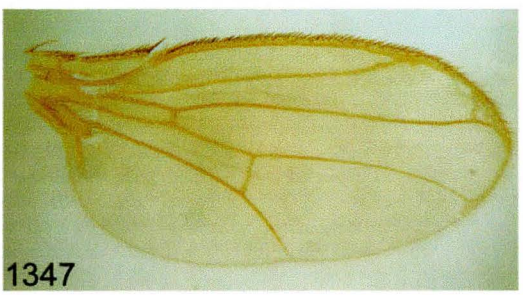
YE/YA; C96/C96

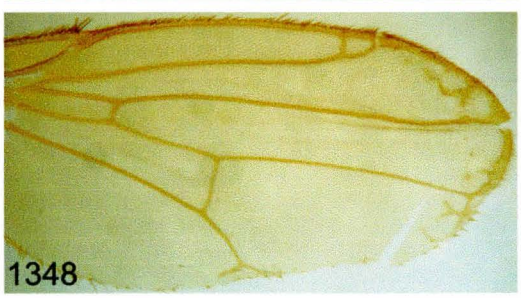


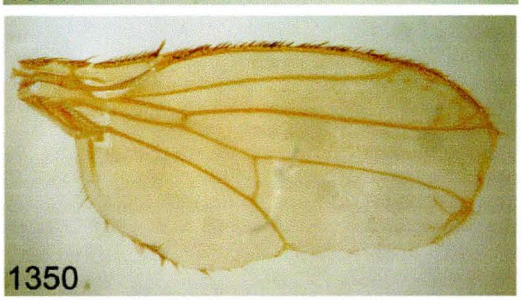


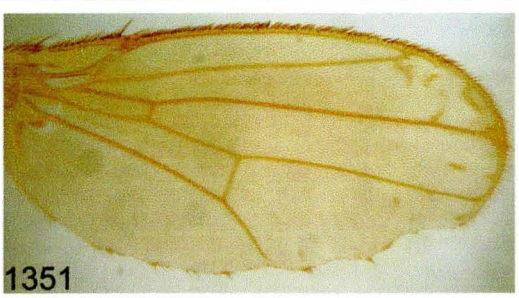


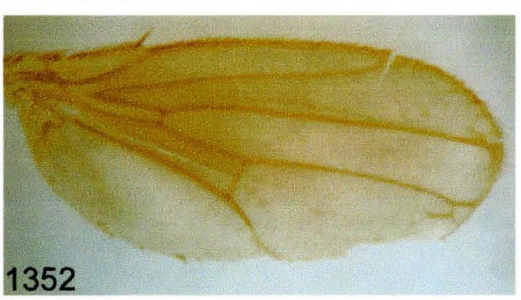








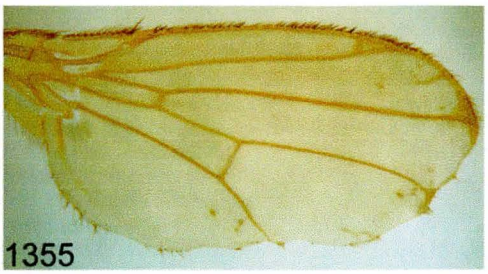


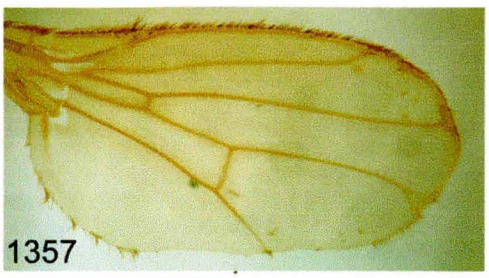


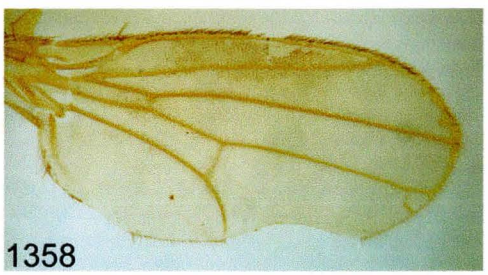
APPENDIX 12:

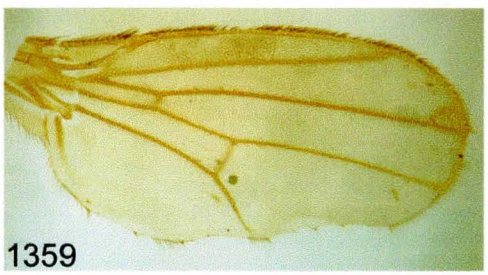
neu^{YA} suppresses the wing phenotypes induced by *neu^{NT}* allele. The left panels show the wings of representing *neu^{NT}* allele while mis-expressed with the *p[C96] GAL4* driver. In right panel, all the wings used herein are heterozygote of the *neu^{YA}*. The bottom left number on each panel indicates the JPEG archive number.

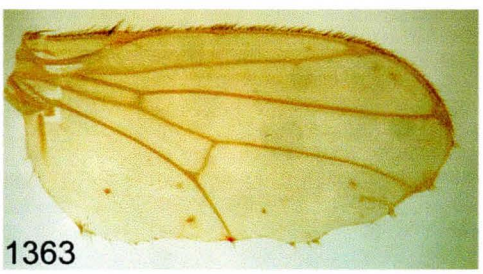
NT/YA; C96/C96

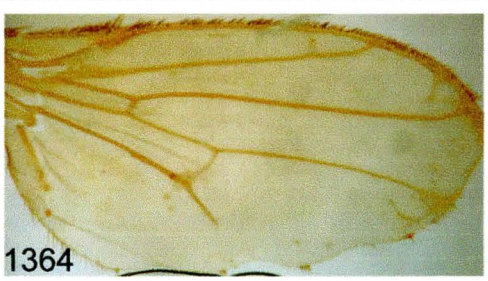


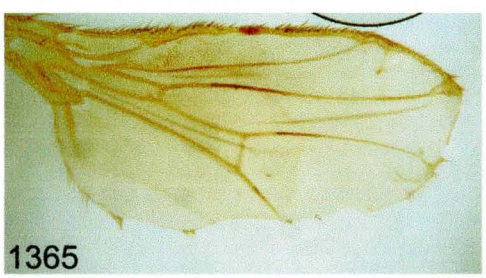


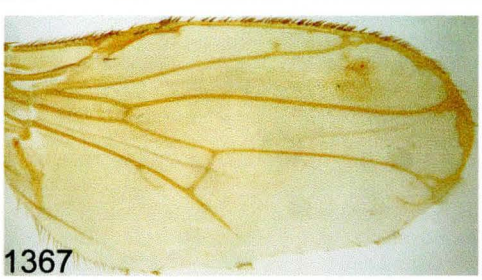








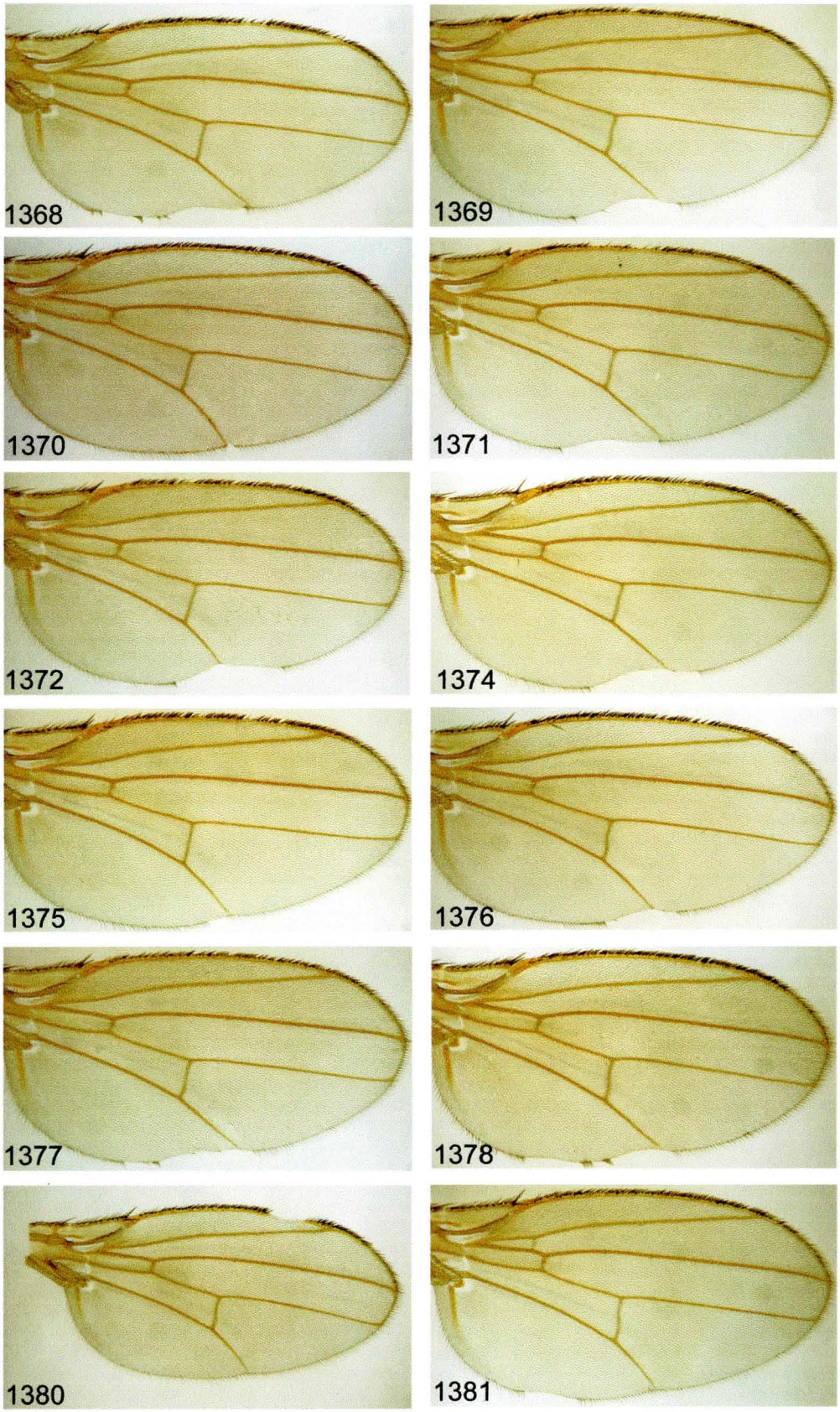




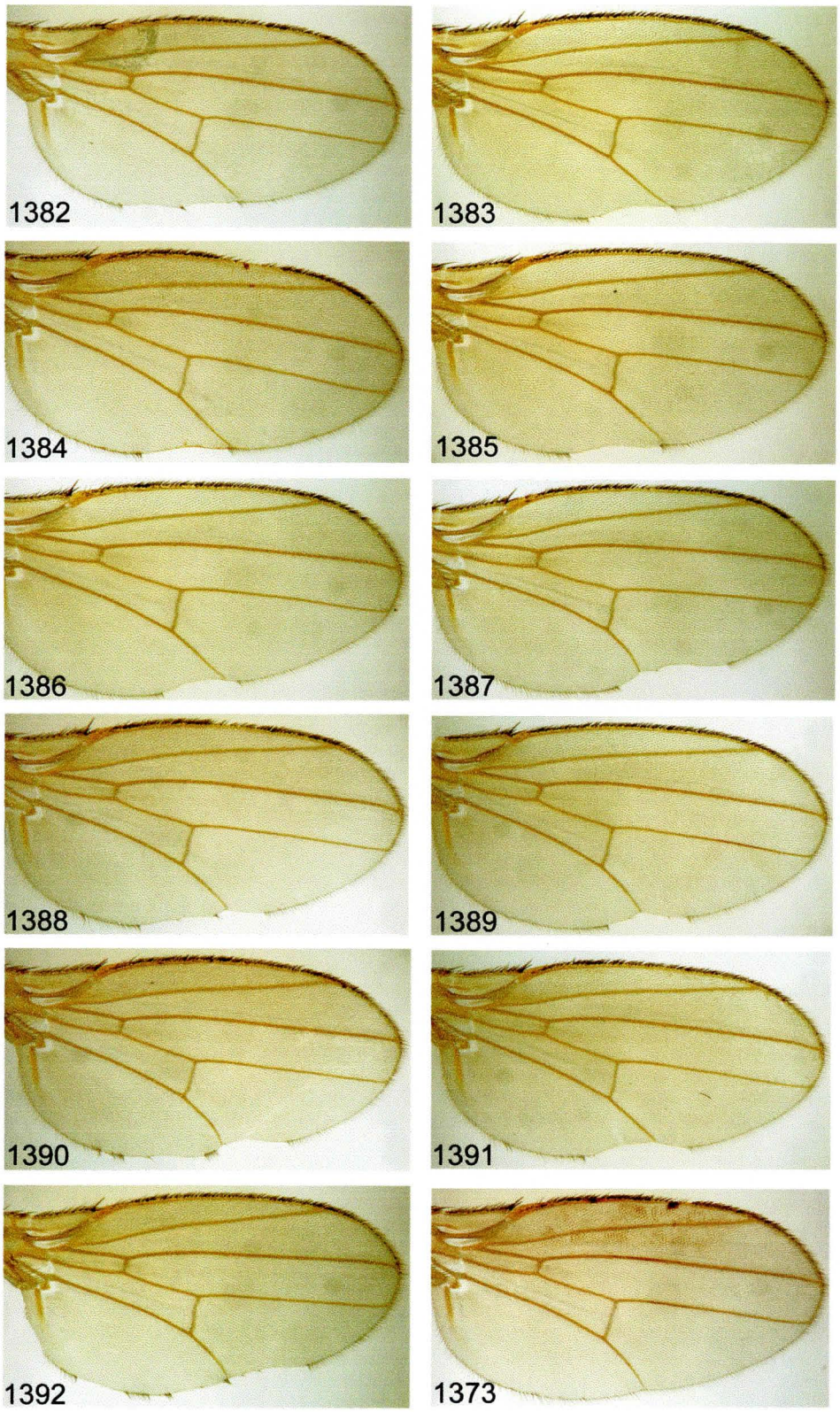
APPENDIX 13:

The mis-expression of DER, at the wing margin, was induced by *p[C96] GAL4*. The wings are from the homozygote of *DER^{A887T}; C96* flies. Mis-expression of DER^{A887T} produced unique phenotype for almost every wing. A total of 24 wings were scored and photographed. 18 out of 24 wings scored had wing margin loss in the DERA887T; C96 flies, while the remaining 6 wings had no significant wing margin loss. The bottom left number on each panel indicates the JPEG archive number.

