Regulation of p53 and hid by Phosphorylated MAP Kinase in developing eye of *Drosophila*

By

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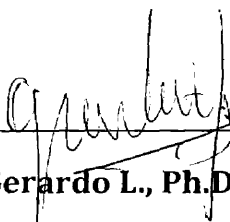
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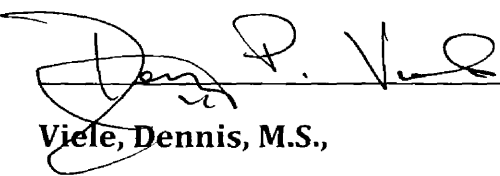
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Abstract

Mitogen Activated Protein Kinase (MAP Kinase) pathway regulates many diverse cellular processes including cell division, growth and differentiation. Many proteins are involved in this complex mechanism of cell cycle regulation. Any mutation in one or more of these proteins may results in abnormal cell phenotypes including tumor formation. Phosphorylated MAPK (dpMAPK/dpERK) is a key protein activated downstream of Ras (proto-oncogene), and regulates cell division and differentiation depending on whether MAPK is localized in the nucleus or cytoplasm respectively. Earlier it was reported that nuclear localization of non-phosphorylated MAPK (ERK) is another novel mechanism which may initiate cell division in the developing eye of *Drosophila*. However, phosphorylation of MAPK is required for proper cell differentiation and growth. Here, we conducted a study on the expression pattern of p53 (tumor suppressor protein) in absence of phosphorylated MAPK. We also studied effect of hid (death activating protein) on MAPK activation and p53 gene expression. Our study shows that active MAPK is required for p53 gene expression. Moreover, we report here for the first time a new mode of negative regulation of p53 gene expression by the nuclear form of non-phosphorylated MAPK (MAPK-nls). Our studies also suggest that presence of hid may induce activation of MAPK via an unknown protein kinase. Mutations in MAPK and p53 have been reported in most human tumors. Inhibition of the Ras/MAPK is used as a mechanism for controlling tumor growth by many cancer drugs. Considering all these together our study might provide valuable contribution to design novel cancer drug.

This thesis is dedicated to

My family

&

My teachers

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List of Abbreviations

BCLxL: <u>B</u>-<u>c</u>ell <u>l</u>ymphoma-<u>ex</u>tra <u>l</u>arge

DPX : <u>D</u>epex-<u>P</u>olystyrene in <u>X</u>ylene

EGFR: <u>E</u>pidermal <u>G</u>rowth <u>F</u>actor <u>R</u>eceptors,

ELAV: <u>E</u>mbryonic <u>L</u>ethal <u>A</u>bnormal <u>V</u>ision

ELISA: <u>E</u>nzyme <u>L</u>inked <u>I</u>mmunosorbant <u>A</u>ssay

Flp: <u>F</u>lippase gene

FITC: <u>F</u>luorescein <u>I</u>so-<u>T</u>hiocyanate

FRT: <u>F</u>lippase <u>R</u>ecognition <u>T</u>arget

GDP/GTP: <u>G</u>uanosine <u>Di</u>phosphate/ <u>G</u>uanosine <u>Tri</u>phosphate,

GFP (<u>G</u>reen <u>F</u>luorescent <u>P</u>rotein)

hid: <u>H</u>ead <u>I</u>nvolution <u>D</u>efective

IAP: <u>I</u>nhibitor of <u>A</u>poptosis <u>P</u>roteins

IL2: <u>I</u>nter<u>l</u>eukin<u>2</u>

IHC: <u>I</u>mmuno<u>h</u>isto<u>c</u>hemistry

MAPK: <u>M</u>itogen-<u>A</u>ctivated <u>P</u>rotein <u>K</u>inase

ERK: <u>E</u>xtracellular signal <u>R</u>egulated <u>K</u>inase

MEK: MAPK Kinase

P: <u>P</u>hosphate (Phosphorylation)

PCD: <u>P</u>rogrammed <u>C</u>ell <u>D</u>eath

pH3: <u>P</u>hosphorylated <u>H</u>istone-<u>3</u>

pMAPK: <u>P</u>hosphorylated <u>M</u>itogen <u>A</u>ctivated <u>P</u>rotein <u>K</u>inase

Raf: MAPK Kinase Kinase

Ras: GTP binding protein

RHG: <u>R</u>eaper, <u>H</u>id and <u>G</u>rim

RTK: <u>R</u>eceptor <u>T</u>yrosine <u>K</u>inases

TRITC: <u>T</u>etramethyl <u>R</u>hodamine <u>I</u>so-<u>T</u>hiocyanate

UAS: <u>U</u>pstream <u>A</u>ctivation <u>S</u>equence

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Introduction

Drosophila melanogaster as an Insect- animal Model

The fruit fly *Drosophila melanogaster (D. melanogaster)* first became prominent in genetic science in 1909, decades before bacteria and fungi. *Drosophila melanogaster* is a species of the order Diptera in the family Drosophilidae. The other names for *D. melanogaster* are "common fruit fly" and "vinegar fly". Starting from Charles W. Woodworth, this species is one of the most popularly used model organisms in biology, including studies in genetics, physiology, and life history evolution. The *D. melanogaster* genome has been sequenced and found to have 165 million nucleotide base pairs, encoding 13,767 genes (Manning, 2006). Moreover, *D. melanogaster* is one of the most studied organisms in biological research, particularly in genetics and developmental biology. There are several reasons as mentioned below;

- Minimal equipment and minimal space are required to take care and maintain culture even at the large scale, and therefore the overall cost is low.

- It is small and easy to grow in the laboratory, and their morphology is easy to identify once they are anesthetized (usually with ether, carbon dioxide gas, or by cooling them).

- It has a short generation time (about 10 days at room temperature) so several generations can be studied within a few weeks.

- It has a high fecundity (females lay up to 100 eggs per day and perhaps 2000 in a lifetime) (Sang, 2001).

- Males and females are readily distinguishable and virgin females are easy to isolate, facilitating genetic crossing.

- The mature larvae show giant chromosomes in the salivary glands called polytene chromosomes—"puffs" indicate regions of transcription and hence gene activity.

- It has only four pairs of chromosomes: three autosomes, and one sex chromosome.

- Recessive lethal "balancer chromosomes" carrying visible genetic markers can be used to keep stocks of lethal alleles in a heterozygous state without recombination due to multiple inversions in the balancer.

- Genetic transformation techniques have been available since 1987.

- Its complete genome was sequenced and first published in 2000 (Adams et al., 2000).

Its importance for human health was recognized by the award of the Nobel Prize in medicine/physiology to Edward B. Lewis, Christiane Nusslein-Volhard and Eric F. Wieschaus in 1995, for their discoveries concerning the genetic control of early embryonic development. Therefore, part of the reason people work with D. melanogaster is historical too.

D. melanogaster: Life Cycle

The adult fly is about 2 mm long. Like other insects, adult *D. melanogaster* has three pairs of legs but, unlike other Dipterans, it only has one pair of wings. The second pair of wings has been modified to small appendages called "Halters", which helps them to balance during flight. The surface of the adult body is covered with sensory hairs and bristles that are connected to the complex nervous system. Other prominent sensory organs, the eye and the antennae, are located in the head.

A female *D. melanogaster* can produce hundreds of eggs. When fertilized, each egg becomes an embryo that hatches out of the egg shell to become a worm-like larva. Larvae feed voraciously for about a week. At intervals during this period it sheds its skin to allow increasing body size. Each larval stage between these molts is called an instar. The third instar is the last stage before metamorphosis in to the imago (adult fly). The skin of the third instar larva hardens in to a case, and the animal becomes a pupa. Packets of cells called, imaginal discs, grow and differentiate into adult structures such as eyes, wings, and legs. In about four days, an adult fly emerges from the pupal case. Figure 1 on Page 4 summarizes the Life cycle of *D. melanogaster*.
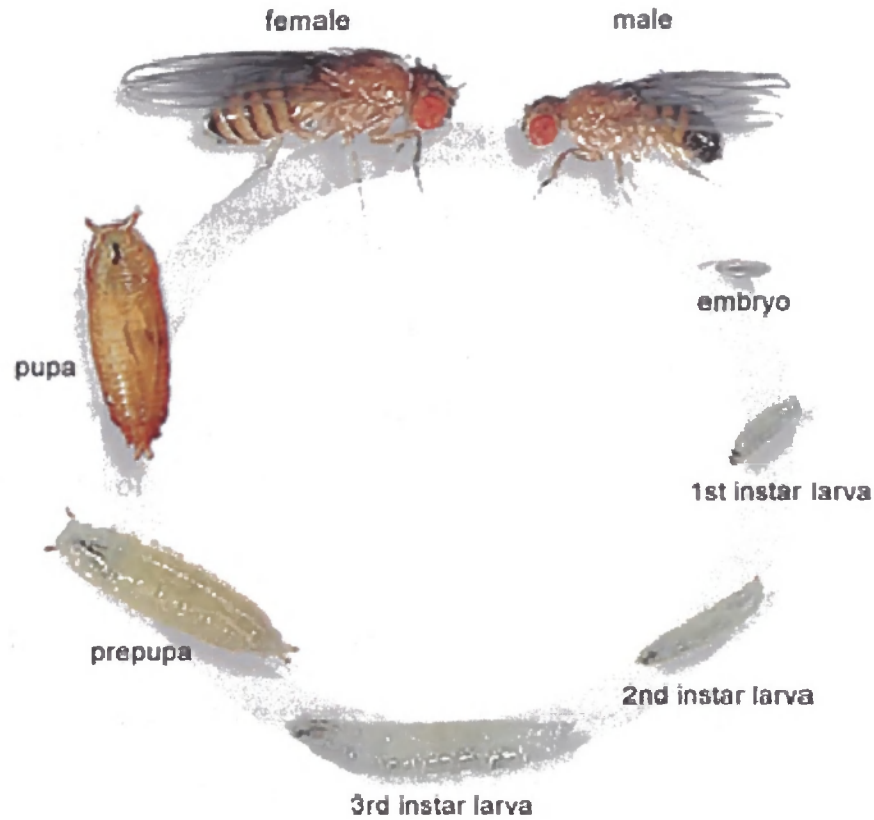
Figure1: Life cycle of *D. melanogaster.* (www.flickr.com/photos/11304375@N07/2993342324/)

D. melanogaster: Genome

The genome of *D. melanogaster* (sequenced in 2000 and housed at the FlyBase database) contains four pairs of chromosomes: an X/Y pair and three autosomes labeled 2, 3, and 4 (Adams *et al.,* 2000). The fourth chromosome is so tiny that it is often ignored. The *D. melanogaster* genome has 165 million base pairs (Gerard, 2006) and contains 13,767 protein-coding genes that comprise ~20% of the genome (Manning, 2006). More than 60% of the genome appears to be functional, non-protein-coding DNA sequence involved in gene expression control (Halligan *et al.,* 2006). Sex determination in *D. melanogaster* occurs by the ratio of X chromosomes to autosomes, not because of the presence of a Y chromosome as in human sex

determination. Although the Y chromosome is entirely heterochromatic, it contains at least 16 genes, many of which are thought to have male-related functions (Carvalho, 2002). For normal female flies this ratio of X chromosomes to autosomes is one, whereas for normal male flies it is 0.5 or less.

D. melanogaster: Retina Development

The compound eye of *D. melanogaster develops* from the retina, which is derived from a monolayer of epithelium called the eye imaginal disc (shown in Figure 2, Panel 'a' as part of the eye–antennal imaginal disc complex). During the final larval instar (the third larval stage), a wave of differentiation, that can be visualized by an indentation in the epithelium called the morphogenetic furrow, sweeps across the disc, transforming an undifferentiated field of cells into a precise tiling of ~800 unit eyes called ommatidia (Kumar, 2001). The construction of an ommatidium involves a series of inductive events that result in the stereotyped recruitment of twenty cells. Among these twenty cells, eight are photoreceptors (R1-8), and twelve are accessory cone and pigment cells (Figure 2 Panel b) (Kumar, 2001). In the adult retina, the photoreceptor neurons make up the core of the ommatidium and project the rhabdomere — a light-gathering organelle — into the central lumen. Above this lumen lie four cone cells that secrete the overlying pseudocone and lens material (Kumar, 2001). Surrounding the photoreceptors and cones are pigment cells that optically insulate each unit eye (Figure 2 Panel b; seen in longitudinal section on the left and in cross-section at different positions on the right) (Kumar, 2001).
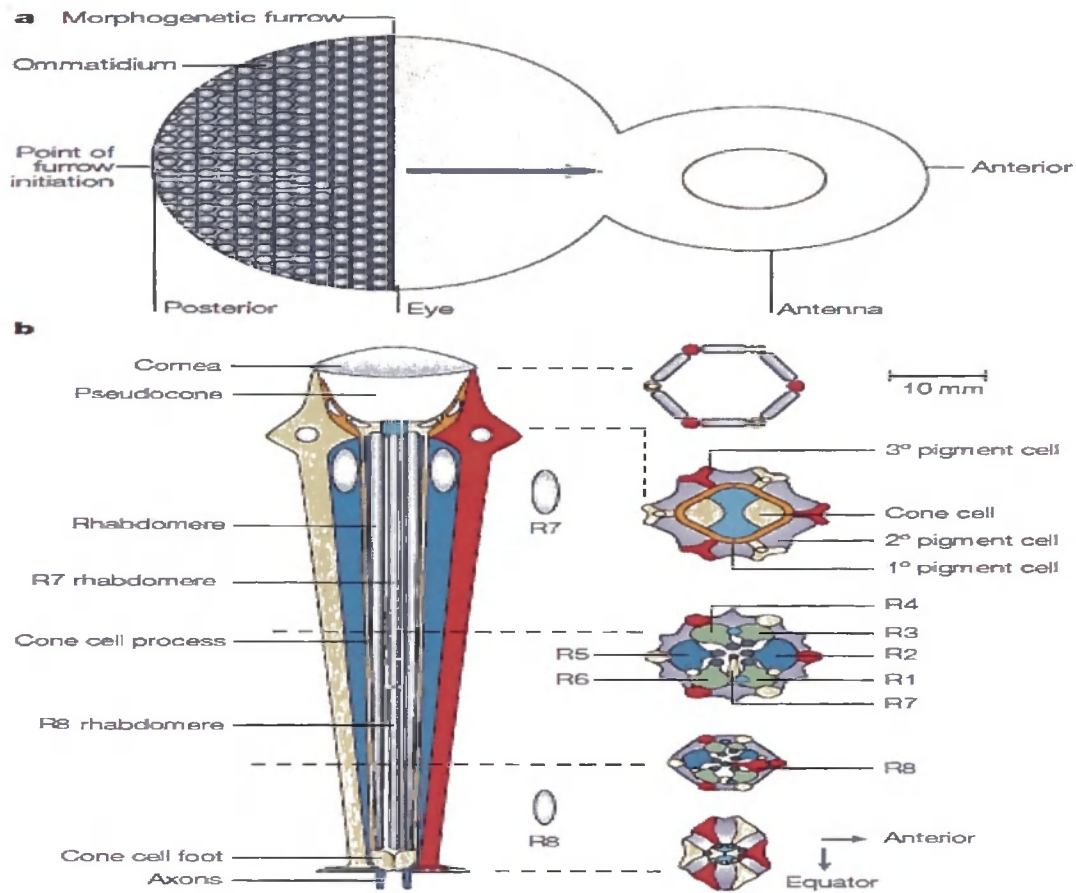
Figure 2: Development and structure of the *D. melanogaster* retinas (Kumar, 2001).

MAP Kinase Pathway

Signal transduction pathways are mechanisms by which cells respond to extracellular stimuli. These stimuli may be chemical (e.g. growth factors, insulin) or physical (e.g. stress, UV radiation). Signal transduction starts with a signal to a receptor, and ends with a change in cell function. Transmembrane receptors span the cell membrane, with part of the receptor outside (the extracellular domain) and part inside the cell (the intracellular domain). The chemical signal (ligand) binds to the extracellular domain of the receptor, changing shape and/or conformation of the intracellular domain to convey another signal inside the cell. Some chemical

messengers, such as testosterone, can pass through the cell membrane, and bind directly to receptors in the cytoplasm or nucleus. These pathways may involve long and complex cascades of signaling that amplifies the initial signal at each step. Thus, a "small" signal can result in "large" response, such as changing the expression of genes or altering the activity of certain enzymes.

The **M**itogen **A**ctivated **P**rotein **K**inase (MAP Kinase, or MAPK) pathway is one of the most important signal transduction pathways and is currently a popular topic in cancer research. The MAP Kinase pathway regulates many diverse cellular processes including cell division, growth and differentiation. In fact, misregulation of the MAPK pathway is associated with approximately 25% of human tumors (Hanahan *et al.*, 2000). A number of peptide factors, including **I**nsulin-like **G**rowth **F**actor **1** (IGF-1), **F**ibroblast **G**rowth **F**actor (FGF), and **E**pidermal **G**rowth **F**actor (EGF), promote cell survival by suppressing the intrinsic cell death program (Botella *et al.*, 2003; Yamada *et al.*, 1997). The mechanisms by which survival factors inactivate the intrinsic cell death program are currently the subject of intensive investigation. The growth factors listed above bind to and activate receptor tyrosine kinases (RTKs) at the cell surface, which in turn stimulate the anti-apoptotic activity of the proto-oncogene Ras. The Ras protein controls the activity of a number of effector pathways, and the MAP Kinase pathway is one of them (Parrizas *et al.*, 1997). Upon ligand binding, EGFR (**E**pidermal **G**rowth **F**actor **R**eceptors) dimerize and auto-phosphorylate. This results in the activation of the GTPase-Ras complex that further phosphorylates and activates Raf (MAPK kinase kinase). Raf phosphorylates and activates MEK (MAPK kinase), which leads to the dual

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phosphorylation and activation of MAPK. MAPK can then either remain in the cytoplasm or move into the nucleus where it phosphorylates transcription factors. This result in activation of multiple transcription factors required for cell growth and inactivation of death signaling proteins, such as hid in case of *Drosophila*. Figure 3 below illustrates the EGFR/Ras/MAP Kinase signal transduction pathway in *Drosophila*.
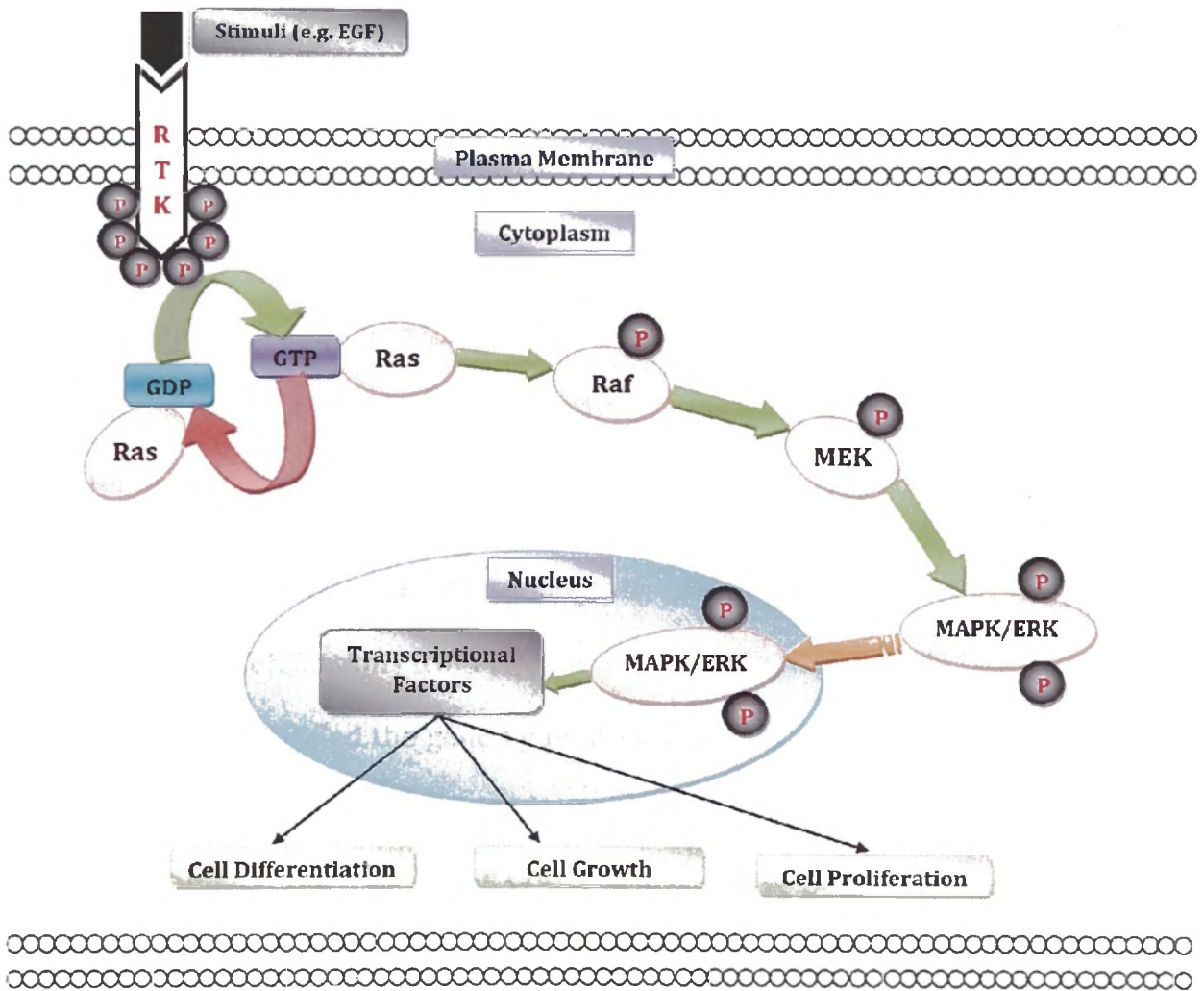
Figure 3: Drawing of the EGFR/Ras/MAPK signaling pathway in *D. melanogaster* and its phosphorylation cascade. **EGFR**: Epidermal Growth Factor Receptors, **RTK**: Receptor Tyrosine Kinases, **Ras**: GTP binding protein, **MAPK/ERK**: Mitogen-Activated Protein Kinase/Extracellular signal Regulated Kinase **Raf**: MAPK Kinase Kinase, **MEK**: MAPK Kinase, **GDP/GTP**: Guanosine Diphosphate/ Guanosine Triphosphate, **P**: Phosphate (Phosphorylation).

p53

The p53 protein is a sequence specific transcription factor that is among the most important tumor suppressors. In fact, most human tumors have been found to have mutations in the p53 gene or its functional pathway. It has also been found to have critical role in safe guarding the integrity of the genome. Hence, it is referred to as "Guardian of the Genome". Under normal circumstances, the level of p53 in the cell is being kept low with relatively short half life. Under cellular stress conditions, such as DNA damage, hypoxia, or abnormal oncogene activation, p53 protein level rapidly increases and it acts to control the cell cycle by inducing either cell cycle arrest or apoptosis. (Shengkan *et al.*, 2003). Induction of apoptosis by p53 is critical for the tumor suppressor function of p53. There appears to be multiple mechanisms through which p53 promote apoptosis. For example, p53 can transcriptionally activate the pro-apoptotic gene Bax in humans.

Characterization of the single *D. melanogaster* p53 (Dmp53) homolog was reported in back-to-back publications from two laboratories (Ollmann *et al.*, 2000; Brodsky *et al.*, 2000). Both identified the gene by homology searches of the expressed sequence tag database of the Berkeley *D. melanogaster* Genome Project. The DNA binding regions of Dmp53 and human p53 (hp5 3) h ave 44% of sequence similarity that includes over 207 amino acids. Also, these regions share 24% of identical nucleotide sequence (Jin *et al.*, 2000). Dmp53 is required for radiation-induced apoptosis in the wing, but not for the normal levels of cell death that occur in the absence of DNA-damaging agents (Brodsky *et al.*, 2000).

Dmp53 encodes a 385-amino acid protein with significant homology to hp53 in the region of the DNA-binding domain, and to a lesser extent the tetramerization domain. Purified Dmp53 DNA-binding domain protein was shown to bind to the consensus human p53-binding site by gel mobility analysis (Sutcliffe *et al.*, 2004). Human p53 is a 393 amino acid protein composed of three main functional domains: (i) an amino-terminal acidic transactivation domain, (ii) a central DNA-binding domain, and (iii) a carboxy-terminal tetramerization domain. Significant similarity between Dmp53 and the vertebrate p53 family is limited to the DNA-binding domain and includes residues identified in human p53 as critical for DNA sequence recognition and coordination of a zinc ion. In human tumors, mutations that inactivate p53 function are clustered in the well-conserved DNA-binding domain. Among the six most frequent sites of mutation in tumors, four are identical in Dmp53 and the other two are similar (Thomas, 1996).

hid

Head Involution Defective is abbreviated as hid. In 1936 Jollos described hid originally as the *Wrinkled* (*W*) gene in his paper entitled "Mutations observed in *D. melanogaster* stock s taken up into the stratosphere." It is one of the three Pre-apoptotic proteins called "Reaper, Hid and Grim" (also referred to as RHG) (Hainning *et al.*, 1999; Wing *et al.*, 1998). In other words they are all death activating proteins in *D. melanogaster.*

There is a general decrease in apoptosis, or programmed cell death (PCD) throughout the homozygous recessive hid mutant embryo. This phenotype is most noticeable in the head region prior to completion of head involution. Striking defects in head morphogenesis occur from a failure of the dorsal fold to migrate to the anterior fold in hid mutants (Abbott, 1991). Flies carrying a single copy of hid expressed from a strong synthetic promoter display a dramatic eye ablation phenotype. Normally, compound eyes consist of about 800 regular units, called ommatidia, each of which consists of several distinct cell types. Flies carrying hid construct only have u ndifferentiated cuticle and a dense band of bristles in the places normally occupied by the compound eyes. hid regulates the expression of caspases such as DREDD and DRONC (Hawkins *et al.*, 2000). Cell death induced by expression of hid can be blocked by inhibitors of caspases such as various members of the inhibitor of apoptosis proteins (IAP) (Wang *et al.*, 1999; Vucic *et al.*, 1998; Yoo *et al.*, 2002). Olson *et al.* (2003) have reported that hid is a substrate for ubiquitination mediated by IAP. The function of hid has been conserved in evolution. A mammalian homolog of hid has not been identified, but expression of hid in mammalian cells induces cell death by apoptosis and this can be blocked by functional interaction with BCLxL ("B-cell lymphoma-extra large") which is one of several anti-apoptotic proteins. Mammalian IAP also inhibits hid function (Haining, *et al.*, 1999). Apoptosis induced by hid in mammalian cells requires caspase-8 and is regulated by the extracellular signal-related kinase (ERK/MAPK) in mammalian cells (Varghese *et al.*, 2002). Varghese *et al.* (2002) have inferred the existence of a hid-like protein in mammalian cells from the observation that IL2 (Interleukin2)

blocks caspase-8 processing and cell death by apoptosis in activated T-cells. The hid is post-translationally regulated by the RAS-MAP Kinase pathway in response to cell survival signals (Bergmann *et al.,* 1998; Goyal *et al.,* 2000). Activated MAP Kinase inhibits the pro-apoptotic function/ activity of hid by phosphorylation (Bergmann *et al.,* 1998; Goyal *et al.,* 2000).

Genetic tools to study *D. melanogaster*

1) Balancers

The idea of using a "Balancer chromosome" was developed by H. J. Muller in 1938. He identified the chromosome C1B as a suppressor of exchange on the X chromosome during meiosis. Since then, the idea of using such a chromosome, especially to study marker mutations linked to it, became a popular tool in genetics.

A balancer chromosome is a genetic tool used to prevent crossing over (genetic recombination) between homologous chromosomes during meiosis. Balancer chromosomes are the products of multiple, nested chromosomal inversions. Hence, they disrupt the synapses between two homologous chromosomes which ultimately suppresses crossing over during meiosis. Recombination in inverted regions leads to dicentric (two centromeres) or acentric (no centromere) chromosomes. Progeny carrying chromosomes that are the products of recombination between balancer and normal chromosomes results in a homozygous lethal situation (as balancer carries recessive lethal gene) and hence are not viable. Balancers are most often used in *D. melanogaster* genetics to allow populations of flies, carrying heterozygous

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mutations, to be maintained without constantly screening for the mutations. Balancer chromosomes have three important properties:

a) They suppress recombination with their homolog during meiosis,

b) Carry dominant markers, and

c) Negatively affect reproductive fitness when carried homozygously.

Balancers are named according to the chromosome they stabilize and also the genetic markers they carry. Their name starts with a letter for their chromosome, i.e. F = First (X-chromosome), S = Second, and T = Third. Along with this an M for Multiply inverted, a number and sometimes a lowercase letter to identify its place in a series. Following are the examples of different Balancers;

FM7c: F = First (X) chromosome, so it is an X-linked balancer which carries the dominant marker Bar (B) and some recessive alleles.

SM6: S = Second chromosome, so it is a second chromosome linked balancer which carries the dominant marker Curly (Cy) as well as various recessive alleles.

2) GAL4/UAS system

The GAL4-UAS system is considered a powerful technique for studying the expression of genes. The system consists of two parts;

I.   The GAL4 gene that encodes the yeast transcription activator protein Gal4,

II.   The UAS (Upstream Activation Sequence), which is a short sequence of the promoter region, to which the Gal4 protein specifically binds to activate gene transcription.

This combined system of GAL4 and UAS provides an aid to study the inducible gene expression. GAL4 is a gene encoding regulatory protein in the yeast *Saccharomyces cerevisiae,* which by binding to the UAS regulates the transcription of GAL10 andGAL1 genes. In 1988 Fischer *et al.* demonstrated that GAL4 was capable of inducing / stimulating transcription of a reporter gene under UAS control in *D. melanogaster.* Further studies reveal that this activity is not limited to just *D. melanogaster,* but also can function in a wide variety of systems to activate transcription from UAS element.

The GAL4 and UAS-Target gene are the two components of a bipartite system for inducing gene expression. GAL4 is referred to as the driver whereas the target gene is referred to as the responder, which is under UAS control. Both the driver and the responder are maintained as separate parental lines. As the transcription of responder requires presence of driver the responder lines are mated/ crossed to/with flies expressing driver. Hence, in the absence of driver responder lines maintains the target in a transcriptionally inactive/ silent state. For example, by fusing a gene encoding a visible marker such as GFP (<u>G</u>reen <u>F</u>luorescent <u>P</u>rotein) to UAS, the expression pattern of the driver genes can be determined (Duffy, 2002). GAL4 and the UAS are very useful for studying gene expression in *D. melanogaster* as they are not normally present and their expression does not interfere with other processes in the cell.
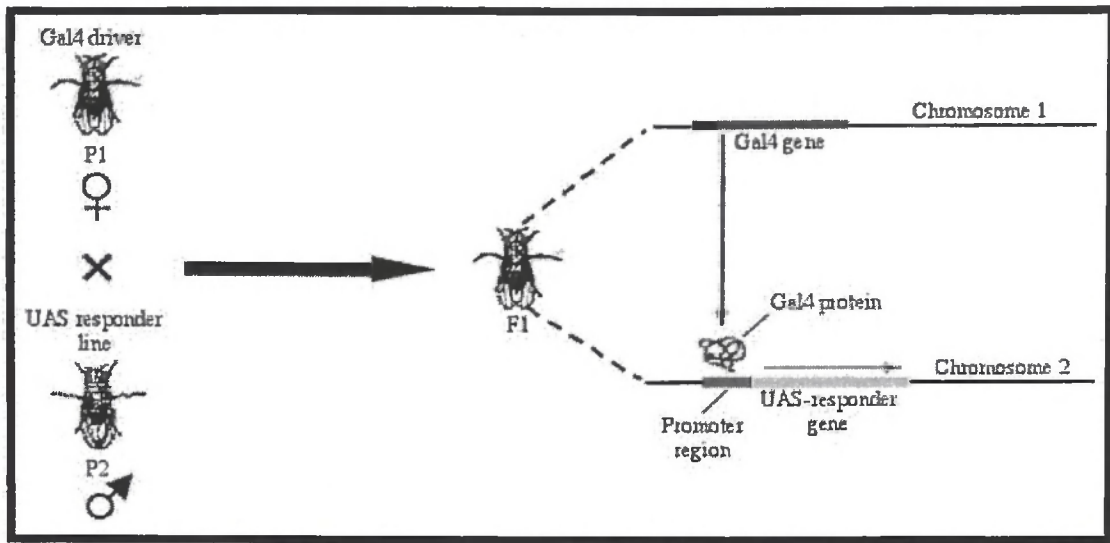
Figure 4 : GAL4-UAS System in *D. melanogaster:* Top left is female fly carrying Gal4 driver and lower left is male fly carrying target/responder gene under UAS promoter control. (Lanata, clas.ufl.edu)

3) Fly stocks

a. dSor1 : FRT 19A
   FM7c

The gene **Downstream of raf1** is referred to in FlyBase by the symbol Dmel\Dsor1\dSor1 (CG15793, FBgn0010269). It is a protein coding gene from *D. melanogaster.* Its sequence location is X:9141828-9144070 (Figure 5). Based on sequence similarity, it is predicted to have molecular function of MAP Kinase Kinase (MEK) activity. No phosphorylation of MAPK can take place in cells that get two copies of dSor1. It is under balancer, FM7c, control that carries dominant Bar-Eye phenotypic marker which helps to identify flies carrying dSor1 construct (genotype). There is experimental evidence that dSor1 is involved in the following biological processes: border follicle cell migration, hemocyte differentiation and signal transduction. The phenotypes of this allele are

associated with: external compound sense organ; peripheral nervous system; nervous system; organ system; egg; adult segment; pigment cell; adult mesothoracic segment. It has one annotated transcript and one annotated polypeptide. A homozygous condition for this allele can be achieved by an enzymatically forced genetic recombination event in somatic tissues. Thus, the enzyme Flippase (FLP) forces recombination between the specific target sites (FRT) on specific chromosomes. This is called P vector mediated site-specific recombination, which works as described below.

In 1989, Golic and Lindquist first mentioned this system. This system is particularly useful for generating mosaics. One P element (transposable element) carries the FLP recombinase gene driven by a heat shock promoter, and a second element has a gene with two FRT sites embedded. When heat shock is applied to such flies, FLP-mediated recombination causes somatic loss of the gene carrying FRT sites. More recently, FLP has been used to generate somatic mosaics with regions homozygous for an entire chromosome arm (Xu and Rubin, 1993). A homozygous P element near the base of a chromosome arm and bearing an FRT site undergoes mitotic recombination when FLP is expressed. The result is a somatic region that is homozygous for all genes distal to the FRT-bearing P element. Such regions can be identified by the absence of a cell-autonomous marker present on one of the homologs. This method allows identification and analysis of genes that are lethal when homozygous in the whole organism (http://engels.genetics.wisc.edu/Pelements/Pt.html).
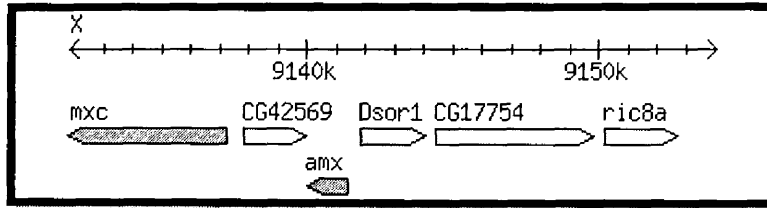
Figure 5 : Genomic location of the gene Downstream of raf1 (dSor1) in *D. melanogaster.*

(http://flybase.org)

b. dSor1 ; FRT 19A ; hs:rl
FM7c   cyo

These flies also have the MAP Kinase gene ("rl"= "rolled", *Drosophila* homolog of human MAPK) construct on chromosome 2 and it is under heat shock promoter control. Therefore, upon heat shock at 37 °C for one hour, MAP Kinase is overexpressed. However, MAP Kinase remains non-phosphorylated and stays in the cytoplasm. In addition to dominant bar-eye marker it also carries recessive curly-wings (cyo) marker which together helps to identify flies carrying this construct.

c. dSor1 ; FRT 19A ; hs:rl[nls]
FM7c   cyo

Here, a nuclear localization signal (nls) has been engineered on to "rl" (under control of a heat shock promoter). This forces MAP Kinase to migrate to nucleus even though it is not phosphorylated/activated. This helps to determine importance of the sub-cellular localization In addition to dominant bar-eye marker it also carries recessive curly-wings (cyo) marker which together helps to identify flies carrying this construct.

d. W[1118] ; P {w[+mc]=GUS p53}2.1

This is a construct having a P element with p53 gene on chromosome 2 and it is under Glass Multimer Reporter (GMR) promoter control. This construct also has a balancer SM6 with curly-wings (cyo). Thus the construct expresses p53 in the eye from the GMR promoter and allows expression of p53 in the presence of a GAL4 driver.

e. P {w[+mc]=GMR hid}G1/cyo

This is a construct having P element with hid gene on chromosome 2 and it is under GMR promoter control. This construct also has a balancer SM6 with curly-wings (cyo). Thus it expresses hid in the eye from the GMR promoter and allows expression of hid in the presence of a GAL4 driver.

4) Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of identifying antigens (e.g. proteins) in cells of a tissue section (Ramos-Vara, 2005). In basic research, IHC is widely used to identify the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. This tchnique takes its name from the roots "immuno", in reference to antibodies used in the procedure, and "histo", meaning tissue. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a color-producing reaction (*e.g.* ELISA – Enzyme Linked Immunosorbant Assay).

Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein, rhodamine, DyLight Fluor or Alexa Fluor.

There are two strategies used for the immunohistochemical detection of antigens in tissue, the *direct* method and the *indirect* method. The *direct* method is a one-step staining method, and involves a labeled antibody (e.g. FITC conjugated antiserum) reacting directly with the antigen in tissue sections. The *indirect* method involves an unlabeled primary antibody (first layer) which reacts with tissue antigen, and a labeled secondary antibody (second layer) which reacts with the primary antibody (Figure 6). This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody.
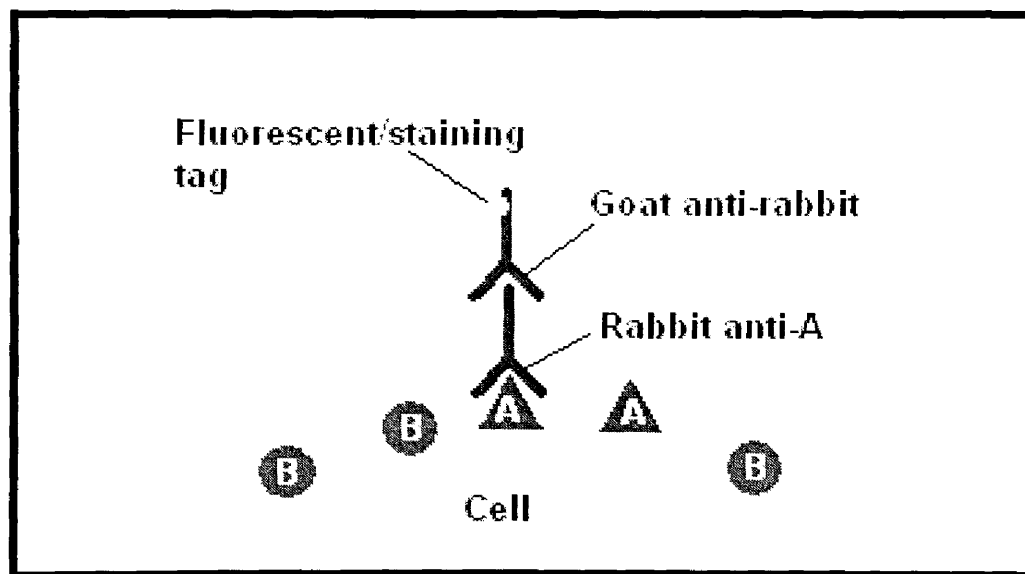
Figure 6 : The indirect method of immunohistochemical staining uses one antibody (Primary antibodcey) against the antigen being probed for, and a second (Secondary antibody), labeled, antibody against the first. (http://en.wikipedia.org).

OBJECTIVE

Our studies focus on the critical role of p53 and hid in cell division, cell growth and differentiation, specifically when MAPK is forced to move into the nucleus without phosphorylation. In humans and other primates, phosphorylation mediated activation of p53 by MAPK (ERK) has been reported (Wu, 2004; Lin *et al.*, 1998; Serrano *et al.*, 1997). Also, the p53 homologue in *D. melanogaster* has been identified based on the biochemical properties, sequence homology and conserved cellular functions, suggesting similar activation may occur.

Is there any connection between Dmp53, hid and MAP Kinase? What happens to the expression of p53 when MAP Kinase is non-phosphorylated (inactive)? Does heat shock mediated overexpression of non-phosphorylated MAP kinase alter the expression of p53? If yes, how does it affect the expression? Recently, it has been reported that non-phosphorylated MAPK is sufficient to induce cell division, but not cell growth, once inside the nucleus of the cell (Marenda *et al.*, 2006; Paez *et al.*, 2010). Therefore, it would be interesting to study the expression pattern of p53 (tumor suppressor) when non-phosphorylated MAP Kinase is forced to enter the nucleus. We hypothesize that, as in human and other primates, activated MAPK is required for p53 gene expression.

Earlier research has shown that activated MAPK directly targets hid and inhibits its activity (Haining, 1999; Bergmann *et al.*, 1998). Therefore, it also would be interesting to study how hid, under P element control, affects p53 expression when MAPK is inactive.