$DER^{A887T}/+; C96/+$



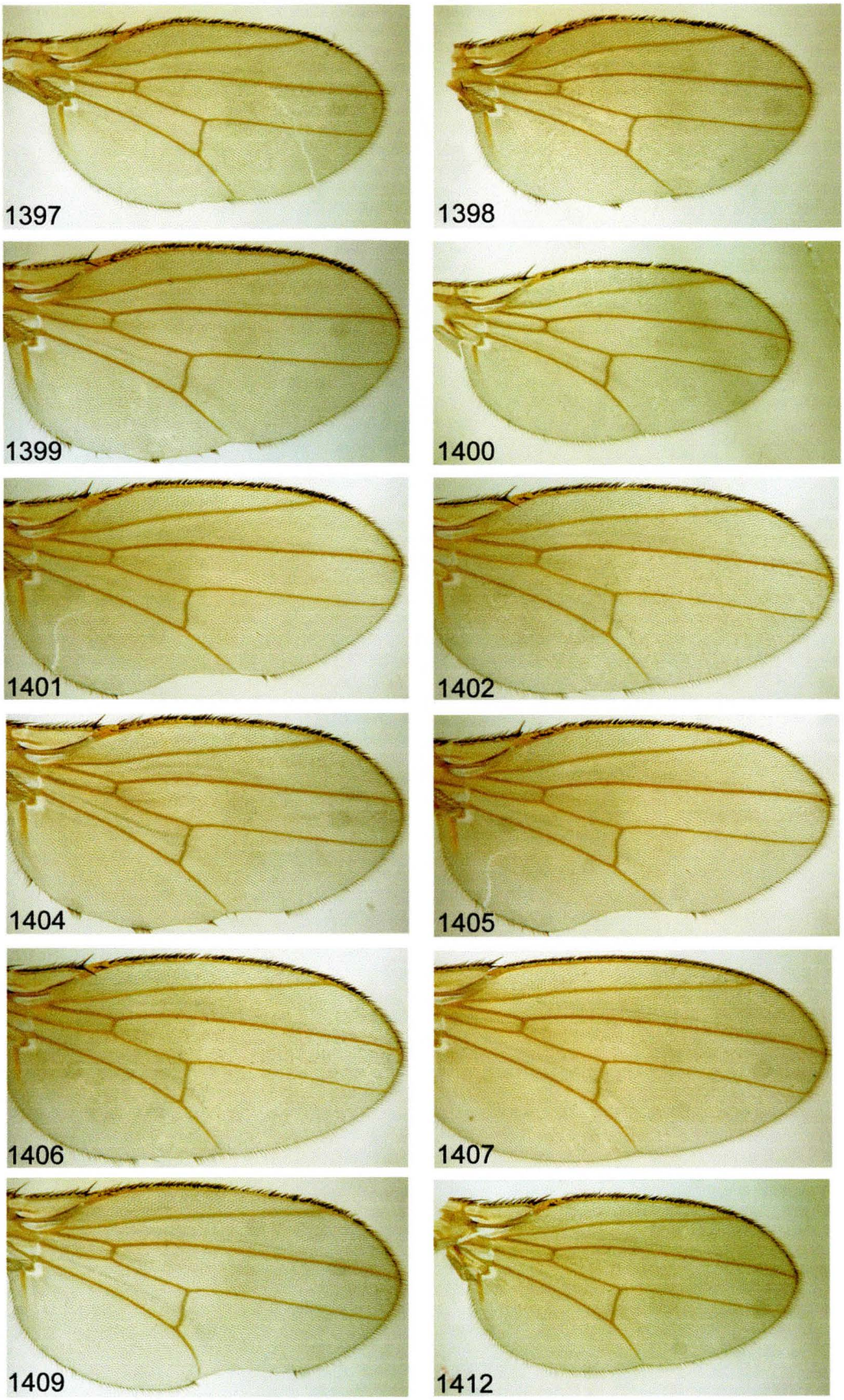
DER^{A887T}/+; C96/+



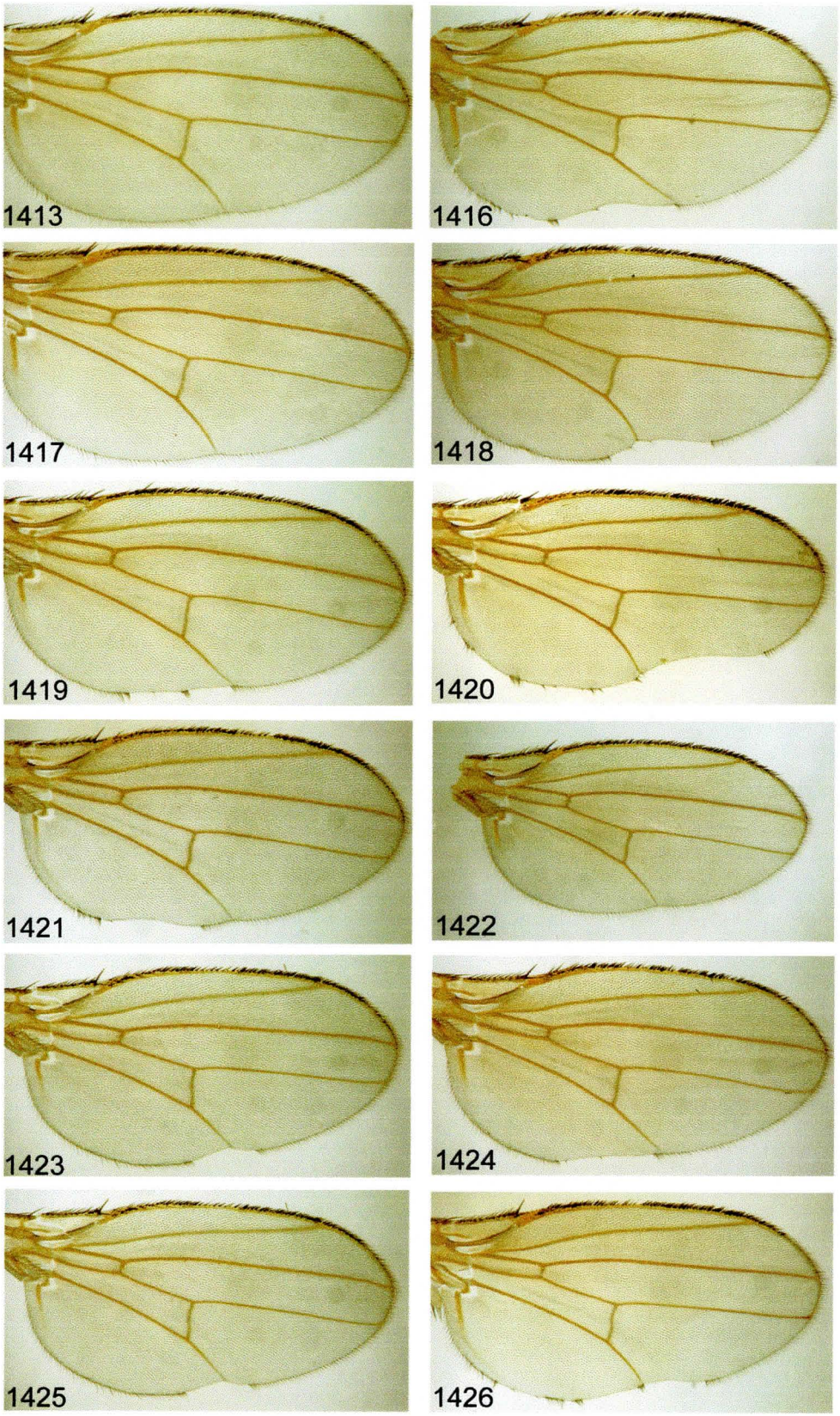
APPENDIX 14:

neu^{YA} did not suppress the wing phenotypes induced by the activated *Drosophila* EGF receptor. The mis-expression of DER, at the wing margin, was induced by *p[C96] GAL4*. The wings are from the heterozygote of neu^{YA} and *DER^{A887T}*. A total of 24 wings were scored and photographed. 15 out of 24 had the same level of margin loss in the *neu^{YA}* heterozygote, where as 9 out of 24 wings had no remarkable wing margin loss. The bottom left number on each panel indicates the JPEG archive number.