MATERIALS AND METHODS

D. melanogaster stocks

The *D. melanogaster* stock dSor1:FRT19A/FM7c was used as a standard control. Other genotypes that were used are described as follows : dSor1:FRT19A/FM7c ; hs;RL/cyo , dSor1;FRT19A/FM7c ; hs;[nls]/cyo, w[1118]; P{w[+mC]=GUS-p53}2.1 (stock # 6584; Bloomington *D. melanogaster* Stock Center; Bloomington, IN) and P{w[+mC]=GMR-hid}G1/CyO (stock # 5771; Bloomington *D. melanogaster* Stock Center). All dSor1 flies were received, as a gift, from Dr. Paez and Dr. Marenda. Flies in all stocks and genetic crosses were maintained at 25° C. The adult flies and larvae were fed on standard cornmeal-molasses-agar medium with yeast. Proprionic acid was used as a food preservative and all cooked food in vials was stored in a refrigerator. Ingredients of fly food are summarized in Table 1 below. Also, the food vials were allowed to reach room temperature (25° C) prior to use for flies.

Ingredients	Uses
Corn meal, molasses and dry Yeast powder	Source of Carbohydrates, proteins and Energy
Tegosept (ethyl 4-hydroxybenzoate), Proprionic acid	Fly food preservatives
Agar powder	Solidifying agent
Water	Solvent

Table1: Fly food ingredients and their uses.

Genetic crosses

To test our hypothesis we performed genetic crosses between flies deficient for MEK/MAPK kinase (dSor1 ; FRT 19A /FM7c), (dSor1 ; FRT 19A /FM7c; hs:RL/cyo), (dSor1 ; FRT 19A /FM7c; hs:[nls]/cyo) and flies carrying wild type ORF of p53 under the control of "GUS" balancer (W[1118] ; P {w[+mc]=GUS p53}2.1) and flies carrying mutation in hid gene (P {w[+mc]=GMR hid}G1/cyo). As MEK mutation is X linked, in all above genetic crosses, virgin females were selected from dSor1 lines to mate with males from W[1118] ; P {w[+mc]=GUS p53}2.1 and P {w[+mc]=GMR hid}G1/cyo. Figure 7 on page 26 represents scheme of all genetic crosses we did.

Dissection

Third instar larvae were dissected to obtain retinal discs. In order to overexpress both MAPK and MAPK-nls within dSor1 clones, late third instar larvae were heat shocked at 37°C for 1 hour and then allowed to recover at room temperature for at least 1 hour, before dissecting them. Dissected retinal discs were immediately fixed in PLP-fix solution (**Paraformaldehyde-Lysine-Periodate**) for 20 minutes and then proceed to immunostaining.

Eye Analysis and Clones

For each genetic cross third instar larvae were selected and kept in a phosphate solution for 10-15 minutes before beginning actual dissection. Then male larvae were isolated based on the gonad size. The gonad is located in near 5th abdominal segment and it is a transparent ball like organ embedded within the fat body. The male gonad is much larger than female's and leaves a clear spherical hole (appears

like transparent light-bulb) in the opaque fat body. In female larvae it is not apparent as in males.

The larvae were d ecapitated and eye discs were separated. Separated eye discs were incubated for 20 minutes in PLP-fix solution in order to harden (fix) the tissue. Finally eye discs were cleaned of other tissues and incubated for 10-15 minutes in wash solution (Triton X-100). Isolated eye discs were then subjected to Immuno staining. The retina discs were mounted in Vectashield (Vector Labs, H-1000, Burlingame, CA).

With the aid of immunohistochemistry, eye discs were analyzed for cell division and cell growth. Primary antibody (rat anti-ELAV) was used to observe cell differentiation. The numbers of clones per retina were counted for an average of 8 retinas or more for each three genetic crosses. The signals for phosphorylated/activated MAPK (dpERK), PH3 (cell divisions) and p53 were also examined through immunohistochemistry. Every time retinas were immunostained with two primary antibodies and two corresponding secondary antibodies. This technique is called double-immunostaining. Every time one of the two primary antibodies was rat anti-ELAV which helps to visualize dSor1 clones (area of our interest that indicates MAPK Kinase mutation). The second primary antibody, used along with ELAV, was either rabbit anti-p53, rabbit anti-PH3 (cell division) or mouse anti-dpERK (activated MAPK). Each experiment was done in triplicate or more. A clone is a group of *identical* cells that share a *common ancestry*, meaning are derived from the same mother cell. Here, the term clone is referred to those groups

of cells which are genetically identical in terms of carrying mutant dSor1 gene construct.

Immuno stained eye discs were observed under phase contrast microscope (Nikon Eclipse TE2000-S). The magnifications used were 20X and 40X. Images were captured with the help of camera attached to microscope and Image-Pro Express software (Media Cybernetics, Inc., Bethesda, MD). As mentioned above, each retina was stained with two primary antibodies and hence had two images for signal of each antibody used. One of the two images was of cell differentiation (ELAV signals) and other was of either p53, PH3 (cell division) or dpERK (activated MAPK). Finally to locate p53 and dpERK signals inside the dSor1 clones we superimposed two images with the help of photo editing software. The photo editing software we used is FotoFlexer (Arbor Labs, Inc., Berkeley, CA), a free online photo editing software available at http://fotoflexer.com/app/index.php?option=18. Figures 8, 9 and 14 - 18 on pages 27, 28 and 39-43 represent some of the images taken and edited as mentioned above.

Immunohistochemistry and Antibodies

Details of primary and secondary antibodies used are described in Table 2 on page 30.

Antibody	Source	Stock ID	Company	Dilution	Used for
Primary Antibodies					
anti-ELAV	Rat	7E8A10	Developmental Studies Hybridoma Bank, Iowa City, Iowa.	1:1000	To detect photorece ptor differentia tion
anti-pMAPK (anti-dpERK)	Mouse	M8159	Sigma-Aldrich, St. Louis, MO.	1:50	To identify phosphory lation of MAPK
anti-pH3	Rabbit	9701	Cell Signaling Technologies, Danvers, MA.	1:1000	To identify mitotic events
anti-p53	Rabbit	SC-25767	Santa Cruz Biotechnology, Santa Cruz, CA.	1:50	To detect presence of Dmp53
Secondary Antibodies					
anti-rat TRIT C	Goat	111-116-144	Jackson Immuno-Research Laboratories, West Grove, PA.	1:1000	To identify anti-rat primary antibody signals
anti-mouse FITC	Goat	115-095-003		1:50	To identify anti-mouse primary antibody signals
anti-rabbit FITC	Goat	111-095-003		1:50	To identify anti-rabbit primary antibody signals

Table 2: Summary of all primary and secondary antibodies used.

Results

Experiment strategy

To check the expression patterns of p53 and regulatory interactions between MAPK, p53 and hid, we crossed female flies of dSor1 mutants (dSor1 alone, dSor1 with MAPK under heat shock promoter control and dSor1 with MAPK-nls under heat shock promoter control) with two different male flies: one containing p53 gene under P element control, and the other containing hid gene under P element control. Then, from F1 progeny third instar larval retina were dissected and subjected to study for cell division (number of mitotic events/ PH3 signals), cell differentiation (ELAV signals) and p53 expression (P53 signals). Figure 7 below illustrates the scheme of our experiment setup.
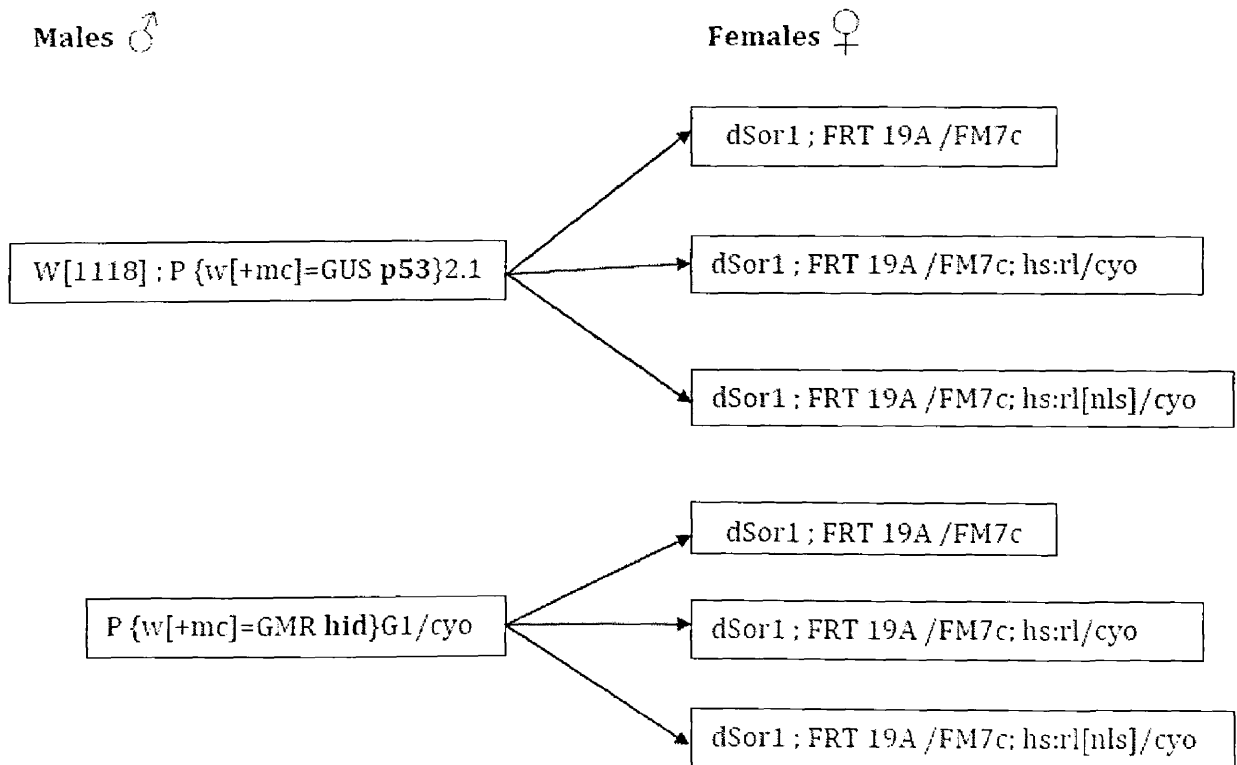
Figure 7: Schematic representation of experiment setup.

Expression of mutant MAPK Kinase (MEK) /dSor1 resulted in clones in developing eye (retina) of *D. melanogaster*.

The dSor1 is referred to as **Downstream of raf1** which is MAPK Kinase (MEK). Following Raf mediated activation, MAPK Kinase (MEK) phosphorylates and activates its downstream target MAP Kinase (ERK). As mentioned earlier in the introduction, phosphorylation is crucial for cell cycle progression. In dSor1 transgenic flies, when expressed in homozygous condition, MAPK Kinase (MEK) is mutated such that it cannot phosphorylate its downstream target, MAP Kinase. Therefore, this mutation stops cell cycle progression that results in cluster(s) of non-dividing cells, which here we referred to as clone(s). We immunostained retinas with anti-ELAV antibody, which allowed us to visualize cell differentiation upon application of fluorescence secondary antibody (TRITC) specific to anti-ELAV; this helped in locating clones. We found large areas of clones in developing eye/retina which confirmed expression of mutant MAPK Kinase (MEK) in those regions (Figure 8a). As shown in Figure 9 no clones were observed in wild type normal retinas.
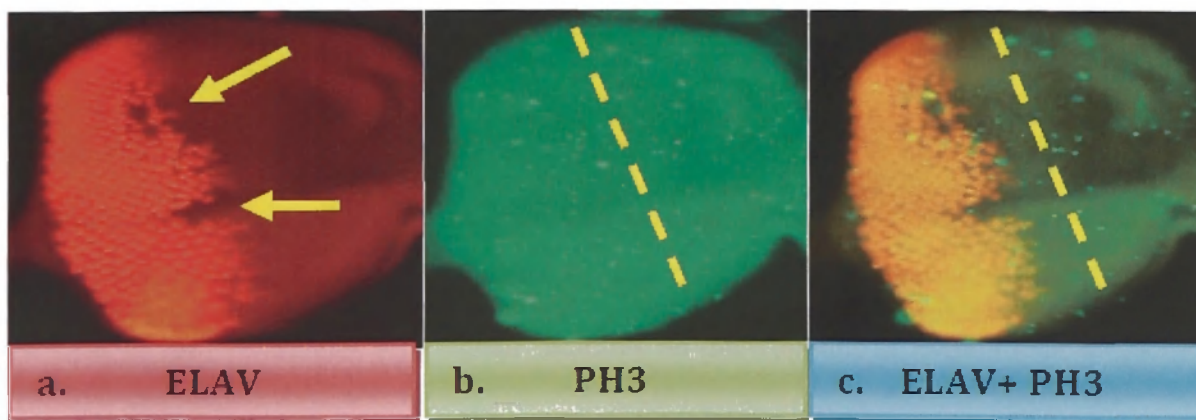
Figure 8: dSor1 X p53 larval retina with somatic mosaic clones of the *Drosophila* gene dSor1, marked with arrows; (b-c) shows interrupted PH3 signals in furrow, marked with dashes [compared to Figure 9b].
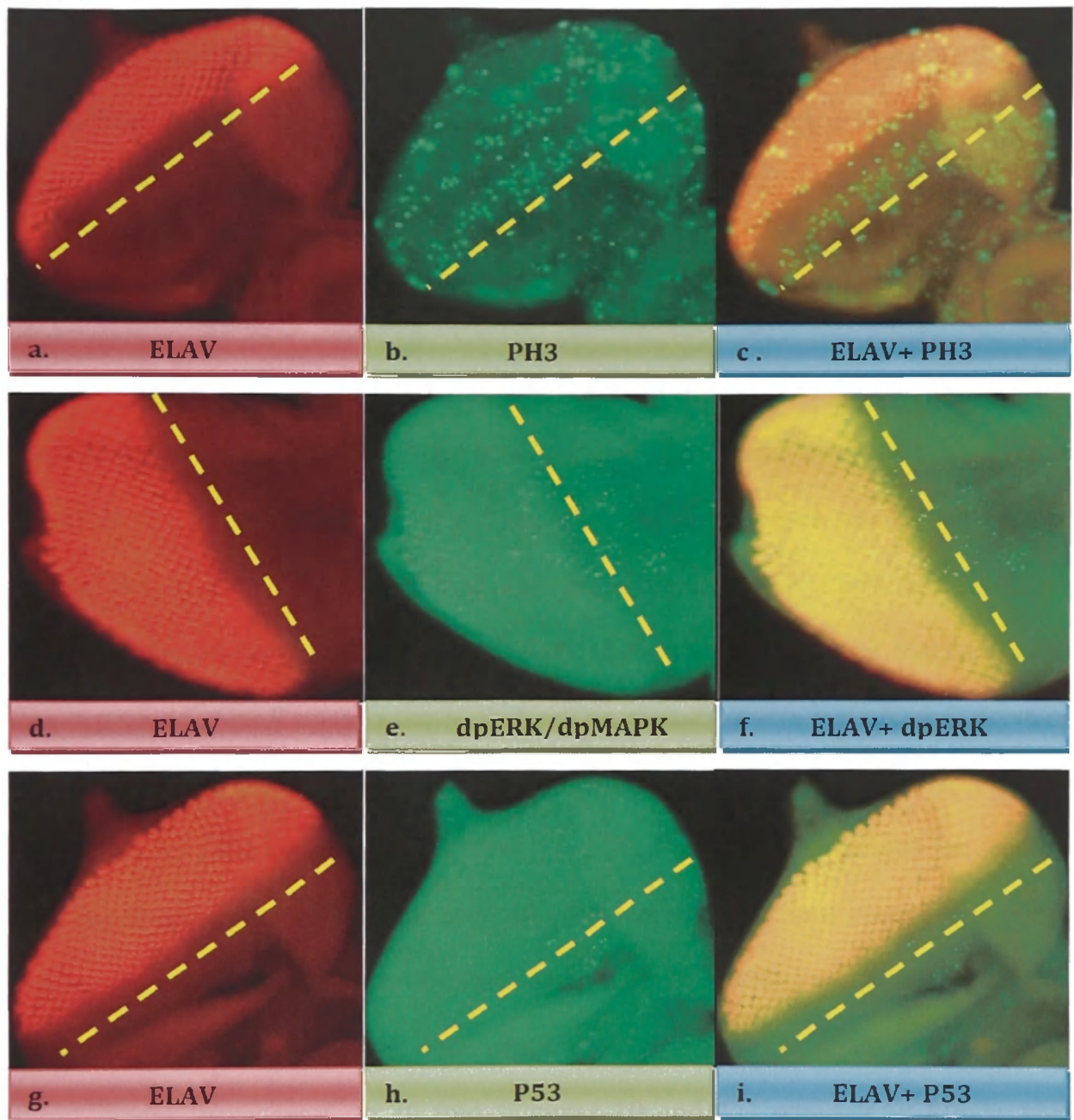
Figure 9: Normal retina development in *Drosophila*: (a, d and g) cell differentiation (ELAV), (b-c) cell division (PH3 signals), (e-f) pMAPK signals and (h-i) p53 signals. Furrow is marked with dashes in each case. In (9b) continuous PH3 signals indicates no interruptions in mitosis (cell division) in furrow.

Nuclear form of non-phosphorylated MAP Kinase is able to initiate cell division but not cell differentiation.

To estimate cell division we counted the number of mitotic events in dSor1 clones via number of pH3 signals inside the clones in developing retina of third instar male larvae of *D. melanogaster*. Phosphorylated **Histone-3** is abbreviated as **pH3**. Histone-3 phosphorylation is an important post-transcriptional modification in nucleosome structure during mitosis. Moreover, phosphorylation of Thr3 (Threonine3) of histone H3 is highly conserved among many mammalian species. Therefore, immunostaining with phospho-specific antibodies reveals mitotic phosphorylation of H3 Thr3 in prophase of mitosis and hence number of mitotic events. We observed and compared pH3 signals inside the dSor1 clones under following three different conditions; i) dSor1 mutant alone, ii) dSor1 mutation with overexpressed MAP Kinase under heat shock promoter control (cytoplasmic form of MAPK) and, iii) dSor1 mutation and overexpressed MAPK-nls (Nuclear form of MAPK) under heat shock promoter control. MAPK overexpression was induced by heat shock treatment at 37°C for one hour followed by one hour recovery at room temperature (as described earlier by Marenda *et al.*, 2006; Paez *et al.*, 2010). As shown in Tables 3 and 4, our data suggest that the number of mitotic events in dSor1 clones increases 5 to 20 fold (Figures 10 and 11) when MAP Kinase was overexpressed and forced to move into the nucleus without phosphorylation, i.e. when MAPK-nls was over expressed. Actual pictures for PH3 immunostaining are shown in Figures 14-18, Panels a, b and c (Pages 39-43). These results support previously reported findings that MAP Kinase phosphorylation is not essential for

cell division, but is for cell growth in *D. melanogaster* (Paez *et al.,* 2010). Although dSor1 clones are results of inactive MAPK due to mutant MEK, we checked the level of active MAPK (dpMAPK/ dpERK) in all cases which were found to be negligible or reduced. This was done by counting the dpERK signals in dSor1 clones. However, the presence of active/phosphorylated MAPK (dpERK) was observed when flies carried hid gene under P element control with dSor1 mutation (Figures 16-18, Panels d, e and f on Pages 41-43).

Cross	dSor1 X p53	hs:rl[nls] X p53
Primary Antibodies used	ELAV + pH3	
# of signals observed inside the clones	7	53
# of Clones observed	16	23
# of pH3 signals per Clone	0.44	2.30

Table 3: Represents Comparison of mitotic events in dSor1 clones alone and MAPK-nls overexpression when both in addition carry p53 gene under P element control.
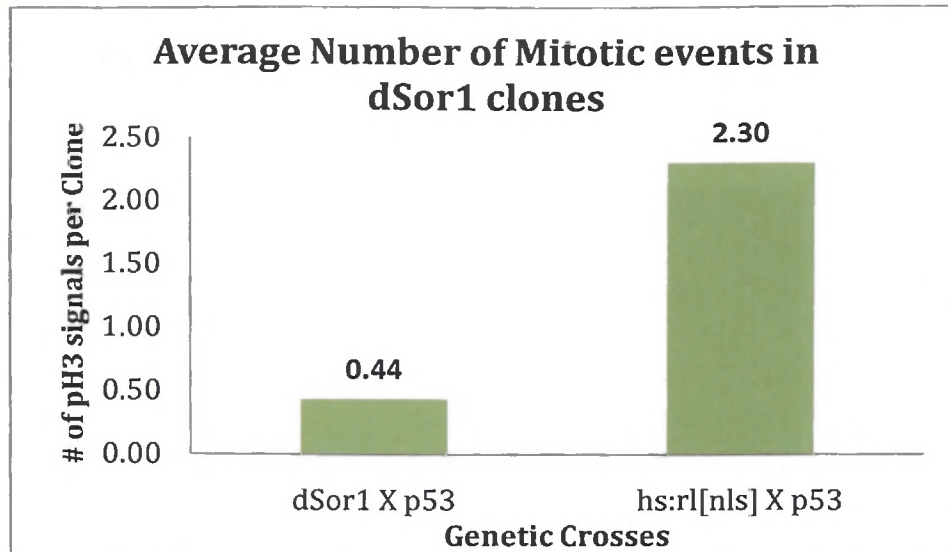
Figure 10: Column graph representing comparison of average number of mitotic events (PH3 signals) in dSor1clones alone and MAPK-nls when both in addition carry p53 gene under P element control.

Cross	dSor1 X hid	hs:rl[nls] X hid
Primary Antibodies used	ELAV + pH3	
# of signals observed inside the clones	5	90
# of Clones observed	19	16
# of pH3 signals per Clone	**0.26**	**5.63**

Table 4: Comparison of mitotic events in *dSor1* clones alone and with MAPK-nls overexpression when combined with hid gene under P element control.
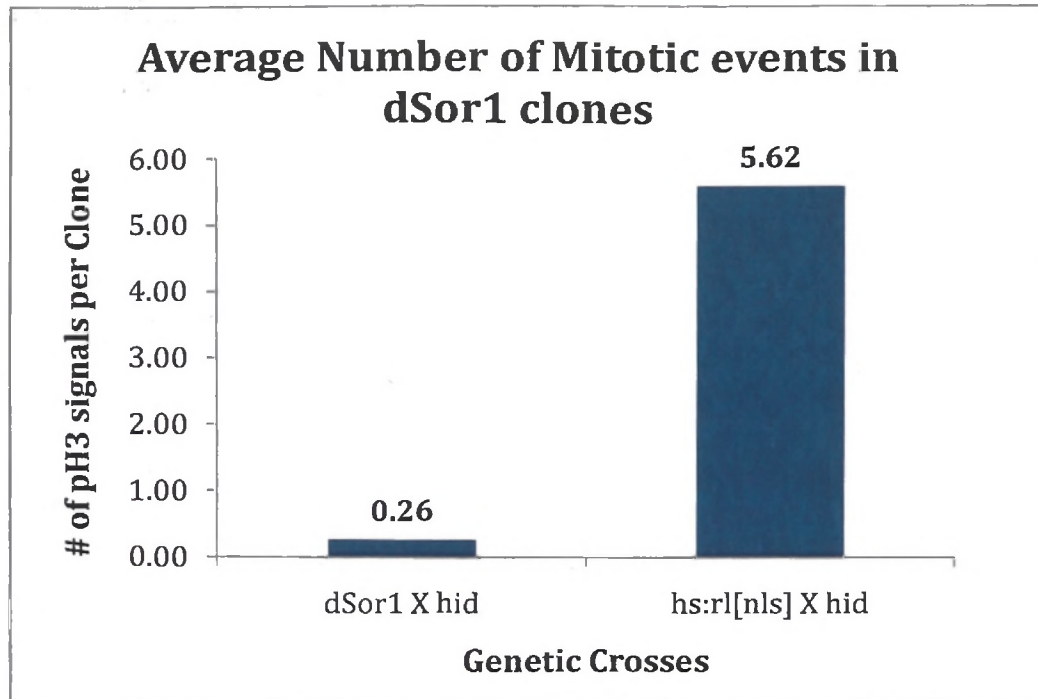
Figure 11: Comparison of average number of mitotic events (PH3 signals) in dSor1clones alone and with MAPK-nls when combined with hid gene under P element control.

Active MAPK is necessary to activate p53 gene expression in developing eye of

D. melanogaster.

In 2001, Agarwala *et al.,* reported 5 to 8 fold increase in p53 mRNA levels upon introduction of activated Ras into wild type human cells. They also reported a decrease in protein levels of p53 and p21 in wild type human cells (H10) treated with MEK inhibitor. In our study MEK is genetically inactive. We asked what would happen to p53 expression if MAPK is inactive and also when that inactive MAPK is forced to move into the nucleus. To test this we crossed the male flies having p53 gene (under P element control) with female flies carrying MEK mutation (dSor1). We dissected retinal discs from third instar male larvae of F1 generation and Immuno-stained with p53 antibody. We counted the number of signals for p53

inside the dSor1 clones. We observed and compared the number of p53 signals with overexpressed MAPK and MAPK-nls. We found no or negligible counts of p53 signals inside dSor1 clones, even when MAPK was overexpressed in cytoplasmic as well as nuclear form. However, p53 signals were observed in dSor1 mutants containing hid gene under P element control (Table 7, Figure 13). As mentioned earlier, and shown in Table 8, active MAPK signals (dpERK) were also observed in dSor1 mutants containing hid gene under P element control. These results, together, explain the presence of positive p53 signals inside dSor1 clones. No p53 signals were observed without corresponding active/phosphorylated MAPK (dpERK) signals. Our data show that p53 expression is not active unless phosphorylated/active MAPK is present (Table 5, Figure 12). Thus, w e here for the first time report that phosphorylated/active MAPK is required to activate p53 gene expression in developing eye of *D. melanogaster.*

Cross	dSor1 X p53	hs:rl[nls] X p53
Primary Antibodies used	ELAV + P53	
# of signals observed inside the clones	3	0
# of Clones observed	15	15
# of P53 signals per Clone	**0.2**	**0**

Table 5: Comparison of p53 expression events in dSor1 clones alone and with MAPK-nls overexpression when combined with p53 gene under P element control.
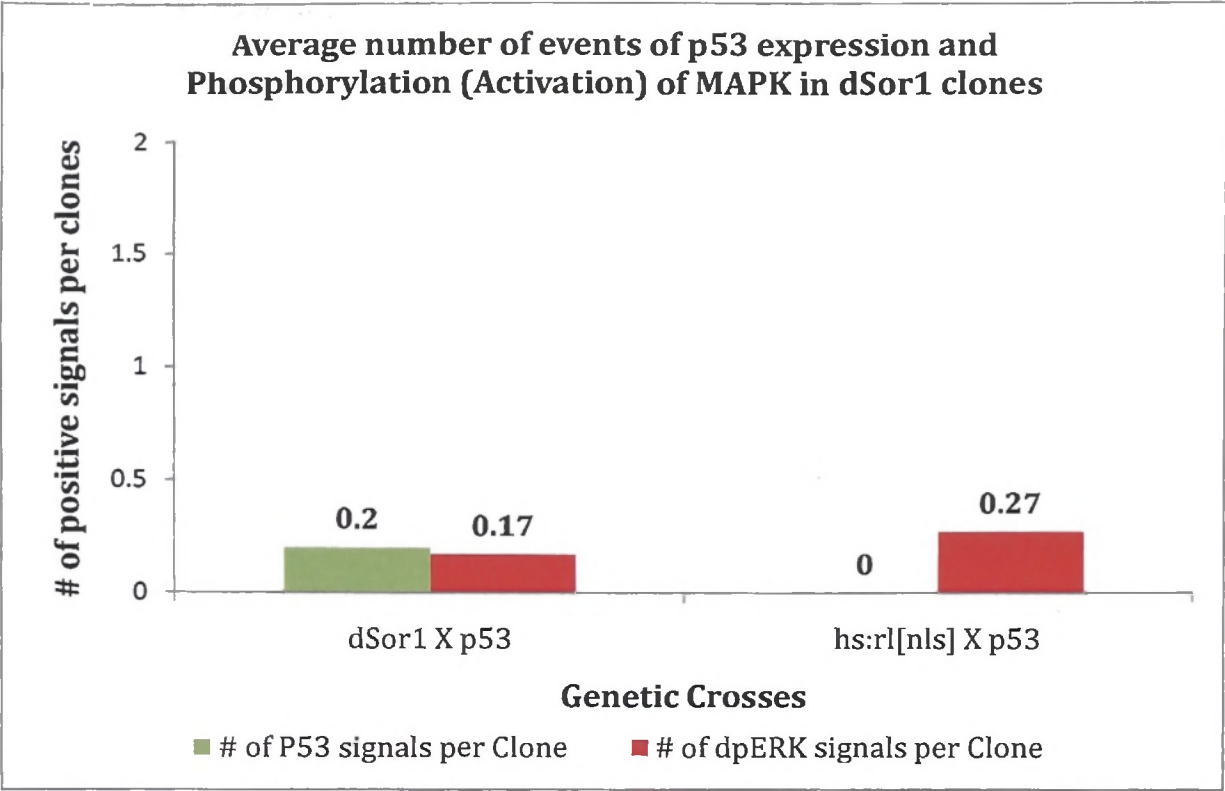
**Figure 12:** Column graph representing comparison of average number of p53 expression events (Green columns) and phosphorylation (activation) of MAPK events (Red columns) in dSor1clones alone and with MAPK-nls when combined with p53 gene under P element control.

Cross	dSor1 X p53	hs:rl[nls] X p53
Primary Antibodies used	ELAV + dpERK	
# of signals observed inside the clones	6	3
# of Clones observed	35	11
# of dpERK signals per Clone	**0.17**	**0.27**

Table 6: Comparison of phosphorylation (activation) of MAPK events in dSor1 clones alone and with MAPK-nls overexpression when combined with p53 gene under P element control.

**Nuclear form of non-phosphorylated MAPK inhibits the expression of p53.**

We compared the p53 signals in clones of dSor1 mutants containing hid gene under P element control and inactive MAPK. Our data show no p53 signals inside dSor1 clones when non-phosphorylated MAPK-nls is overexpressed in flies carrying hid gene under P element control, even though some level of active MAPK is present inside the dSor1 clones (Table 7, Figure 13). However, when the cytoplasmic form of non-phosphorylated MAPK was overexpressed in flies carrying hid gene under P element control, p53 signals were recorded inside the dSor1 clones (Table 7, Figure 13). Also, as we mentioned above, active MAPK can activate p53 gene expression. Here, when we forced inactive MAPK into the nucleus, no p53 signal was observed. Similar results were obtained even when non-phosphorylated MAPK-nls was overexpressed in flies carrying the p53 gene under P element control (Table 5, Figure 12). Overall, these results may indicate negative regulation of p53 gene expression by a nuclear form of non-phosphorylated MAPK. Thus, for the first time, we report here that a nuclear form of non-phosphorylated MAPK inhibits the expression of p53. Figures 14-18, on pages 39-43, show actual pictures for PH3 (Panels a-c) and p53 (Panels g-i) immunostaining.

Cross	dSor1 X hid	hs:rl X hid	hs:rl[nls] X hid
Primary Antibodies used	ELAV + P53		
# of signals observed inside the clones	31	36	0
# of Clones observed	32	11	17
# of P53 signals per Clone	**0.97**	**3.27**	**0**

Table 7: Comparison of p53 expression events in dSor1 clones subjected to MAPK alone and with MAPK-nls overexpression when combined with hid gene under P element control.

Cross	dSor1 X hid	hs:RL X hid	hs:rl[nls] X hid
Primary Antibodies used	ELAV + dpERK		
# of signals observed inside the clones	65	19	38
# of Clones observed	32	5	12
# of dpERK signals per Clone	**2.03**	**3.8**	**3.17**

Table 8: Comparison of phosphorylation (activation) of MAPK events in dSor1 clones subjected to MAPK alone and with MAPK-nls overexpression when combined with hid gene under P element control.
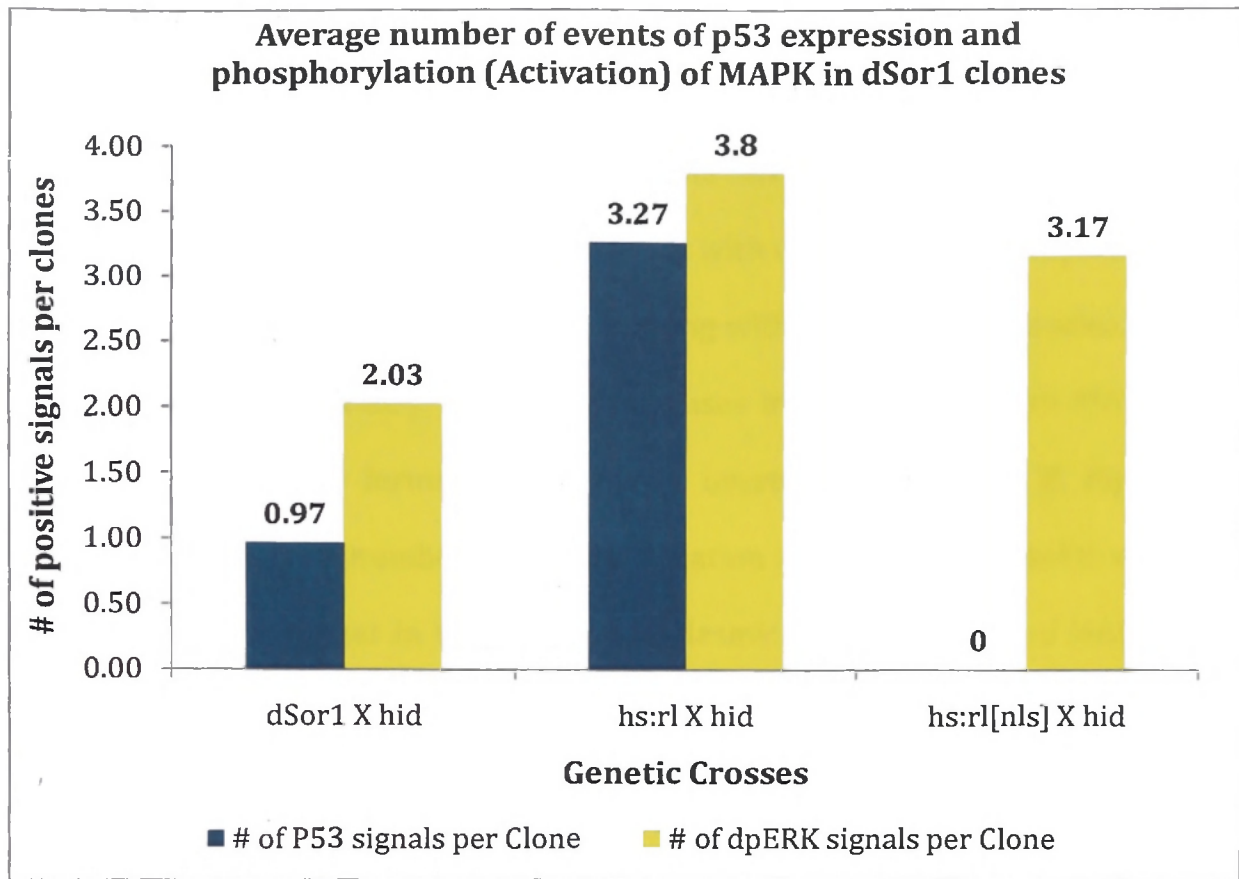
Figure 13: Comparison of average number of p53 expression events (Blue columns) and phosphorylation (activation) of MAPK events (Yellow columns) in dSor1 clones subjected to MAPK alone and with MAPK-nls overexpression when combined with hid gene under P element control.

Presence of hid gene under P-element control may lead to activation/phosphorylation of MAPK via an unknown protein kinase.

In 1998, Bergmann *et al.* reported that an activated MAPK directly phosphorylated *Drosophila* hid which resulted in inhibition of hid-mediated apoptosis. *Drosophila* hid is a pro-apoptotic target of p53 (Steller, 2000; Brodsky *et al.*, 2004; Bilak, 2009). The hid is one of the RIPD (**R**adiation **I**nduced **p**53 **D**ependent) genes (Akdemir *et al.*, 2007). The hid is also a major effector of Dmp53-induced apoptosis in the *Drosophila* eye and it is transcriptionally induced when Dmp53 is active (Fan *et al.*,

2009). We counted the number of activated/phosphorylated MAPK (dpERK) signals and found the presence of active MAPK inside the dSor1 clone, at different levels, when the hid gene under P element control was carried in dSor1 mutant flies with i) only dSor1 mutation, ii) dSor1 mutation along with overexpressed cytoplasmic form of inactive MAPK, and iii) dSor1 mutation along with overexpressed nuclear form of inactive MAPK (MAPK-nls). Noticeable increases in signals for active MAPK were recorded when either forms of MAPK were overexpressed (Table 8, Figure 13). However, an average number of phosphorylation (activation) of MAPK events in dSor1 clones was higher in the case of cytoplasmic overexpression of MAPK when compared to MAPK-nls (Figure 13). This indicates that not all MAP Kinase gets phosphorylated because of its forced entry into the nucleus via nuclear localization signal (nls). Also, no p53 expression was observed when MAPK-nls was overexpressed. This supports our earlier observation of negative regulation of p53 gene expression when non-phosphorylated MAPK is forced to move in to the nucleus.

Figure 14: dSor1 X p53 larval retina with somatic mosaic clones of the *Drosophila* gene dSor1, marked with arrows. dSor1 encodes the mutant MAPK kinase (MEK) protein, leading to inactive MAPK. (a, d, g) ELAV measures the differentiation of photoreceptor neurons and indicates a loss of differentiation in these clones. (b-c) shows loss of mitotic events inside the dSor1 clones (PH3 signals) indicating a defect in MAPK phosphorylation and cellular division within these clones, (e-f) shows loss of pMAPK inside the dSor1 clones, (h-i) shows loss of p53 expression inside the dSor1 clones. c, f and i are superimposed images of their corresponding images presented on their left side. Superimposing was done using free online photo editing software called "FotoFlexer".

Figure 15: hs:rl[nls] X p53 larval retina with somatic mosaic clones of the *Drosophila* gene dSor1, marked with arrows. hs:rl[nls] encodes MAPK protein that is overexpressed under heat shock promoter control and [nls] (nuclear localization signal) forced nuclear translocation of MAPK. (a, d, g) ELAV measures the differentiation of photoreceptor neurons and indicates a loss of differentiation in these clones. (b-c) shows mitotic events inside the dSor1 clones (PH3 staining), (e-f) shows loss of pMAPK inside the dSor1 clones, (h-i) shows loss of p53 expression inside the dSor1 clones. c, f and i are superimposed images of their corresponding images presented on their left side. Superimposing was done using free online photo editing software called "FotoFlexer".
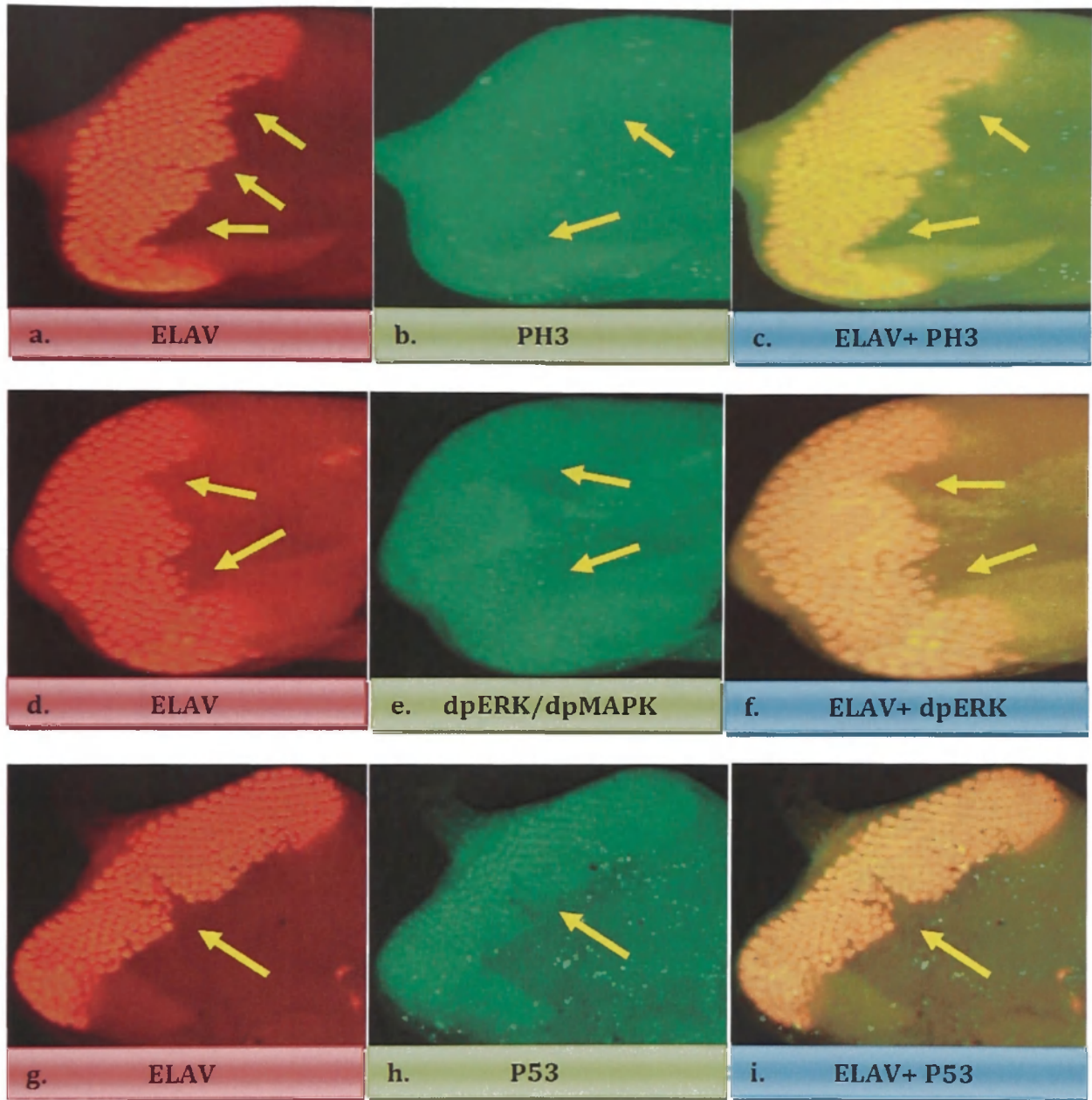
Figure 16: dSor1 X hid larval retina with somatic mosaic clones of the *Drosophila* gene dSor1, marked with arrows. *dSor1* encodes the mutant MAPK kinase (MEK) protein, leading to inactive MAPK. (a, d, g) ELAV measures the differentiation of photoreceptor neurons and indicates a loss of differentiation in these clones. (b-c) shows loss of mitotic events inside the dSor1 clones (PH3 staining) indicating a defect in MAPK phosphorylation and cellular division within these clones, (e-f) shows pMAPK signals inside the dSor1 clones, (h-i) shows p53 expression inside the dSor1 clones. c, f and i are superimposed images of their corresponding images presented on their left side. Superimposing was done using free online photo editing software called "FotoFlexer".
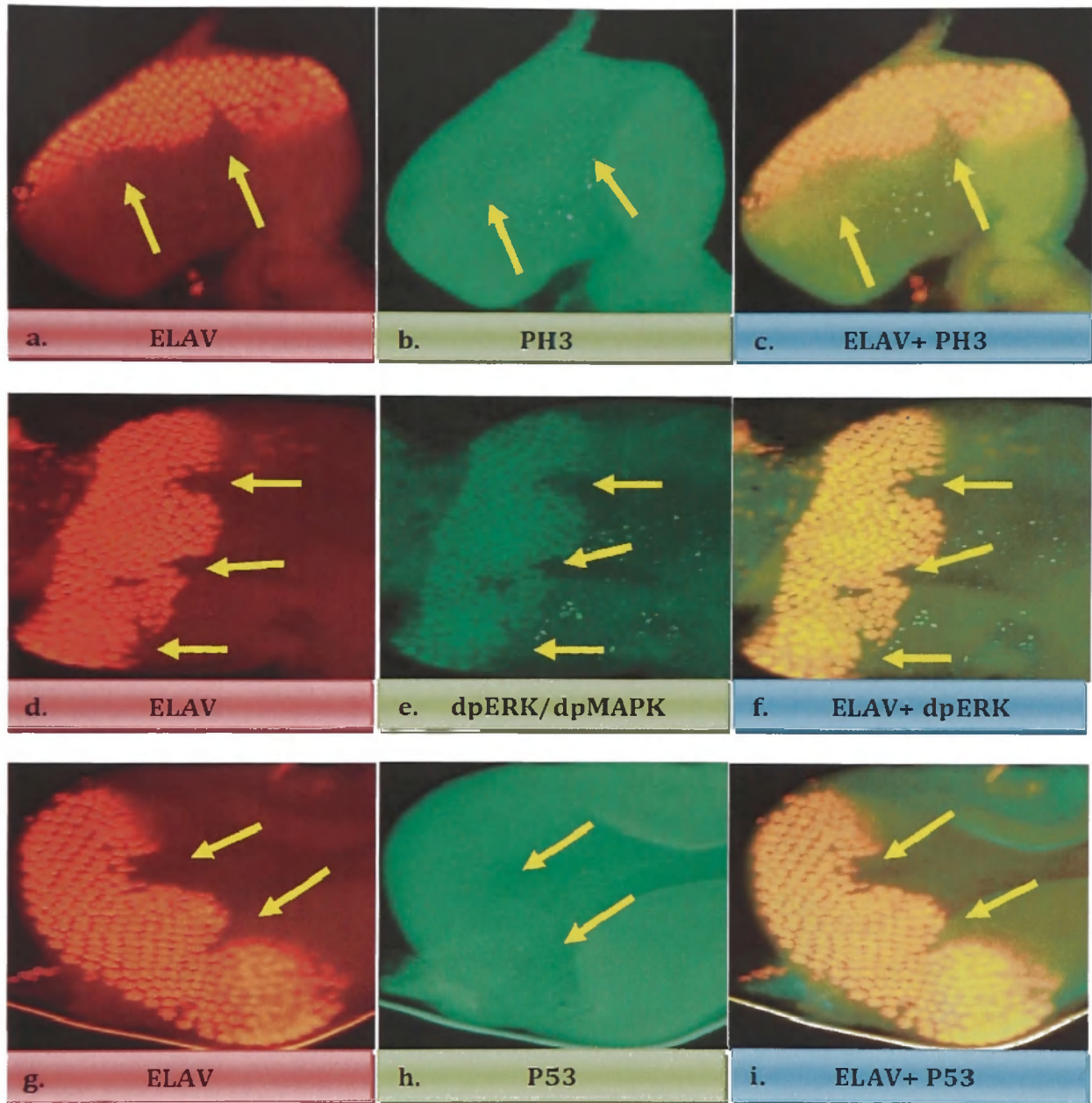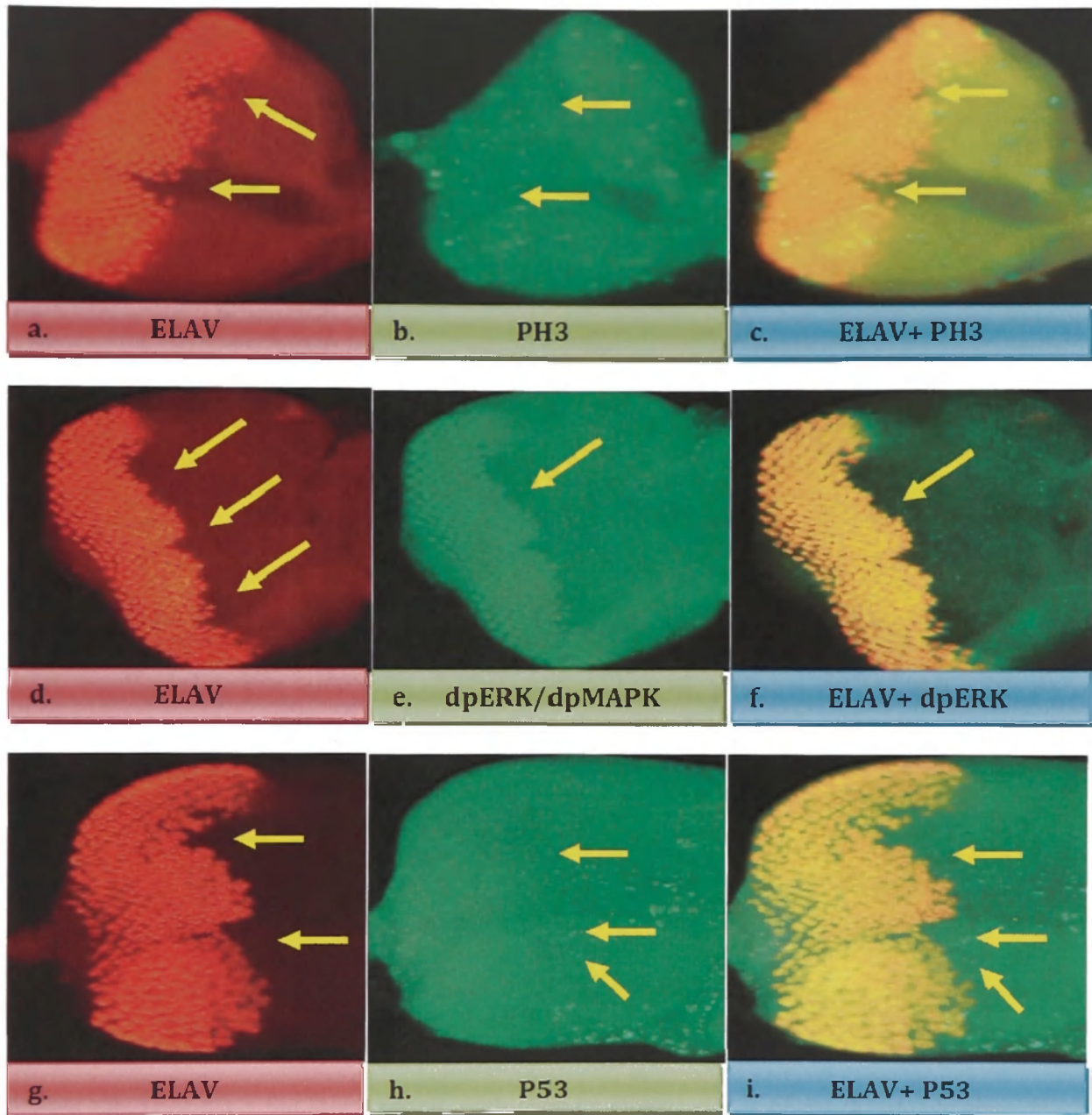
Figure17: hs:rl X hid larval retina with somatic mosaic clones of the *Drosophila* gene dSor1, marked with arrows. hs:rl encodes MAPK protein that is overexpressed under heat shock promoter control. (a, d, g) ELAV measures the differentiation of photoreceptor neurons and indicates a loss of differentiation in these clones. (b-c) shows loss of mitotic events inside the dSor1 clones (PH3 signals) indicating a defect in cellular division within these clones, (e-f) shows pMAPK inside the dSor1 clones, (h-i) shows some p53 signals inside the dSor1 clones. c, f and i are superimposed images of their corresponding images presented on their left side. Superimposing was done using free online photo editing software called "FotoFlexer".
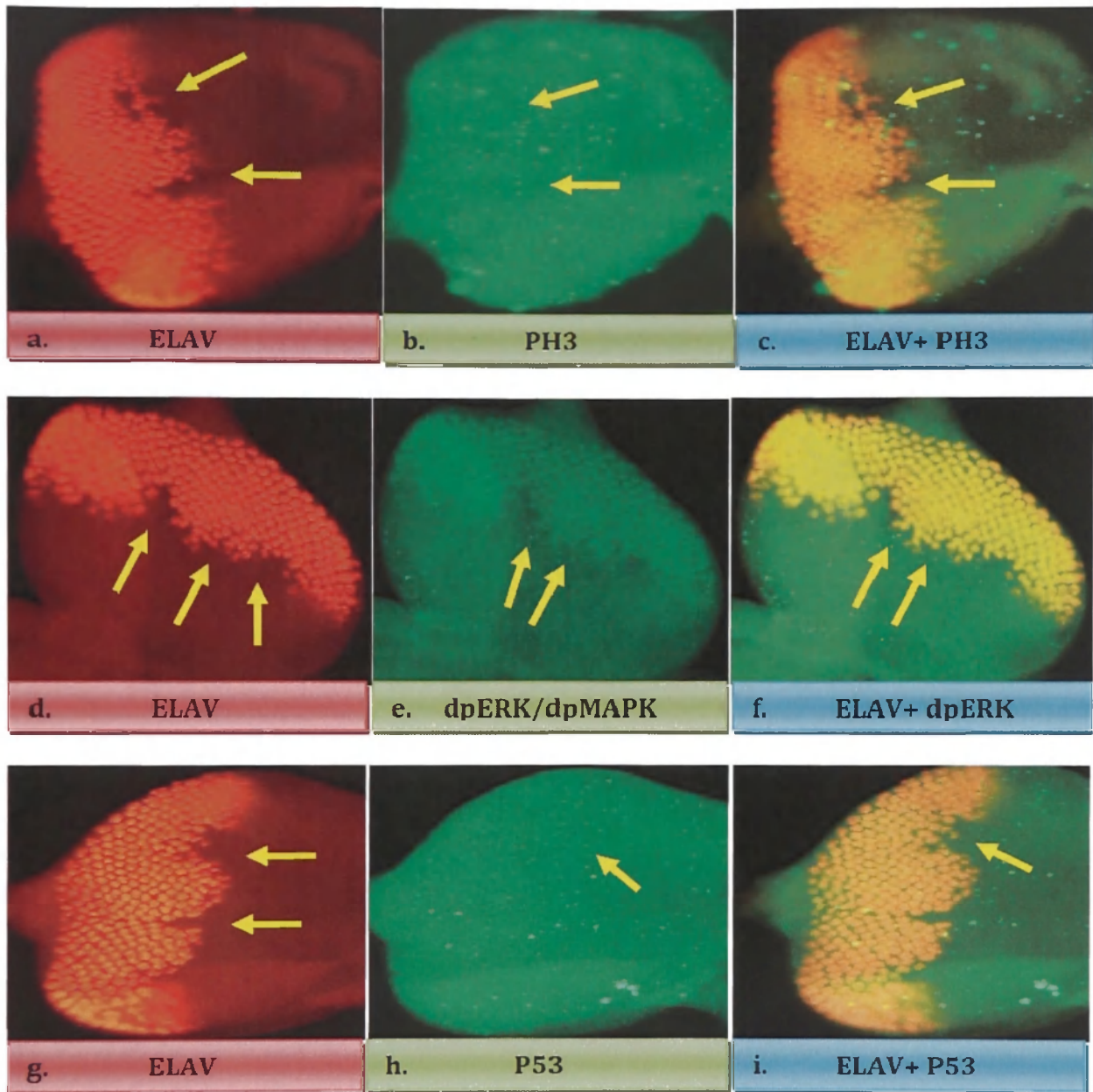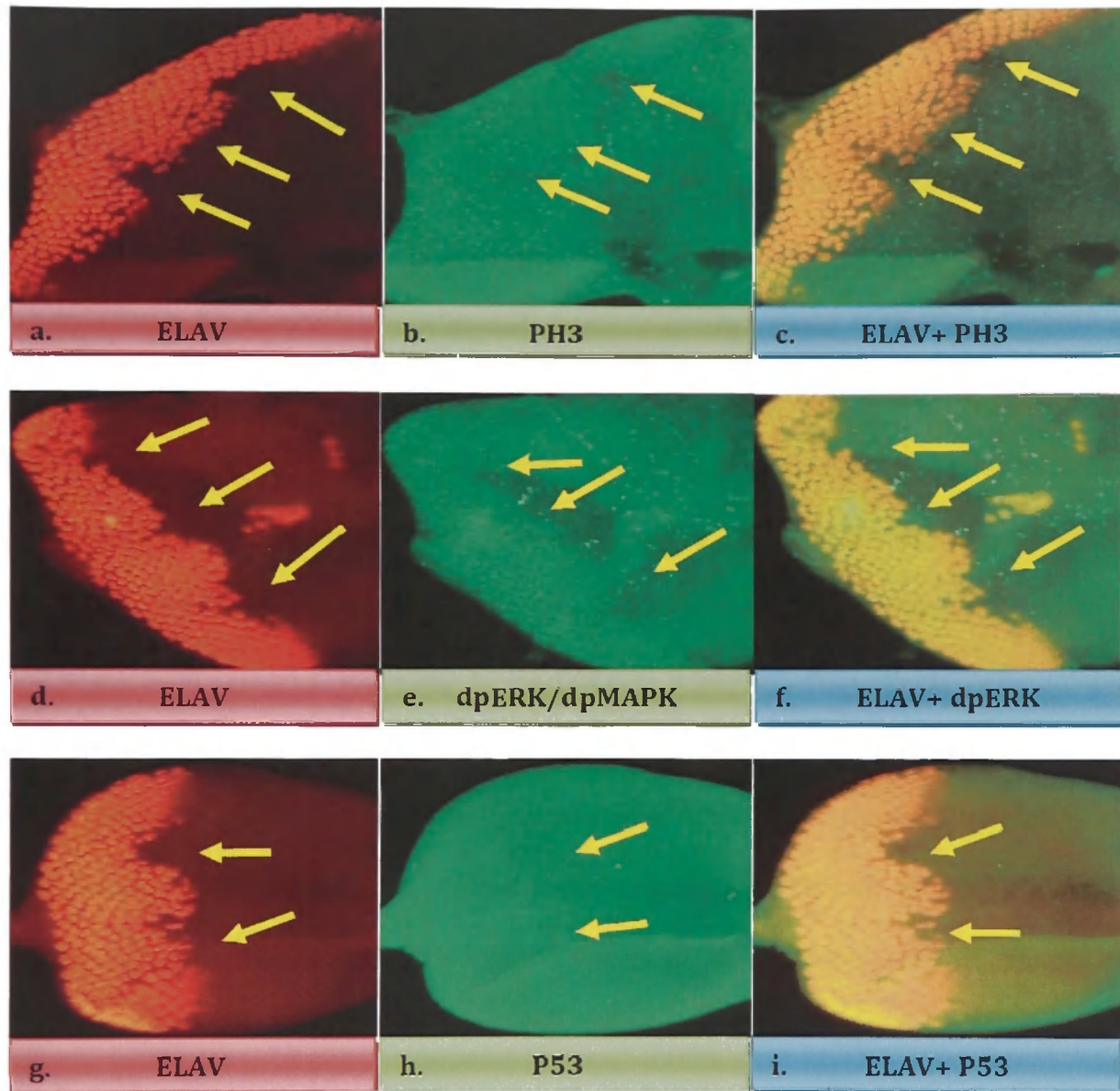
Figure 18: hs:rl[nls] X hid larval retina with somatic mosaic clones of the *Drosophila* gene dSor1, marked with arrows. hs:rl[nls] encodes MAPK protein that is overexpressed under heat shock promoter control and [nls] (nuclear localization signal) forced nuclear translocation of MAPK. (a, d, g) ELAV measures the differentiation of photoreceptor neurons and indicates a loss of differentiation in these clones. (b-c) shows mitotic events inside the dSor1 clones (PH3 staining), (e-f) shows pMAPK inside the dSor1 clones, (h-i) shows loss of p53 expression inside the dSor1 clones. c, f and i are superimposed images of their corresponding images presented on their left side. Superimposing was done using free online photo editing software called "FotoFlexer".

Discussion

MAP Kinase pathway regulates many diverse cellular processes including cell division, growth and differentiation. In fact, misregulation of MAPK pathway is associated with approximately 25% of human tumors (Hanahan *et al.*, 2000). A deeper understanding of cell cycle regulations is required to design more effective treatment for human tumors. Although much is known about MAP Kinase pathway, it is not fully understood yet. Scientists are discovering novel regulatory mechanisms involved in MAPK regulation every year. Our data suggest that nuclear localization of non-phosphorylated MAPK (ERK) is another novel mechanism that may initiate cell division in the developing eyes of *Drosophila* as reported earlier by Marenda *et al.*, (2006) and Paez *et al.*, (2010). However, this non-phosphorylated MAPK is not able to initiate expression of all the genes required for cell growth and differentiation. Therefore, phosphorylation of MAPK is still required for complete cell growth and differentiation (Marenda *et al.*, 2006; Paez *et al.*, 2010). Earlier research in flies and mammals suggests that both cell division and cell growth are regulated by MAPK activation and its nuclear localization (Brunet *et al.*, 1999; Prober *et al.*, 2000). We found that nuclear localization of MAPK is sufficient to initiate cell division. However, this non-phosphorylated MAPK is unable to initiate cell differentiation and cell growth. Overall, this indicates limited regulatory power of non-phosphorylated MAPK. In other words, non-phosphorylated MAPK cannot activate expression of all genes required for complete cell growth.

Earlier research also revealed that phosphorylation-mediated activation leads MAPK to dimerize and this dimerized, active MAPK enters the nucleus by active transport which is energy dependant (Cobb *et al.*, 2000; Brunet *et al.*, 1999). How does the non-phosphorylated MAPK localize in to nucleus? Nuclear localization signal (NLS/nls) sequence facilitates MAP Kinase nuclear translocation. NLS facilitates this transport by Ran (**Ra**s-related **N**uclear protein) dependent active transport mechanism (Matsubayashi *et al.*, 2001). This supports nuclear transport of non-phosphorylated MAP Kinase, when carrying the NLS sequence, in our model.

How does non-phosphorylated MAPK cause activation of transcription factors required for cell division? Recent studies have shown that ERK2 (MAPK) can directly bind to DNA and function as a transcriptional repressor for a γ-interferon induced gene (Hu, S., *et al.*, 2009). This suggests that MAPK, once in the nucleus, can directly interact with DNA. However, direct interactions with DNA do not always result in negative regulation. Therefore, we suggest that a similar mechanism may be followed to activate transcription factors required for cell division.

Another interesting result of our study is that MAPK gets phosphorylated in dSor1 clones when hid P element is present. Also, this phosphorylation level was higher in case of cytoplasmic overexpression of MAPK compare to MAPK-nls (nuclear form of MAPK). This indicates that phosphorylation of MAPK occurs in cytoplasm. In the case of MAPK-nls, MAPK moves into the nucleus before getting phosphorylated as it carries nuclear localization signal. How can MAPK get phosphorylated when its upstream activator MEK is inactive? Does hid activate MAPK or some other protein

kinase which further activates MAPK? Earlier research has shown that activated MAPK directly targets hid and inhibits its activity (Haining, 1999; Bergmann *et al.*, 1998). Therefore, direct activation of MAPK by hid is least likely. Here, we suggest that hid activates another protein kinase (other than MEK) that activates MAPK. However, further studies are necessary to reveal this novel mechanism.

Perhaps the most important result of our research is that the active MAPK is required for p53 (tumor suppressor protein). Moreover, non-phosphorylated MAPK, when localized into the nucleus, showed negative regulation of p53 gene expression. How does non-phosphorylated MAPK negatively regulate p53 gene expression? Recent studies have shown that ERK2 (MAPK) can directly bind to DNA and function as a transcriptional repressor for γ-interferon induced gene (Hu *et al.*, 2009). Hence, we suggest that a similar mechanism could play a role in p53 gene expression regulation by non-phosphorylated MAPK-nls (ERK-nls). In other words, we suggest that non-phosphorylated MAPK, once in the nucleus, may bind directly to p53 regulatory gene sequence on DNA and function as a transcriptional repressor. Agrawal *et al.*, (2001), reported decreases in protein levels of p53 and p21 in wild type human cells (H10) treated with MEK inhibitor. In our case MEK is genetically inactivated. Therefore, having the similar condition we would also expect decrease in p53 protein level. This also suggests the possibility that active MAPK provides p53 protein stability. However, further studies are needed to examine these possibilities.

In conclusion, based on an earlier model proposed by Paez *et al.,* (2010, Figure 19.a), we proposed that expression of p53 gene is an important step next to the nuclear localization of active/phosphorylated MAPK for cell growth (Figure 19.b).
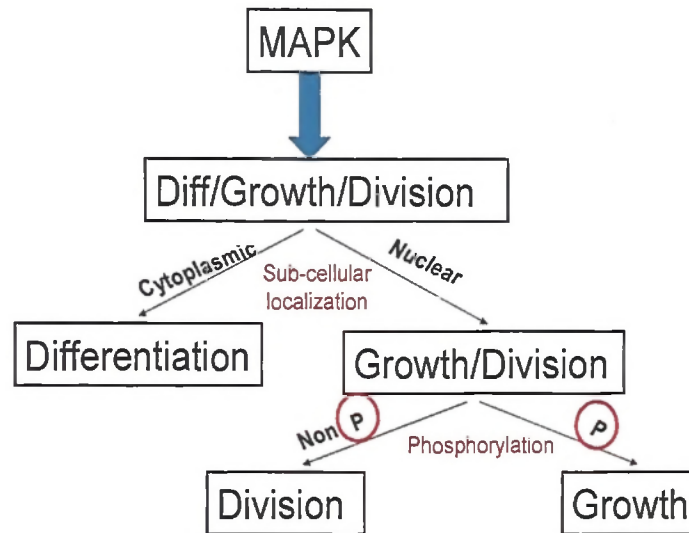

Figure 19.a: Previous MAPK signaling model suggested that MAP Kinase phosphorylation is dispensable for cell division, but not for cell growth in *D. melanogaster* (G.L.Paez ,*et al.,*2010).
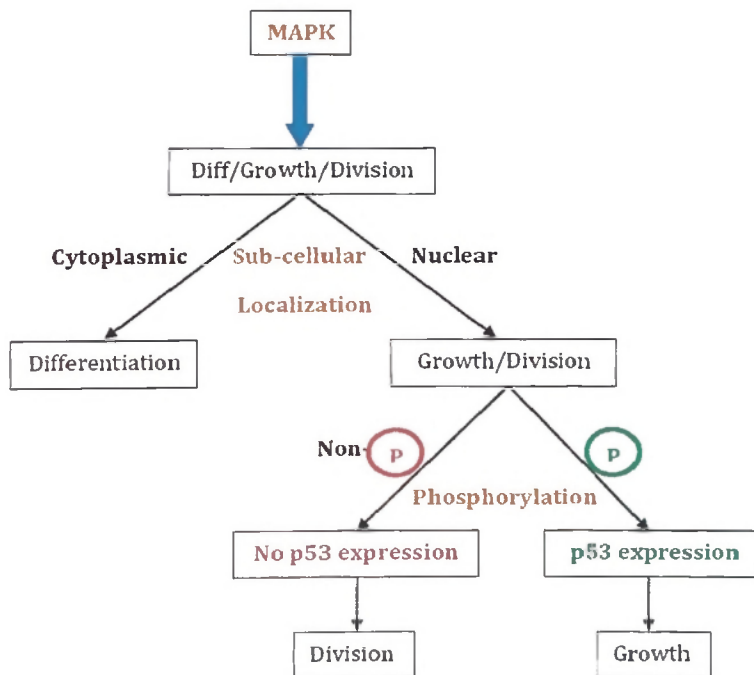

Figure 19.b: The final model for the regulation of MAPK as proposed by our study suggesting p53 gene expression is required for cell growth.

Conclusion

- *Drosophila melanogaster* is a very useful animal model to study signal transduction pathways and wide variety of biological mechanisms including genetic diseases in humans.

- Immunohistochemistry (IHC) is very convenient and effective technique to identify the distribution and localization of biomarkers, and differentially expressed proteins in different parts of a biological tissue.

- Nuclear localization of non-phosphorylated MAPK (ERK) is another novel mechanism that may initiate cell division in developing eye of *Drosophila*.

- Active/phosphorylated MAPK is required to activate p53 gene expression in developing eye of *Drosophila*.

- Nuclear form of non-phosphorylated MAPK inhibits the expression of p53 in developing eye of *Drosophila*.

- Presence of hid gene under P-element control may leads to activation (phosphorylation) of MAPK via unknown protein kinase other than MEK in developing eye of *Drosophila*.

- As mutation in MAP Kinase and p53 (tumor suppressor) are found to be associated with human cancer, deeper studies on their regulatory interactions will provide valuable contribution to design novel cancer therapy.

Future studies

- Nuclear localization of non-phosphorylated MAPK (ERK) may initiate cell division in developing eye of *Drosophila*. However, completion of cell division is still questionable. Does nuclear localized non-phosphorylated MAPK (ERK) activate transcription of all genes required for complete cell division? Chromatin Immunoprecipitation (ChIP) and RNA immunoprecipitation (RIP) can be used to determine this, both in flies and mammalian systems. DNA footprinting is another good option to determine DNA protein interactions.

- Nuclear form of non-phosphorylated MAPK inhibits the expression of p53 in developing eye of *Drosophila*. However, the exact mechanism of this negative regulation is not clear from our study. Recent studies have shown that ERK2 (MAPK) can directly bind to DNA and function as a transcriptional repressor for a γ-interferon induced gene (Hu *et al.*, 2009). Hence, we suggest that similar mechanism would play role in p53 gene expression regulation by non-phosphorylated MAPK-nls (ERK-nls). DNA footprinting and microarray techniques can be used to determine this mechanism.

- Our data also suggest that presence of hid gene under P-element control may leads to activation/phosphorylation of MAPK. However, the level of hid expression needs to be examined in all crosses we studied. Immunostaining with hid specific antibody can help to find out hid expression.

- Finally, all protein levels should be subjected to total protein assay and western blot analysis in order to match protein levels with the expression pattern we observed with Immunohistochemistry.

- After confirming protein levels with expression patterns, if they match, the same studies can be done in phylogenetically diverse species such as mice.

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