DER^{A887T}/YA; C96/+

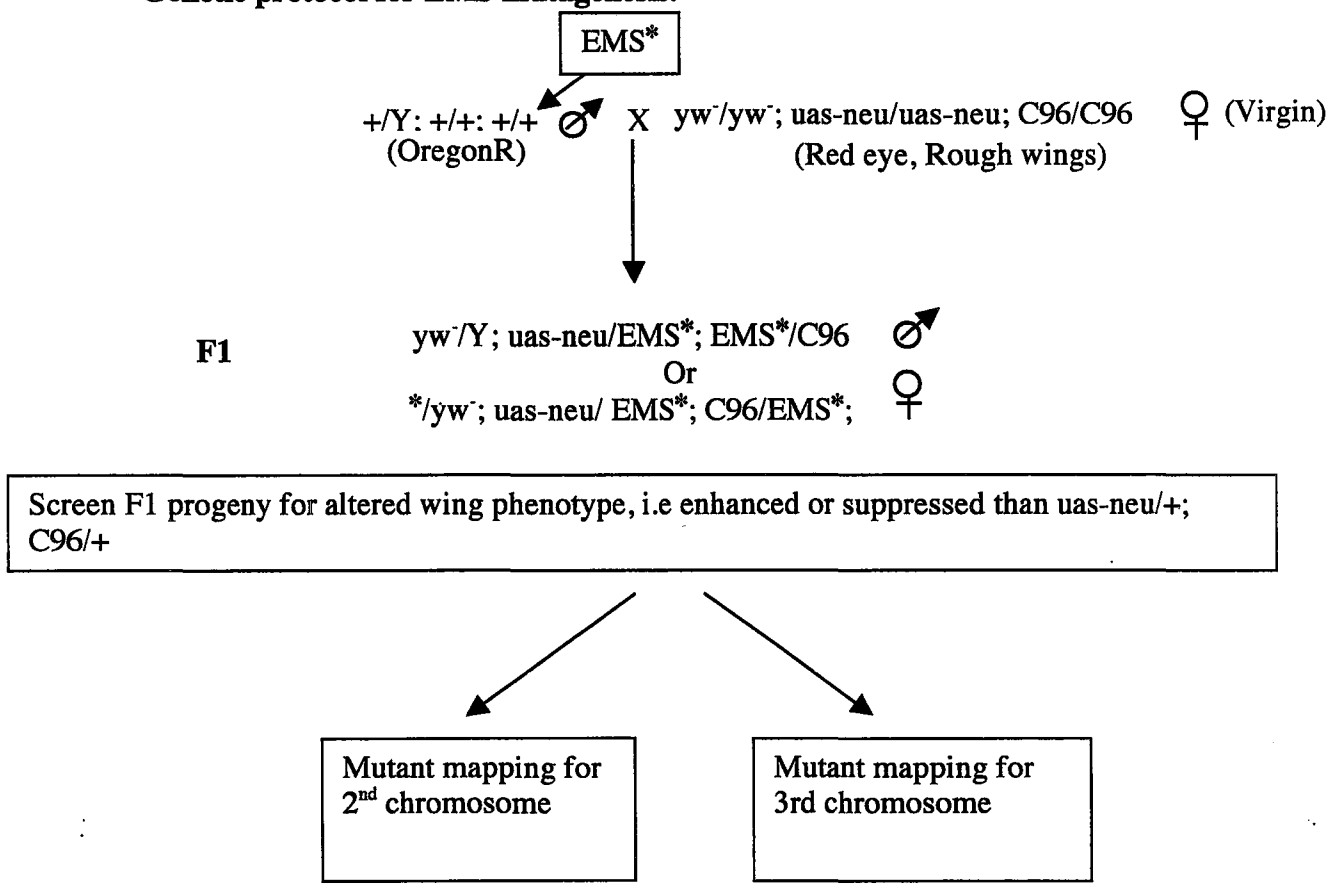


DER^{A887T}/YA; C96/+



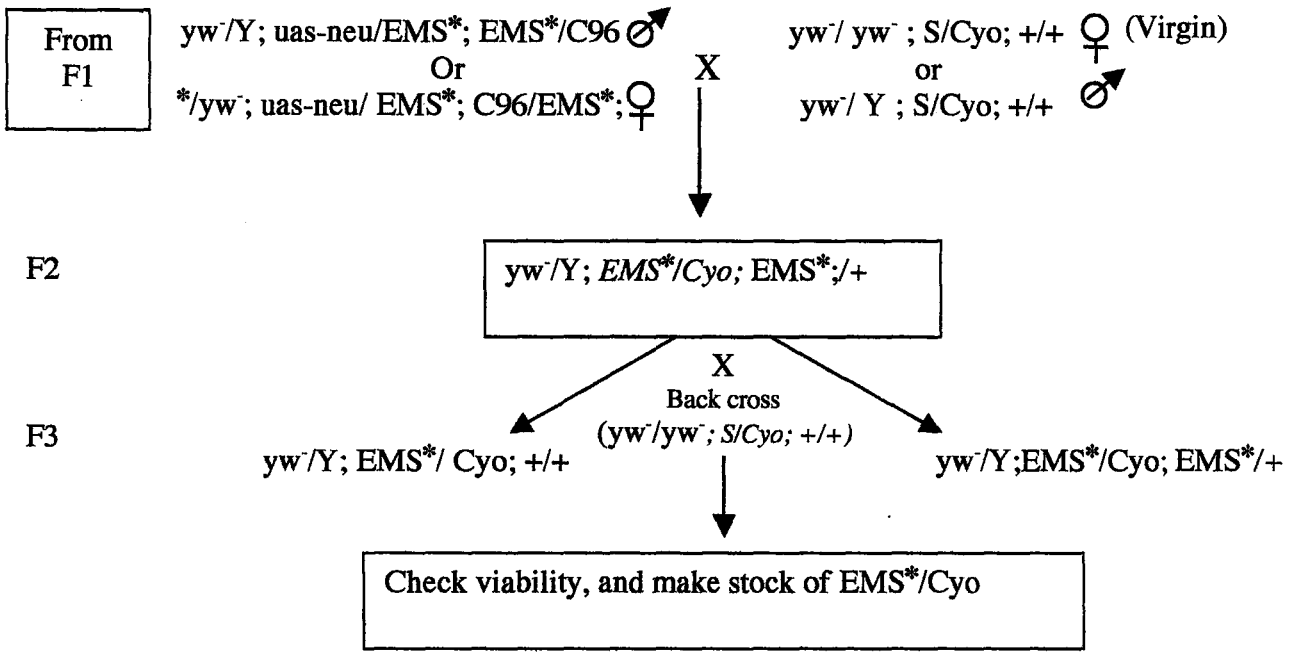
APPENDIX 15:

Genetic protocol for EMS mutagenesis:

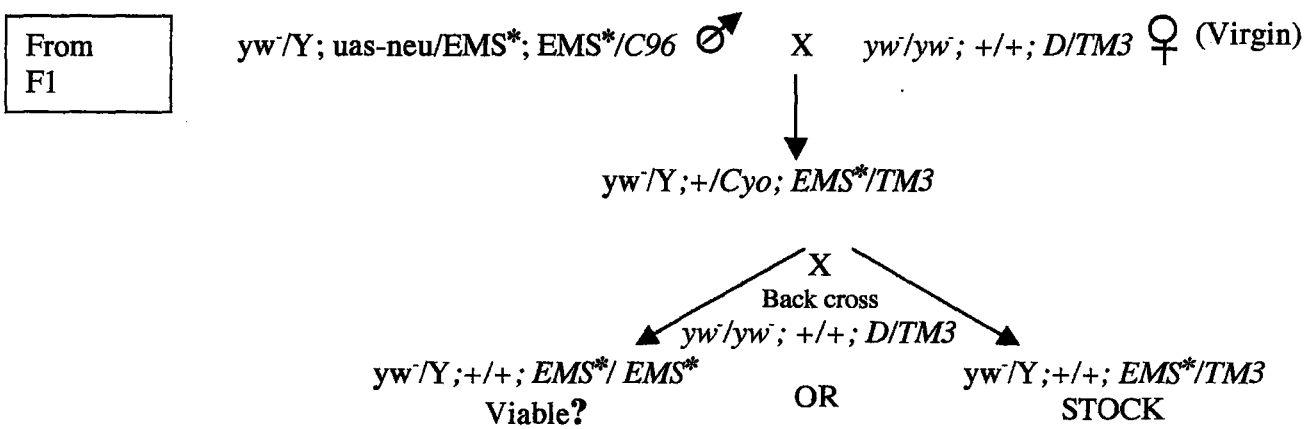


APPENDIX 16.

A. Mutant mapping in the 2nd cromosome:



B. Mutant mapping in the 3rd chromosome:



APPENDIX 17.

Genetic scheme for generation of homozygous *neu;C96* lines: