ROLE OF *Drosophila melanogaster* MOF (MALE-ABSENT-ON-THE-FIRST) IN MAINTENANCE OF GENOME STABILITY

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CERTIFICATE

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LIST OF ABBREVIATIONS

AO	Acridine Orange
APS	Ammonium per sulphate
Ark	Apaf-1 related killer
ATM	Ataxia telangiectasia mutated
bp	Base Pair
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
Chk1	Checkpoint kinase1
CS	Canton S
DAPI	4 ’,6-Diamidino-2-phenyl indole dihydrochloride
DDR	DNA damage response
dMOF	*Drosophila* MOF
dNTPs	Deoxynucleoside 5’ triphosphates
DEPC	Diethyl pyrocarbonate
DrICE	*Drosophila* Ice
DroNc	*Drosophila* Nedd2-like caspase
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
EP	Enhancer-and-promoter element inserted
GMR	Glass Multiple Reporter
Gy	Gray
H4K16Ac	Histone H4 Lys16 Acetylation
HAT	Histone acteyltransferase
Hid	head involution defective

hMOF	human MOF
JNK	Jun-N-terminal Kinase
IR	Ionizing Radiation
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PI	Propidium iodide
PMSF	Phenyl methyl sulfonyl fluoride
RHG	Reaper, Hid, Grim
SEM	Standard Error of Mean
RNAi	RNA interference
RNase A	Ribonuclease A
rpm	revolutions per minute
Sir	Silent information regulator
TE	Tris EDTA
TEMED	N, N,N’,N’-tetramethyl ethylene diamine
TrIP	transgenic RNAi Project
UAS	Upstream Activation Sequence

SYNOPSIS

Title: Role of *Drosophila melanogaster* MOF (Male-absent-on-the-first) in maintenance of genome stability

Background

The males-absent-on-the-first (MOF) histone acetyltransferase is evolutionary conserved from yeasts to humans. In *Drosophila melanogaster*, this protein was identified (dMOF), in a genetic screen to identify regulators of dosage compensation, on the basis of the male specific lethal phenotype. Subsequently, it was established that this member of the multiprotein *Drosophila* dosage compensation complex is responsible for acetylating histone H4 at Lys16 on the male X chromosome for a 2-fold hypertranscription and equalization of X-linked gene dosage between the two sexes.

Considerable similarity exists between the amino acid sequences of dMOF and other MYST family of histone acetyltransferases: the human HIV-1 Tat interactive protein Tip60, human MOZ (monocytic leukemia zinc finger protein) and SAS1 and SAS2 (yeast silencing proteins). Despite this, their functions in various nuclear processes are diverse. Both *Drosophila* and human MOF (hMOF) share the same substrate specificity and enzymatic activity and conserved interacting partners in multisubunit complexes. Surprisingly, hMOF has no reported participation in human dosage compensation. Chromatin organisation can be regulated by MOF mediated H4K16 acetylation in two ways. Chromatin packing may be loosened by decreased affinity of basic histones towards negatively charged DNA, favouring binding of various factors. Modified histones may also themselves attract chromatin modifying proteins. Till date, however, the significance MOF's chromatin modifier role in regulating vital cellular processes such as DNA damage repair, transcriptional regulation, cell cycle progression and apoptosis have remained limited only to few, scattered reports.Nevertheless,H4K16Ac mediated by MOF acting to regulate such mechanisms, has been positively implicated in maintenance of overall genome stability and faithful transmission of genetic information.

Frequent losses in the H4K16Ac epigenetic marks, correlated with reduced levels of MOF have been observed in human cancer cell lines. Further, MOF knock-

down cell lines (HeLa and 293T cells) display tumorigenic nuclear morphological defects, G2-M arrest and an impaired DNA repair process upon IR-induced DNA damage. hMOF has been reported to activate ATM in response to DNA damage, though the actual mechanism has not been worked out. These reports point towards a multifunctional role of MOF in crucial cellular processes. This study has looked into MOF's involvement in cellular events aimed at achieving genome stability and body homeostasis, utilising the wealth of genetic tools available in the model organism *Drosophila*.

Results:

1) The role of MOF in the ionizing radiation (IR) response is conserved in *Drosophila*

Our study reports that *Drosophila mof* mutations in males and females, as in *mof* knockdown in SL-2 cells, reduce post-ionizing radiation survival. MOF depletion in SL-2 cells results in an elevated frequency of metaphases with chromosomal aberrations, suggesting that MOF is involved in DNA damage repair (DDR). A functional G2-M phase checkpoint delays entry of cells into mitosis when DNA is damaged and sets of a multitude of DNA repair pathways into action. When tested immunohistologically in actively developing cells in imaginal discs of *mof* mutant larvae, a defective mitotic checkpoint was observed, allowing irradiated damaged cells to escape to cell division. Also, extremely high basal levels of apoptotic cells in mutant discs preclude any DNA damage induced apoptosis. Post-irradiation, *mof* mutants displayed a defective p53 response, as Western blots of larval protein extracts revealed enhanced basal levels of p53 and no obvious change on IR exposure.

Corroborating with results from hMOF knock down cell lines and SL-2 cells, mutant *mof* larvae showed minimal increase in the levels of IR-induced H4K16Ac in contrast to wild type controls where IR exposure significantly enhanced H4K16ac levels. These results are the first to demonstrate a requirement for MOF in the whole animal in IR response and suggest that its role in DNA damage response is conserved between *Drosophila* and mammals.

2) *Drosophila* MOF controls Checkpoint protein2 and regulates genomic stability during early embryogenesis

Genome stability is ensured to a large extent by proper functioning of cell cycle checkpoints operating during organism's development. In *Drosophila* embryos, checkpoints delay cell cycle progression so that DNA damage repair can occur or replication completed. Early syncytial nuclear divisions of *Drosophila* embryos are very rapid with surveillance mechanisms to effectively cull abnormal nuclei from the dividing population of cortical nuclei. During the last 4 syncytial blastoderm stage divisions, a DNA replication checkpoint comes into play, delaying mitosis in the event of damaged or unreplicated DNA.

The expression levels of *Drosophila* MOF are quite high during early embryogenesis. It was found that that haplo-insufficiency of maternal MOF leads to spontaneous mitotic defects like mitotic asynchrony, mitotic catastrophe, chromatid bridges and disruption of cytoskeleton in the syncytial embryos. Such abnormal nuclei are eliminated and digested in the yolk tissues by nuclear fallout mechanism.MOF negatively regulates *Drosophila* checkpoint kinase2 tumor suppressor homologue. In response to DNA damage the checkpoint gene *Chk2* (*Drosophila mnk*) is activated in the *mof* mutants, thereby causing centrosomal inactivation suggesting its role in response to genotoxic stress. A drastic decrease in the fall out nuclei in the syncytial embryos derived from $mof_{1}/+;\ mnk_{p6}/+$ females further confirms the role of DNA damage response gene *Chk2* to ensure the removal of abnormal nuclei from the embryonic precursor pool and maintain genome stability. The fact that *mof* mutants undergo DNA damage has been further elucidated by the increased number of single and double stranded DNA breaks.Such spontaneous defects in *mof* mutant embryos resemble those induced by X-ray irradiation or chemical treatment of wild type embryos.

3) *Drosophila* MOF regulates transcription of DIAP1 (*Drosophila* Inhibitor of Apoptosis Protein 1) and controls apoptosis through JNK (Jun N-terminal kinase) dependent pathways.

Irreparable deleterious mutations are naturally eliminated through induction of apoptosis. Our early observations that *Mof* null mutant discs exhibit reduced size and extremely high levels of apoptosis urged us to look into role of MOF in regulating

programmed cell death. The developing eye imaginal discs of *Drosophila* were chosen as the ideal *in vivo* tissue system to genetically study the mechanisms of interactions of MOF with cell death mediators in the apoptotic signal transduction pathways. Suppressors of MOF knockdown apoptosis phenotype induced by RNAi include-baculovirus caspase-3 inhibitor p35, knockdown alleles of caspases -Drice and DroNc, Ark (*Drosophila* homolog of mammalian Apaf-1 related killer) and suppressor alleles of *diap1* (*Drosophila* Inhibitor of Apoptosis1). In both *Drosophila* and mammals, the ubiquitin ligase IAP1 is a critical negative regulator of apoptosis, directly inhibiting caspases, and itself inhibited by the apoptosis triggering RHG proteins- Rpr, Hid and Grim. We found, through chromatin immunoprecipitation assays in wild type larvae,that MOF localizes to the DIAP1 promoter at about 800 bp upstream of the transcription start site .This enrichment (coinciding with histone modification H4K16Ac) is significantly higher (upto 2 folds) than in *mof1* mutants , where the localisation is barely detectable and confirms that MOF directly induces transcriptional upregulation of DIAP1.Genetically, the adult eye ablation phenotype produced by ectopically expressed Hid, Rpr ,Grim, could be relieved through overexpressing MOF; entirely in case of Rpr and partially with Hid and Grim. Furthermore, our results predict that apoptosis triggered by loss of MOF proceeds through a caspase-dependent, p53- independent, JNK (Jun –N-terminal kinase) mediated pathway. Finally, pull-down assays show a strong physical *in vitro* interaction of dMOF with Sir2 (a histone deacetylase implicated in regulating JNK mediated apoptosis), reinforcing MOF's critical role in regulating cell death and survival in the whole organism.

Significance

MOF appears to be involved in the DDR primarily through the effect of H4K16 acetylation on chromatin structure. DNA damage checkpoints are major mechanisms of DDR, serving to pause the cell cycle for allowing repair machinery to operate before DNA replication or cell division, or initiating apoptosis for eliminating irreparably damaged cells.MOF depleted human cancer cell lines show a prominent G2-M arrest and upon irradiation, a delayed kinetics of DNA repair. Similarly higher basal levels of Mei-41 and p53 in *mof* mutant *Drosophila* larvae are indicative of an abnormally activated checkpoint. Along with it, a faulty G2-M response to IR might be explained by the fact that hypoacetylated histones impairs normal DNA damage

and hence prolongs activation of G2-M checkpoint. Another possibility might also be reduced transcription of MOF-regulated genes involved in cell cycle progression. *Mof* mutant embryos exhibited genomic instability as evidenced by the occurrence of frequent mitotic bridges in anaphase, asynchronous nuclear divisions, disruption of cytoskeleton and inactivation of centrosomes finally leading to DNA damage. Our findings are consistent to what has been reported earlier in mammals; that reduced levels of MOF resulted in increased genomic instability while total loss resulted in lethality.

Our view is that the important, caspase-dependent step to triggering apoptosis is decided by a fine balance between DIAP1 inhibitors (Hid, Rpr, Grim) and positive inducers-MOF and inducer-MOF. IAP (such as XIAP) levels in mammals are known to be highly elevated in cancer cells. The interaction between HATs (MOF) and HDACs (Sir2) in regulating cell killing and survival proposes a plausible model involving previously unexplored interacting players in *Drosophila* apoptosis. This opens up possibilities of generating molecules with MOF inhibitory or degrading properties, to serve as potent anti-cancer drugs. The study can be further extended using *Drosophila* as model system to carry out the interaction of MOF with the known components of the DNA damage and apoptotic pathway.

LIST OF PUBLICATIONS

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- *Drosophila* MOF regulates transcription of DIAP1 and controls apoptosis through JNK -dependent pathway (manuscript in preparation).

CHAPTER-1
INTRODUCTION

1. INTRODUCTION

Preserving the integrity of genome over successive cellular generations is vital to all organisms, ranging from the simplest prokaryotes to the highly evolved metazoans. Cells should safeguard the genome information at all times, both during normal course of life, as well as when they become exposed to external insults like radiation that can damage the DNA. To combat the effect of such hazardous events, cells have evolved to house an endogenous repository of genes, non-coding RNAs and proteins that can detect damage and activate repair pathways or induce cell cycle arrest and/or apoptosis when the lesion is beyond repair.

Failure to maintain genomic stability could pave the way for death in unicellular organisms or lead to complex developmental defects, loss of viability and cancer in multicellular organisms (Ciccia, A.2010; Zhou, BB 2000; Shiloh, Y.2003; Langerak, P 2011; Flynn, R.L2011; Lengauer, C1998 Kolodner, R.D et al 2002; Vessey,C.J et al 1999).Several regulatory events such as cell-cycle checkpoints controlling aberrant cell proliferation, error- free DNA replication for faithful genome propagation, and DNA repair machinery functioning to eliminate DNA damages, work in concert to ensure genome integrity. The importance of such mechanisms in preserving accurate information coded in our genomes is now well established. Throughout the past two decades, studies in organisms from budding and fission yeasts, the fungus *Aspergillus*, nematodes, *Drosophila*, mouse, to mammalian systems, have contributed tremendously to our understanding of the molecular mechanisms and players in such regulations. Apart from the aforesaid regulatory processes, it is becoming increasingly evident that epigenetic alterations to the chromatin is an additional layer in regulating the processes that preserve genomic fidelity (Goldberg,A.D. et al.,2007). Such alterations include predominantly chromatin remodelling processes such as ATP-dependent chromatin remodeling, dynamic histone modifications, exchange of histone variants and DNA methylation (Wang et al., 2007).Of particular interest are histone modifiers, which act in combination to establish a 'histone or epigenetic code'(Jenuwein and Allis, 2001)-epigenetic signatures in chromatin microenvironments, helping to recruit special proteins mediating important chromatin mediated biological processes like transcriptional regulation, DNA repair and replication as well as chromosome condensation and segregation. Among these, acetylation is the first described histone modification (Phillips, 1963).

The *Drosophila* MYST acetyltransferase MOF, one of the key components of the Male specific lethal (MSL) complex, has been been extensively studied for its crucial role in mediating 2-fold hyperactivation of male X-linked genes to equalize their dosage in females. This study focuses on how this chromatin remodeler enzyme, through its chromatin organisation activity, mediates cellular functions linked to main like regulation of gene expression, DNA damage response , cell cycle progression and apoptosis.

1.1 Surveillance mechanisms for genome stability in different organisms

1.11 Mechanisms in Prokaryotes.

In *E.coli* and most other bacteria except Spirochaetes (Erill I.et al., 2007), DNA damage induced by agents like UV radiation, mitomycin C, methyl methane sulphonate (MMS),and other chemicals can elicit cell cycle arrest, accumulation of single stranded DNA(ss DNA)and elicit a global SOS response. First reported by Radman M(1975) as a repair system induced in *E.coli* after extensive DNA damage, this error- prone response mechanism is regulated by two major proteins: LexA, and RecA, mutations in which make cells highly sensitive to UV radiation.(Janion C,2008). LexA is a transcriptional repressor protein, that under normal conditions negatively regulates about 48 SOS response genes by binding to their operator consensus sequences or SOS boxes, preventing RNA PolII from binding and blocking their transcription.(Little et al.,1982,Lewis LK et al., 1994).Accumulation of ssDNAs due to stalled DNA polymerases at replication forks signals the coprotease RecA to form filaments around the regions in an ATP dependent manner and activate Lex A's self cleavage activity, causing its dissociation and depression of SOS-genes.(Shinagawa H., et al., 1988).Nucleotide excision repair is the first repair mechanism to be initiated.(Sancar A., 1996).In the event of an extensive damage, LexA is further repressed by RecA inducing expression of the late expressing genes with stronger LexA boxes -sulA, umuD, umuC .RecA protein dependent cleavage of UmuD protein is necessary for the assembly of the mutagenic DNA repair polymerase V.(Shinagawa H.,et al. 1988). The SOS response has been proposed as a mechanism of evolution of certain antibiotic resistant bacterial populations, specially against ciprofloxacin or rifampicin, thus promising the device of novel therapeutic strategy (Cirz RT., et al., 2005).

1.12 Mechanisms in yeast.

For years the unicellular budding yeast *Saccharomyces cerevisiae* has been a key model in studies of eukaryotic cell cycle checkpoints and DNA damage repair. Studies in yeast deletion mutants have shown that about 30% of genes are involved in mediating repair, post damage with DNA damaging alkylating agents. In fact, specific deletion strains exist for combating recovery for particular damaging agents (MMS, 4-NQO, t-BuOOH and UV), signifying separate pathways committed to maintaining genome stability (Svensson JP et al., 2012).

Early genetic screens in yeast analysed genes involved in affecting survival to UV and ionizing radiation and placed them into distinct epistatic groups. The *rad* mutants were the deletion strains most extensively covered then. The epistasis group RAD3 encodes components of the nucleotide excision repair pathway, the major pathway for repairing UV-induced lesions. The RAD52 group encodes components of the homologous recombination pathway and is required for the repair of ionizing radiation-induced damage. RAD6 postreplication repair pathway has been less extensively studied. It encodes factors required for the bypass of damages that block replicative DNA polymerases. Components of the base excision repair pathway were mostly identified biochemically as they were absent among early *rad* mutants.(Weinert, T.A 1988,Hartwell 1989, Foiani, M et al., 2000).Other epistasis groups identified were *mec* alleles (defective in mitotic enty checkpoint) (Weinert et al., 1994), *ddc1* alleles(suppressed mutations in various checkpoint genes)(Longhese, M.P 1997) and *chk1* alleles (bearing sequence homology to *S.pombe* checkpoint genes).The three canonical checkpoints identified in *S.cerevisiae* can sense damaged DNA and pause progression of cell cycle.(Hartwell, L.H,1989).These are: G1/S checkpoint or START senses damaged DNA and presence of stresses and halts cell cycle prior to DNA replication(Morgan D, 2007);the intra S checkpoint detects DNA damage within S phase and stalls replication and initiation events at late replication origins(C et al., 1998).Thirdly, the G2-M checkpoint checks entry into cell divisional phase to prevent to prevent aberrant segregation and faulty information transmission to the next transmission.

On the onset of DNA damage, sensor molecules comprising predominantly RAD9, RAD17, RAD24, MEC3 and DDC1 induce a checkpoint response through activating MEC1, a signal transducer kinase homologous of human ATM (Jazayeri, A.et al,2006).This in turn activates several protein kinases including RAD53 and

CHK1.RAD53,once activated, regulates G1 cyclin transcription, progression of replication forks and initiation of new forks at late origins and targets DUN1 and CDC25,both if which function at G2-M transition (Longhese, M.P, 1997). Activated chk1 phosphorylates Wee1 and Cdc25, leading to a G2 arrest (Raleigh, J. M.2000).

1.13 Mechanisms in plants.

Apart from the photosynthetic algae, all plants are obligate phototrophs, fixing $C0_2$ from light as the vital source of carbon and utilise sunlight derived energy to split water generating oxygen and reducing power. This process spontaneously generates ROS (reactive oxygen species), the prime cause of single strand breaks (SSBs) in the DNA (Freidberg et al.,1995). Coupled to this, their sedentary nature, leads to prolonged exposure to environmental genotoxic mutagens like UV radiation. Intrinsically, plants also run the chances of accumulating mutations from errors in multiple DNA replication in their undifferentiated meristematic cells destined to generate gametes. Surprisingly the fact that frequency of mutations observed in higher plants are not alarmingly high provides evidence for the existence of an efficient and robust genomic surveillance and repair mechanism(Hays et al., 2002).Several molecular mechanisms exist in plants to sense, signal and repair DNA in case of genotoxic stress. A well defined G2-M checkpoint is present in plants ;this is well evidenced by the increase in cell numbers in G2 phase upon gamma irradiation of *Arabidopsis* plants (Preuss & Britt, 2003).Plant homologues of human ATM (ataxia telangiectasia-mutated, Mec1 in *S. cerevisiae*), ATR (*ATM*-Rad3-related) and the mammalian DNA-dependent protein kinase catalytic subunit(DNA-PKcs) exist and play crucial roles in response induced by DNA damage and meiotic double strand breaks(DSBs)(Bray C.M et al., 2005)

1.14 Mechanisms in mammals.

A highly sophisticated array of DNA repair pathways and cell cycle control processes exist in higher eukaryotes to maintain genome stability. In mammalian cells, 2 major phosphatidylinositol 3-kinases, ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) ,are checkpoint activators in response to DNA damage (Abraham, R.T. 2001, Shiloh, Y. 2001).ATM predominantly responds to ionizing radiation induced double strand breaks(DSBs),with subsequent signalling by ATR; the latter also directly signals UV damage and stalls DNA replication. The G1

checkpoint prevents entry into S phase in the event of DNA damage. Post IR damage, ATM phosphorylates and activates downstream kinase Chk2 which in turn phosphorylates residue S20 of p53 (Matsuoka, S. *et al.* 2000). This serves to block p53-MDM2 interaction, which in normal states serves to keep p53 levels low and mark it for proteosome mediated degradation.(Matsuoka, S. *et al.* 2000).ATM also directly phosphorylates MDM2 on S395. Besides, ATM and ATR mediated phosphorylation of p53 at a critical residue, S15 enhances its transcriptional transactivity (Dumaz, N.,1999) serving to upregulate expression from target genes such as MDM2, GADD45a, and p21/Cip. In particular, increase in levels of p21, a cyclin-dependent kinase inhibitor, suppresses Cyclin E/Cdk2 kinase activity thereby arresting cells in G1(Bartek J., 2001).

In vitro study data from cancer cells of patients affected with ataxia telangiectasia (AT) or Nijmegen breakage syndrome (NBS), implicates signalling by ATM and NBS1 in the S-phase checkpoint signalling (Falck, J. *et al.* 2002) to stall DNA synthesis following IR damage. ATM phosphorylates and activates Chk2 kinase, which in turn, phosphorylates Cdc25A phosphatase for its ubiquitination and degradation.(Falck J et al., 2002). This prevents CDC25 mediated activation of Cdk2/Cyclin E and Cdk2/Cyclin A complexes, leading to a replication arrest. In addition, several ATM targets like NBS1, BRCA1(coded by the breast cancer susceptibility gene), and SMC1 (structural maintenance of chromosome protein 1),also get phosphorylated at multiple sites, failing which drives the cell with unchecked damage into S phase.(Falck J et al., 2002, Kim, S.T. *et al.* ,2002).

The G2 checkpoint in mammals is well defined, regulated primarily by the state of activity of cyclin dependent kinase Cdc2 (Nurse, P.1990). Sensing DNA damage, the downstream kinases Chk1 and Chk2 (activated by ATR- and ATM-dependent phosphorylation, respectively) phosphorylate Cdc25C at S216.(Peng, C.Y. *et al.* 1997).This allows Cdc25 binding to 14-3-3 proteins, and being prevented from dephosphorylating Cdc2 at T14 and Y15 residues. Cdc2/Cyclin B1 complexes remain inhibited and the cell cycle is arrested prior to chromosomal segregation.

Similar to checkpoints, an equally advanced arsenal of DNA repair proteins thrive in mammalian cells, specially humans. These include: Activation of a large number of transcription factors aimed for repair (Jelinsky,S. A,1999),excision repair brought about through induction of the *p48* gene(Hwang, B,1999),induction of ATM and the Nbs1-hMre11-Rad50 complex for non-homologous end joining (NHEJ) and

homologous recombination repair of DSBs in DNA(Carney, J. P.et al,1998) and repair through homologous recombination involving proteins BRCA1 complexed with ATM and Nbs1-hMre11-Rad50 (Moynahan, M. E, 1999).

1.2 Genotoxic stresses in multicellular organisms

Genomic stability can be compromised by both spontaneous endogenous events as well as external agents, bringing about damage to DNA, faulty replication or aberrant cell divisions (Papamichos-Chronakis M, et al., 2013). Endogenous cellular events causing DNA alterations include erroneous DNA replication miscorporating dNTPS, deamination of DNA bases causing interconversion between purines and pyrimidines, DNA depurination events creating basic sites and alkylation of bases producing modified products (Lindahl and Barnes, 2000). Cellular metabolism also generates hazardous by-products like reactive oxygen(ROS) and nitrogen species, lipid peroxidation products, endogenous alkylating agents, estrogen and cholesterol metabolites, and reactive carbonyl species (Hoeijmakers, 2009).

External environmental agents damaging DNA can result from both physical and chemical sources. Physical sources include ionizing radiation (IR) (from cosmic radiation and medical treatments like X-rays or radiotherapy) and ultraviolet (UV) light from sunlight. It is estimated that a cell per day can undergo up to 10^5 DNA lesions (pyrimidine dimmers and photoproducts) from UV exposure per day (Hoeijmakers, 2009).Chemical genotoxic agents can result from cancer chemotherapy or cigarette smoking. Chemotherapeutic drugs include alkylating agents (methyl methanesulfonate or MMS and temozolomide), intra strand and interstrand cross linking agents (mitomycin C or MMC), cisplatin, psoralen, and nitrogen mustard) and topoisomerase inhibitors like camptothecin(CPT) and etoposide that inflict DNA lesions by trapping topoisomerase-DNA covalent complexes. (Ciccia et al., 2010).A wide variety of adducts and (4-6) photoproducts can be produced in the lungs and other tissues of smokers. An alarming 10^3 carcinogenic DNA adducts per cell could be present in the lung of smokers following 1-2 cigarette packs per day for 40 years (Philips et al., 1998) A single radiation dose of approximately 1800-2000 mSV administered generally in cancer therapy alone can generate 72-80 DSBs per cell (Ciccia et al., 2010).

1.3 Chromatin regulatory factors in maintenance of genomic integrity

In eukaryotic cells genomic DNA is compacted with an equal mass of protein to form chromatin **(**van Holde 1988, Zlatanova and Leuba, 2004). Nucleosomes, the fundamental repeating building blocks of chromatin, are each formed of a canonical histone octamer core with 145-147 bp of DNA wrapped around it (Luger et al., 1997) and connected by short stretches of linker DNA. The canonical core histone octamer consists of 2 molecules each of H2A, H2B, H3 and H4 (Arents and Moudrianakis, 1993). This represents the 10 nm 'primary structure' of chromatin organisation, called the beads-on–a–string organization of nucleosomes (Luger et al., 2012).It is further folded into the 30 nm 'secondary structures'(either the 'solenoid ' or 'zigzag' model) via short range interactions between nucleosomes, and then further into higher order 'tertiary structures'.

Fundamental repair, transcription and signalling processes in the cell requires the reversible unwrapping of chromatin and localized changes in chromatin architecture to make the underlying DNA accessible to the action of enzymatic machinery. Nucleosomal protein composition and dynamics can vary locally with certain factors. Such changes can occur through ATP-dependent chromatin remodeling (Flaus and Owen-Hughes, 2004), by posttranslational modifications of histones (Kouzarides, 2007; Bernstein et al., 2007), and/or by altering the biochemical composition of nucleosomes by replacing canonical histones with nonallelic histone variants (Redon et al., 2002; Henikoff and Ahmad, 2005; Kamakaka and Biggins, 2005; Kusch and Workman, 2007; Boulard et al., 2007)

1.31 ATP–dependent chromatin remodelling factors.

Highly conserved from yeasts to humans , these are multiprotein complexes characterized by the presence of a catalytic ATPase subunit (Saha A,2006).Utilizing energy from ATP hydrolysis, these are able to reposition nucleosomes, expel histones away from DNA or facilitate exchange of histone variants, making DNA amenable for repair or accessible for transcription (Wang et al.,2007).4 major families of well studied mammalian chromatin remodelling complexes exist: the SWI/SNF (switching defective/sucrose non-fermenting) family that mainly promote gene activation, the ISWI (imitation SWI)and NuRD (nucleosome remodeling and deacetylation)/Mi-2/CHD (chromodomain, helicase, DNA binding) families enabling transcriptional

repressors binding, and the INO80 (inositol requiring 80) family (Becker et al., 2002, Bao et al., 2007) which can both activate or repress genes.

1.32 Post translational histone modifications

Histone tail residues may undergo post translational modifications in response to DNA damages. The changes include phosphorylation, acetylation, methylation, ubiquitination, poly(ADP-ribosyl)ation,(PARylation), and SUMOylation.They serve mainly as signaling sites, drawing chromatin remodeler proteins to the site for DDR and less importantly, may also alter the packing of chromatin (Smeenk et al.,2013).

1.33 Phosphorylation at H2AX activates DDR

At the site of DNA double strand breaks in mammalian cells,, sensor molecules-MRN complexes (MRE11-RAD50-NBS1), recruit ATM (ataxia telangiectasia–mutated) protein kinase to facilitate phosphorylation of H2AX at Ser139 on the C-terminal SQL motif to form γH2AX (Savic et al., 2009). Another protein kinase, ATR(ATMandRad3-related) ,specifically phosphorylates H2AX in response to UV and replication stress signals (Durocher D., et al, 2001). Such rapid phosphorylation of H2AX at the DSB is the major epigenetic signal for accumulation of DNA damage repair protein MDC1(mediator of DNA damage checkpoint protein 1) (Stucki M., et al., 2005). H2AX knockout mice are viable and still exhibit retention of retention of Rad51 into IR-induced foci, the hallmarks of DSBs. However they display increased sensitivity to IR, abundant M-phase chromosomal defects and impaired G2/M checkpoints (Fernandez-Capetillo O., et al., 2002). All this evidence points to the need for H2AX in maintaining overall genome stability. The phosphorylation of H2AX is tightly regulated and various phosphatases like PP2ACα,PP2ACβ, PP4C, PP6C, and WIP1, in mammals ,can revert it back to unphosphorylated H2A(Keogh M., et al ,2006; Douglas P et al., 2010., Cha et al., 2010).

1.34 Histone Methylations

The best understood modifications of the histone code are methylations, which are usually associated with transcriptional repression, including genomic imprinting and developmental programs, and can occur both at lysines and arginines. H3K4 and H3K36 have always been associated with transcriptional activation (Lachner M,

2002) while methylation of lysines H3K9 and H3K27 is correlated with transcriptional repression (Rosenfield et al., 2009). H3K9me3 is correlated with constitutive heterochromatin (Hublitz 2009).The well characterized HMTases in mammals include: H3-K9 methyltransferases-Suv39h1 and Suv39h2 (Rea S, 2000), ESET/SetDB1and Eu-HMTase1 (Schultz, D et al 2002; Ogawa, H et al, 2002) and Heterochromatin Protein 1(HP1) (Lachner M, 2001). This act in concert with DNA cytosine methyltransferases- Dnmt1, Dnmt3a and Dnmt3b (Bestor et al., 1992) and Methyl-CpG-binding proteins- MECP2, MBD1, MBD2, MBD3, MBD4 and Kaiso (Li,2002).

1.35 Histone variants in maintaining chromosome stability

Genome wide studies reveal that histone variants, highly conserved among eukaryotes, are incorporated into specific genomic sites via DNA replication independent mechanisms and these have evolved to perform various functions. Crucial among them, is modifying local nucleosomal structure to alter its interactions with repair and transcription machinery. The 3 major H2A variants associated with DNA damage response are–H2AX, H2A.Z and MacroH2A. Upon induction of DSBs in mammals, H2A is rapidly phosphorylated to generate variant γH2A.X (Rogakou et al., 1998). This serves as molecular landmark signalling recruitment of various checkpoint and repair proteins like MDC1 (Mediator of DNA damage chekpoint 1) and 53BP1 (p53 binding protein 1). γH2A.X marks also spread both ways from the locus of DSB (Yuan et al., 2010).The histone acetyltransferase Tip60,a homolog of MOF, acetylates phospho-H2Av (an H2A.X/H2A.Z ortholog) in *Drosophila* and mediates its exchange for H2Av (Kusch et al., 2004).

1.4 DNA damage checkpoint responses and repair mechanisms in *Drosophila*

Drosophila has emerged as a genetically tractable model organism to study DNA damage responses in an *in vivo* context.

1.41 In developing embryos.

The early stages during Drosophila embryogenesis display 3 major naturally occurring checkpoints that respond to DNA damage: delay of entry into mitosis/replication checkpoint, centrosome inactivation, and metaphase arrest. The early cycles of zygote development consists of 13 rapid synchronous nuclear divisions

within a maternally contributed cytoplasm. These syncytial cycles contain only alternating S and M phase with no detectable gap phases. Whenever DNA replicates in S phase, a replication checkpoint becomes activated and prevents onset of M phase before completion of replication (Smits et al., 2010). Maintenance of the correct time length of S phase and alternate S and M phases requires checkpoint kinases ATR (mei-41) and Chk1 (grapes).mei-41 also regulates checkpoints induced by DSBs during all phases of the cell cycle (Brodsky et al., 2000b; Garner et al., 2001; Hari et al., 1995; Jaklevic and Su, 2004; Sibon et al., 1999).Embryos devoid of mei-41or grapes enter mitosis prematurely without completing replication, ultimately causing aberrant segregation and death.mei-41 mutants have been shown to be hypersensitive to agents that block replication such as hydroxyurea, alkylating chemicals, and ultraviolet radiation , and those that induce DSBs (Boyd et al., 1976; Sibon et al., 1999).

As in other eukaryotic cells, mechanisms also exist to allow only cells that have successfully completed M phase to enter S phase and build replication complexes. This is ensured by complete degradation of mitotic cyclins and Geminin (which inhibit pre replication complex or pre-RC formation) on exiting M phase (Li and Jin ,2011;Tanaka et al., 2010). Mitotic cyclin A/Cdk1 majorly inhibits pre-RC formation in *Drosophila* embryos. Presence of non degradable cyclin A hinders association of MCM and chromosomes during mitotic cycles (Su and O Farell 1997).

Mitotic defects such as anastral mitotic spindle assembly, mitotic delays, and aberrant chromosome segregation occur in the event of cell division ensuing with damaged or incompletely replicated DNA (Sibon et al., 2000). To eliminate defective cells, aberrant nuclei are spontaneously eliminated from the cortex (‘nuclear fallout’). In the divisional stages1-13, embryos utilize inactivation of centrosome, while during the cellular blastoderm cycles that contain a G2 phase, mitotic entry delay is mediated by a metaphase arrest (Su et al., 2000). This checkpoint is suppressed in *Chk2* mutant (but not in *p53* and *atm* mutants) embryos, indicative of a possible role of Chk2 in regulating this checkpoint in embryos (Takada et al., 2003).

1.42 In adult flies.

Several abnormal phenotypes are associated with *atm* (*Drosophila* homolog *tefu*) mutants-lethality in pupae and rough eyes, misshapen wings, missing or abnormal bristles, abnormalities in telomeres and uncontrolled apoptosis in

hypomorphic mutants in adults (Oikemus etal., 2004; Silva et al., 2004; Song et al., 2004).Products encoded by *atm*, *mre11* and *rad50* help prevent end joining between chromosomes and fusion of telomeres through targeting HP1 and HOAP proteins (Song 2005). *Dmnk*, the *Drosophila* maternal nuclear kinase (Oishi et al., 1998), is the homolog of mammalian protein kinase chk2, was first identified in the ovaries and is thought of as an upstream regulator of p53.Chk2 inactivation, but not Grp, can help rescue the apoptotic rough eye phenotype induced by expressing p53 in the adult eye (Brodsky et al., 2000).

The major components of DNA damage response (DDR) in eukaryotes: damage sensors-MRN and the 9-1-1 complexes, signal transducing protein kinases-ATM, ATR,Chk1 and Chk2 and effectors-p53 and Cdc25,are all conserved in *Drosophila* (Su 2006).In addition, novel genes required for damage mediated G2/M checkpoint activation , *mus101*(encoding an ortholog of mammalian TOBP1) and *mus312* (encoding an ortholog of mammalian BTBD12)(Kondo et al, 2011)have been identified through a genome wide RNAi screen. Mutants for *grp* and *lok* (*Drosophila* homolog of *chk2*) have aberrant replication and DNA damage checkpoints (Brodsky et al., 2004, Royou et al., 2005). Mei-41, like its mammalian counterpart ATM kinase, functions in cell survival post IR ,independent of its role in checkpoint activation.G2-M checkpoint-defective *mei-41* and *grp* mutants treated with doses of IR , get killed in absence of mei-41 ,but not grp (Jaklevic and Su,2004).Also, an elevated frequency of spontaneous and IR-induced chromosome breaks were obtained in *mei-41*mutants but not in those of *grp* (Oikemus et al., 2006).All this evidence clearly point towards the fact that similar to mammals, ATM/ATR mediated roles in DNA damage response and repair through activation of checkpoints is conserved in *Drosophila*.

1.5 Histone acetyltransferases and diverse members

The connection between acetylation of histones in the chromatin and transcriptional upregulation was first implicated by Allfrey and co workers (Allfrey V. G. et al, 1964) and the idea was further reinforced by subsequent studies (Vettese-Dadey M., 1996, Sealy L. 1978, Hebbes T.R 1994). Diverse Histone acetyltransferases (HATs) have been discovered ever since – for example, yeast HAT1 (Kleff S.,1995), Gcn5/PCAF (Berger S. L.,1992), CBP/p300 (Bannister A.

J..1996), TAFII250 (Mizzen C. A, 1996), SRC-1(Spencer T. E., 1997) and Esa1 (Allard, S.1997).The targets of HATs are the e-amino group of specific lysine residues on histone tails (Wolffe A. P.1992).

1.51 MYST family of Histone acetyl transferases and MOF

Highly conserved in eukaryotes, the histone acetyltransferases belonging to the family of MYST possess highly homologous ~370residue MYST domains deriving their name from 4 founding members- human MOZ (monocytic leukemia zinc finger protein) (Borrow et al., 1996), yeast Ybf2 or Sas3 (something about silencing 3, yeast Sas (Reifsnyder,C.1996) and mammalian TIP60 (HIV Tat-interacting 60 kDa protein) (Kamine, J et al., 1996).Well studied members in humans include: MOZ and TIP60, hMOF (ortholog of *Drosophila* Mof), HBO1 (HAT bound to ORC1, a Chameau ortholog) (Iizuka, M et al., 1999) and MORF (MOZ-related factor). *Drosophila* consists of 5 members from this family: Mof (male-absent on the first) (Scott, E.K et al., 2001, Hilfiker,A.1997), Enok (Enokimushroom) (Scott, E.K et al., 2001),Chameau (Grienenberger,A.,2002) and two uncharacterized MYST proteins (CG6121 and CG1894). The MYST HAT enzymes exist as conserved, multisubunit complexes and are involved in regulating diverse biological processes like gene-specific transcription regulation, DNA damage response and repair, as well as DNA replication.

In *Drosophila*, MOF (male absent on the first) is well known for its role in acetylating Histone H4 at Lys 16 (H4K16Ac) on the male X chromosome, thus mediating a 2 fold hyperactivation of male X linked genes to equalize their dosage in females(Hilfiker et al., 1997). It was first discovered through a genetic screen using EMS(ethyl methane sulfonate) to induce mutations and identifying novel genes involved in dosage compensation through the male specific lethal phenotype. In a mutation of mof(male absent on the first), dying mutant *Drosophila* males lack the X-associated isoform of H4Ac16(Hilfiker et al., 1997). *mof* mutant males can survive only upto third instar larval stage; mutant females are viable.

Drosophila MOF is a component of the dosage compensation complex, which is also known to include the male-specific lethal proteins 1–3 (MSL1, MSL2, MSL3), the maleless (MLE) protein, and two noncoding RNAs called roX1 and roX2 (Straub and Becker 2007).The DCC is specifically targeted to the male X chromosome, binding to about 35 “high affinity binding sites” and then spreading in *cis*, coating the

entire X chromosome (Kelley 1999). Except MOF, all other components of the DCC, including the non coding RNs are synthesized at very low amounts because of inhibition of translation of MSL-1 subunit by Sxl protein(Sex lethal)(Bashaw GJ,1996).

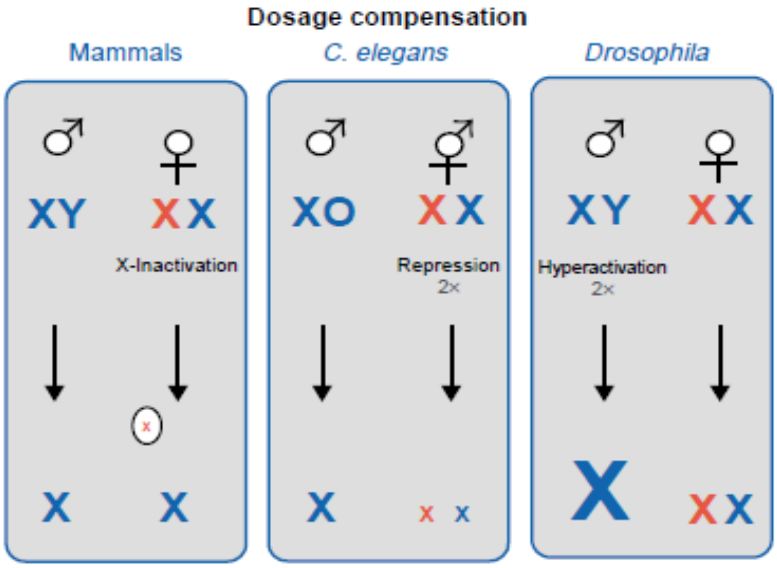


Fig1.1 Dosage Compensation (Akhtar A et al, 2003)

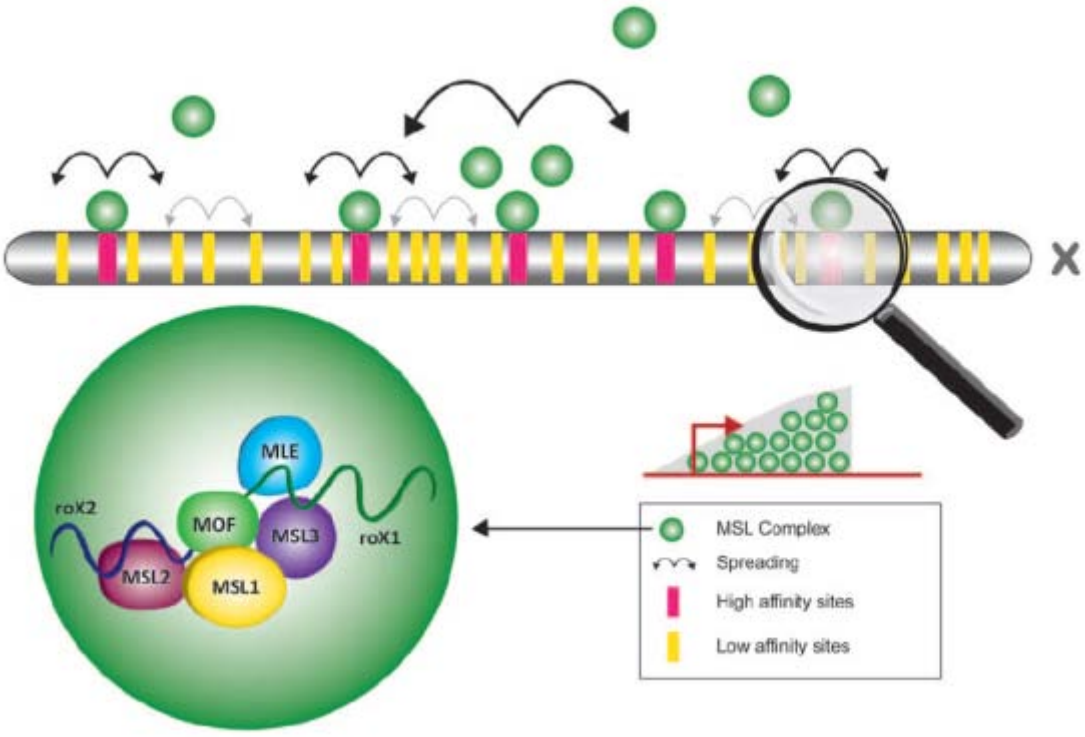


Fig1.2. X chromosomal targeting of male X chromosome in *Drosophila*. The ribonucleoprotein containing MSL complex (green)targets the male X chromosome on several high(red) and low(yellow) affinity sites (Georgiev et al., 2011).

It has been proposed that MOF mediated H4K16 acetylation loosens the chromatin, allowing efficient transcriptional elongation (Ercan S et al.,2008).In addition ,since MOF can also bind RNA through its chromodomain , it has been proposed that it might help in targeting the complex to its binding sites via rox RNAs (Akhtar A, 2000).

Since endogenous MOF is expressed at the same level in males as well as females, there have been speculations about its other unknown functions, apart from dosage compensation. Further, MOF homologs are conserved from yeasts to humans, and its various counterparts - Tip60, SAS2 and SAS3, have reported roles in transcriptional regulation. Like all histone acetyltransferases, MOF in both humans and *Drosophila* exists in multiprotein complexes within the cell, and majority of the interacting components are conserved (Smith et al., 2005) For example, in mammalian cells, human MOF (hMOF) exists in 2 distinct complexes:in a complex with MSL1v1/hNSL1 ,hMOF acetylates p53 K120 and H4K16 at the 5′ end of target loci. When present within mammalian MSL complex, it acetylates H4K16Ac at the 3′ end of target loci (Laverty et al., 2010). Similar to humans, dMOF has also been purified biochemically from the non-specific lethal (NSL) complex (NSL1, NSL2, NSL3, MCRS2, MBD-R2, and WDS), which functions as a major transcriptional regulator in *Drosophila* (Raja et al., 2010).

Genome wide studies in *Drosophila* reveal that in autosomes or DCC independent X chromosomal genes in both males and females, MOF binds to promoters of genes.It thus acts as a general transcription factor, as expression levels of a number of autosomal genes are lowered on MOF depletion (Kind J et al., 2008).Thus emerging data from genetic and interaction studies point towards unexplored roles of dMOF in several cellular processes aimed at maintaining overall genome stability.

1.52 MOF mediated H4K16 Acetylation and chromatin organization

Studies of the crystal lattice structure of nucleosome particles by Luger et al., 1997 first reported that a stretch of positively charged basic residues (14-19) in the tail region of histone H4 is involved in interaction with an acidic pocket of highly conserved negatively charged residues on the H2A-H2B surface. Subsequent studies revealed that packing chromatin into higher order structures relied heavily on this internucleosomal interaction (Caterino T.L., 2007).The four lysines at positions 5,8,12

and 16 on the H4tail can undergo reversible acetylation *in vivo* in most eukaryotes (Allfrey V.G., 1968). According to the charge neutralization model, addition of acetyl group on Lys16 of H4 neutralizes the positive charges, weakening the interaction with H2A, thus loosening the nucleosome array from its tight 30 nm solenoid coil (Shogren-Knaak, M et al., 2006). To a lesser extent, such modification also has the effect of impeding long range internucleosomal interactions mediated through linker histones (Kan, P. Y.,2009).

An alternative model called the 'histone code hypothesis', suggests that acetylated histone tails could be read and recognized by protein motifs (Turner et al., 1992 ; Strahl and Allis 2000).The H4K16Ac epigenetic mark could ,in fact, inhibit the binding of other chromatin condensing remodelers.Sas2 (the yeast ortholog of MOF) mediated H4K16Ac prevents binding of Sir3 at telomeres and thus blocks heterochromatin spreading inwards into the chromosome (Ehrenhofer-Murray*et al.*, 1997 ;Kimura et al., 2002). In *Drosophila*, it inhibits binding of ISWI ATPase, a protein involved in the compaction of chromatin (Corona *et al.*, 2002).

Thus the overall effect of this post translational modification in DDR was implied in unwrapping chromatin to an open configuration, thus facilitating signalling to binding of appropriate repair proteins at the site of damage. The *in vivo* role of MOF in chromatin remodelling in response to DNA damage has been barely studied in *Drosophila*, and still date, not extensively covered in higher eukaryotes.

1.53 Reported Roles of MOF in DNA repair mechanisms: hMOF in the DNA damage response

It has been proposed that MOF mediated loosening of chromatin via H4K16Ac could allow better access of damaged chromatin to repair machinery. An early indication came from DSBs induced by HO endonuclease in yeast, where the frequency of actylations at H4K16 at the area around the breaks increases and accumulates of HATs (Bird et al., 2002).

MOF knockdown in human (HeLa and 293T) cell lines leads to activated G2/M cell cycle arrest. hMOF-depleted HeLa cells show an activated G2-M checkpoint ,with major percentage of a population of knockdown cells accumulating in the G2/M phase compared to wild type control cells, as revealed by FACS (fluorescence-activated cell sorter) analysis (Taipale et al., 2005). Given that 2 major signaling cascades regulate the G2/M arrest-the caffeine insensitive, cytoplasmic,

activated p38 kinase pathway (Bulavin D.V. et al., 2007) and the caffeine sensitive, nuclear , ATM/ATR/DNA-PK pathway(Sancar A et al., 2004), it was interesting to observe which one of these was activated in response to a *mof* knockdown. Treating MOF-depleted and control HeLa cells with inhibitors for the ATM/ATR pathway, caffeine and wortmannin, could rescue the G2/M arrested cells back to normal. Besides, the *mof* knockdown cells also revealed a much greater percentage (about 40%) of immunostained phospho-ATM foci colocalizing with γH2A.X domains, compared to normal cells. Time course studies conducted to study the kinetics of the DNA repair process post ionizing radiation (doses of 1 Gy ,6 Gy) of *hmof* knockdown and control cells indicate a marked delay in accumulation of phospho-ATM and γH2A.X foci. Also, DNA damage repair in response to IR was markedly attenuated in cells expressing a dominant negative form of hMOF (Gupta et al. 2005).The data suggests a mechanism for the activation of G2/M checkpoint in *mof* depleted cells. It seems that it is actually post DNA damage and occurrence of DSBs, that hypoacetylation of H4 delays the DNA repair process and activates checkpoint delaying entry into mitosis, rather than hypoacetylated histones actually rendering chromatin susceptible to breakage (Taipale et al., 2005).However the exact role of MOF in the DNA damage response and repair pathway still remains obscure.

1.54 MOF mediated H4K16 acetylation is required for recruitment of DNA damage repair protein Mdc1 and other downstream factors at DSBs.

The mammalian damage repair protein MDC1/NFBD1 can bind directly to phospho-H2A.X(γH2A.X) via its BRCT(BRCA1 carboxy-terminal) domain and aggregrates in large nuclear clusters termed IRIFs(IR induced nuclear foci as seen in fluorescence microscopy) in cells exposed to ionizing radiation (Stucki M.et al., 2005).

Mof depleted MEF (mouse embryonic fibroblast) cells, subjected to a dose of 10Gy of IR and subsequently monitored for rapid IRIF formation by immunostaining with antibodies against against Mdc1, 53bp1, and Brca1 , showed almost complete loss of foci formation for these factors. However, the treatment failed to affect γH2A.X IRIF aggregates. Further studies conducted on cell lines carrying conditional *mof* knockout alleles confirmed that indeed the recruitment of MDC1 to γH2A.X domains was mediated through MOF. *Mof* $^{flox/flox}$ *Cre*$^{+}$ MEF cells deleted of endogenous *mof* and recued with transfection with siRNAs for wild type *mof* and

Mof E350Q(a *mof* mutant allele with drastically reduced acetyl group binding capacity)displayed greatly increased percentage of H4 K16ac and Mdc1-positive cells in wild type compared to mutant MEFs in immunofluorescence studies (Li et al., 2010).

It is also reported that both of the cellular mammalian MOF complexes- Mof-Msl and Mof-Msl1v1 regulate Mdc1 recruitment in the DNA damage response. Knockdown of MOF associated msl1 amd msl1-v1 in MEF cell lines showed almost identical profiles of reduced H4K16Ac and abolishment of MDC1 IRIFs post DNA damage. H2A.X containing FLAG tagged mono nucleosomes purified from mof+/+ cells display an enrichment for H4K16 acetylation, while showing depletion for this modification in *mof* knock out cells (Li et al., 2010).

The mechanism by which *mof* mediated H4K16 acetylation mediates MDC1 binding to γH2A.X is yet to be understood. Possibly H4-H2A.X interactions are perturbed by acetylations on the basic stretch of Lys residues of H4.Introducing mutations at 2 crucial residues on the acidic pocket of H2A.X (E91 and E92 to R91L92) and transfecting H2A.X null cells with both wild-type H2A.X and the H2A.X-RL mutant resulted in complete disappearance of IRIF for Mdc1 in mutant cells, while wild type cells retained them post irradiation. Incorporation of phosphorylated H2A.X into DNA damaged chromatin remained unaffected in both. (Li et al., 2010).All this supports the role of MOF mediated H4K16 acetylation in recruiting the DNA repair machinery to the site of damage on chromatin.

1.55 hMOF acetylates the tumor suppressor protein p53.

Hailed as the guardian of the genome, p53 tumor suppressor is crucial in preventing malignancies (Skyes et al., 2006). In response to DNA damaging signals, p53 can either activate tranctiption of checkpoint regulating genes, leading to a cell cycle arrest or promote apoptosis through inducing expression of proapoptotic genes (Vousden 2006).

Mutation in a critical modified residue**,** K120Ac, located within the DNA binding domain of p53 could lead to cancers (Meyers et al 1993; Hashimoto et al., 1999),possibly through specifically blocking transcription of proapoptotic genes such as *BAX* and *PUMA*.p53 acetylated at K120 accumulates at the promoters of proapoptotic genes in response to DNA damage (Skyes et al., 2006).Acetylation at

this site is mediated by hMOF and Tip60 (Skyes et al., 2006,Tang et al., 2006).This indicates a mechanism of DNA damage induced cell cycle progression by MOF through regulating p53 and serving to enhance transcription from growth arrest genes. Further, in case of irreaparable damage, extensive acetylation of p53 by MOF could even pave the way for apoptosis (Rea S 2007).

1.56 Interaction of hMOF and ATM

In all higher eukayotes, ATM protein kinase is the predominant signal transducer molecule, serving to phosphorylate and activate several downstream repair proteins in response to ionizing radiation and other DNA damaging agents. (Pandita TK., 2003; Shiloh Y 2003). It was earlier indicated by Bakkenist and Katan, 2003, that the stimulus causing ATM activation lies in the disturbances in the chromatin structure induced by DNA damaging signals. Reports by Gupta et al., 2005, from yeast two hybrid assays and CoIP studies in human cell lines, show that hMOF directly interacts with ATM through its chromodomain. Further, IR treated cells expressing a dominant negative mutant hMOF or harbouring a silenced endogenous *hmof* gene, show significantly reduced levels of H4K16Ac and ATM autophosphorylation. They also demonstrated increased susceptibility to apoptosis, decreased ATM kinase activity and phosphorylation of downstream effectors of ATM and DNA repair. Cell cycle checkpoint responses to DNA damages were also obliterated in MOF depleted cells.

Several models have been put forward to explain the MOF regulated function of ATM. Apart from the evidence that MOF directly activates ATM, IR induced damage could also increase H4K16Ac, a signal transmitted to ATM via hMOF resulting in the former's activation (Gupta et al., 2005).There might be other unknown MOF targets,including upstream of ATM , and these get acetylated upon DNA damage and transduce signals for DNA repair. Further work will shed light on these possible mechanisms.

1.57 MYST histone acetyltransferases and cancer

Given the widespread role of MYST histone acetyltransfereases in regulation of gene transcription, DNA repair and replication, their defects could lead to cancer. One of the most widespread histone modifications in mammals, loss of this H4K16 acetylation post translation mark could mark off tumorigenesis. In fact, human colon

cancer cells show a significant loss in Lys 16 acetyaltion and Lys 20 trimethylation marks on histone H4 (Fraga MF., et al., 2005). Similar profiles are observed for various other human tumor cell lines and primary tumors (Taipale et al., 2005).

The well known transcriptional activator Tip60 (HIV Tat-interacting protein of 60 kDa) (Kamine et al., 1996) helps up regulate expression of nuclear hormone receptors (Brady et al., 1999), oncogenic nuclear factor-kB (NF-kB) (Baek et al., 2002), c-Myc oncoprotein (Frank et al., 2003) and E2F (Taubert et al., 2004). Acetylation by Tip60 activates the hormone-dependent transcription factor,androgen receptor (AR) ;impaired function of the latter has been implicated in the development of prostate cancer (Brady ME et al., 1999; Halkidou,K et al., 2003). A well characterised functional interaction exists between Tip60 and the widely studied tumor suppressor p53,the former being involved in specifically acetylating a tumorigenic hotspot residue, lysine 120 (K120) of p53, upon DNA damage (Sykes et al.,2006; Tang et al., 2006).In addition, TIP60 mediated acetylation of ATM, post DNA damage helps initiate DNA repair signalling cascades(Sun et al., 2005). A critical outcome of such activation of ATM is the phosphorylation of several downstream proteins, including p53 (Sancar et al., 2004), causing cell cycle arrest or apoptosis and the histone variant H2AX, needed or DNA repair at DSBs (Shroff et al., 2004).

The histone acetyltransferases p300, CBP and PCAF associate with and modify various transcription factors, such as p53, Rb, E2F, H1Fa and E2A .In fact, p300 and CBP have been identified as tumor suppressors and are targets of the viral oncoproteins E1A and large T antigen (Goodman RH et al., 2000 ;Chan H.M., 2001). Involvement of HATs has been indicated in several chromosomal aberrations leading to leukemogenesis (Yang X.J., 2004). Several leukaemia-associated chromosomal translocations result in fusion of the MYST acetylatransferases MOZ, MORF and MLL (mixed lineage leukemia) to several partners, generating oncogenic chimeric products. MOZ (monocytic leukaemia zinc-finger protein, also known as MYST3), located at 8p11,gets fused to CBP in two translocation t(8;16)(p11;p13) (Borrow J., et al.,1996) and to p300 in t(8;22)(p11;q13) translocations (Chaffanet M et al., 2000., Kitabayashi I., 2001). Runt domain Transcription factors Runx-1 and -2, frequently impaired in leukemia patients, have been shown to functionally interact with MOZ and MORF histone acetyltransferases respectively.

1.6 Apoptotic mechanisms and chromatin remodelers in *Drosophila*

The evolutionary conserved phenomenon of apoptosis or programmed cell death (Lockshin and Williams, 1965), has remained a subject of intense research ever since it was described by Carl Vogt, about 100 years ago in 1842.For all metazoans, this phenomenon is vital for overall body homeostasis and stability. While it is a natural consequence for cell and tissue differentiation during embryonic development and metamorphosis, it may also be a end point decision for a cell faced with terminal DNA damage. While an excessive cell death may destroy healthy cells and tissues, as in neurodegenerative disorders and autoimmune disease, too less of apoptosis may also promote uncontrolled cell proliferation and onset of tumorigenesis. Thus, not surprisingly, in order to maintain genomic stability, the entire apoptotic process is under tight regulation (Holcik M., et al., 2001).

1.61 Role of the caspases

The executioners of apoptosis in flies, as well as in *C.elegans* and humans belong to an evolutionary conserved family of highly selective proteases or caspases (Alnemri ES.,et al., 1996). Synthesized as inactive zymogens or procaspases, several proapoptic signalling pathways promote their activation by proteolysis. All caspases studied so far are characterised by the presence of a cysteine residue at their active site and specifically cleave bonds after an Asp in their substrates; the substrate preferences by different caspases is determined by the specific sequence of the 4 residues amino terminal to the cleavage site (Thornberry N.A. et al., 1997). According to crystallographic studies,when activated, caspases function as tetramers, with two large and two small subunits with each heterodimer derivedfrom a single procaspase molecule (Kumar S et al., 1996., Nicholson DW., 1999).

Broadly, caspases have 2 major roles in carrying out programmed cell death. Death inducing stimuli are received and transduced by Apical or ‘initiator’ caspases ,serving to activate downstream ‘effector ‘caspases that can cleave a number of cellular proteins. Such activity culminates in the various biochemical and morphological changes characteristic of cells dying of apoptosis (Vernooy et al., 2000). The active site of caspases is preceded by an N terminal prodomain region, that usually contains specific protein interaction sequence motifs. Prodomains in initiator caspases are long while those in effector caspases are short. The death effector domains [DEDs] or caspase recruitment domains [CARDs]) on the

prodomains of iniator caspases promote oligomerization and activation to act on downstream caspases (Vernooy et al., 2000).

Ectopic expression of Dronc induces cell death in *Drosophila* eye (Hawkins, 2000) as well as in *S.pombe* and mammalian cells (Dorstyn, 1999). It is also important for programmed cell death during embryogenesis, as silencing Dronc by siRNA injection into early *Drosophila* embryos drastically reduced cell death (Hunter C.P. 1999). The ablated eye phenotype generated by overexpression of hid, rpr and grim can be rescued by 50% in a genetic background expressing a dominant negative Dronc mutant allele in heterozygous state (Meier, 2000;Hawkins, 2000). This indicates that Dronc acts downstream of the RHG proteins. Conversely, over expression of Dronc in the eye produces ablated eye that can be suppressed by halving H99 gene dosage (Hawkins 2000) and increased by *diap1*(*thread*) mutations(Meier P., 2000). Coexpression of the baculoviral caspase inhibitor p35 can also rescue Dronc induced apoptosis in the eye. The CED-4/Apaf-1 fly homolog, Ark and Dronc interact both genetically and biochemically; Ark is required for processing of Dronc as protein extracts from *Ark* homozygous mutant flies are unable to properly process Dronc *in vitro*(Quinn LM et al., 2000). It has been demonstrated in *Drosophila* S2 cells that Dronc is continuously processed to 40KDa factor called Pr1,generated by auto processing at amino acid E352. During apoptosis, another 37KDa processed factor,Pr2 is also generated via drICE mediated cleavage of Dronc at at amino acid D135.The first event is absolutely required for Dronc caspase activity and requires the oligomerizing factor, Dark, as is evident from knockdown studies. Interestingly, although the effector caspase drICE is also needed for apoptosis in S2 cells, the 2nd cleavage event mediated by drICE has no effect on Dronc activity (Muro et al.,2004).

Interaction between the prodomain of Dronc with the *Drosophila* inhibitor of apoptosis protein 1(DIAP1) is necessary for Dronc mediated cell death. In fact, expression of prodomain depleted Dronc is unable to rescue the ablated eye phenotype induced by DIAP1.Both in vivo and in vitro, the RING domain of DIAP1 promotes ubiquitination full length Dronc *in vitro* and also auto processed Dronc. However this does not target the full length protein for proteosomal degradation; rather it suppresses DRONC processing and activation in a non-proteolytic manner. It also results in a Dronc protein with reduced stability. In *diap1* mutant S2 cells that are prevented from proceeding to apoptosis by simultaneous expression of p35,an

increased accumulation of Dronc transcripts and protein is observed, thus further reinforcing our understanding of DIAP1mediated regulation of Dronc (Lee et al., 2011)**.**The apical caspase, Dredd appears to act downstream of the proapoptotic proteins, Hid ,Rpr, Grim, as Dredd transcripts accumulate in proapoptotic cells , about to undergo apoptosis but not in H99 cells, where the entire region coding for RHG proteins has been deleted. Further, expression of Hid, Rpr ,Grim in transfected S2 cells can process Dredd to its active form (Chen et al., 1998). Ectopic expression of hid, rpr, grim generates the small rough eyed phenotype;this can be rescued by heterozygosity at the *dredd* locus(Chen et al., 1998).Apart from its role in PCD, Dredd also has an added role in innate immune response, participating in the Toll signalling pathway and helping in activating ,Relish, a member of the NFkB family of transcription factors (Leulier et al., 2000).

The role of effector caspase drICE in inducing apoptotic cell death in *Drosophila* S2 cells has been well studied *in vitro*, though parallel findings have not been confirmed *in vivo*. Cells can be sensitized to apoptotic both by overexpression of full length drICE as well as aminoterminally truncated form of drICE lacking the first 80 amino acid residues. When apoptosis is induced in S2 cells , by overexpression of *rpr* or by cycloheximide or etoposide treatment ,the cytoplasmic lysates derived showed the presence of a putative caspase. This caspase possessed DEVD specificity, can cleave e p35, lamin DmO, drICE and DCP-1 *in vitro* and can induce chromatin condensation in isolated nuclei. Depleting drICE from the lysates using drICE specific antibodies eliminates most of the caspase mediated apoptotic activity. Conversely, restoring drICE protein to the same lysates from exogenous sources, also restores apoptotic activity (Fraser G., et al., 1997).

*Dcp-1*null mutants in Drosophila survive only to the third instar larval stage. Such larvae display normal central nervous system but have fragile trachea, melanotic tumours and do not have gonads and imaginal discs. Such mutants, however, do not show any abnormality during apoptosis in embryonic stages (Song et al., 1997). The reason might be maternally contributed protein that allows normal apoptosis to occur.In order to eliminate such maternal effect in the dissection of dcp-1 function in the early embryos, germ line *dcp-1* mutant clones were generated by the established technique of mitotic recombination using the yeast FLP recombinase/FRT site system (Xu et al, 1993; Chou et al., 1996). Female flies carrying *dcp-1*$^{-}$ germ line clones are sterile due to a defect in the transfer of the nurse cell cytoplasmic contents to the

developing oocytes.Dcp-1 mutant nurse cells display cytoskeletal reorganisation and nuclear breakdown (McCall K et al., 1998). *In vivo*, the apoptotic ablated small eye phenotype can be induced by can be generated only by expression of a Dcp protein lacking the N terminal 28 residues. Such phenotype can be enhanced by co expression of hid, but not rpr and grim, indicating a hid mediated regulation of the activity of dcp-1 *in vivo* (Song Z., 2000).

Overexpression of Damm in the fly eye produces the ablated eye phenotype, but the effect is not very pronounced (Harvey et al., 2001). It is suggested that Damm may function downstream of hid ,because expressing a dominant negative Damm can rescue the ablated eye phenotype generated by *hid* overexpression ,but not *rpr*.(Harvey et al., 2001). The apoptotic functions of Strica and Decay has not been well demonstrated in mammalian cells and Drosophila S2 cells, as they generate very mild apoptotic effects (Doumanis et al., 2001).

Table 1. Summary of *Drosophila* caspases and their functions (Richardson H et al.,2002; Kumar S et al., 2000).

Caspase	*Function*	*Prodomain*
Dronc	Functional homolog of CED-3/caspase-9; Initiator caspase acting downstream of Ark and H99 proteins	Long, contains CARD domain
Dredd	Genetically interacts with Hid,Grim, Rpr. Also functions in immune response.	Long; Contains two DEDs
Strica	Apoptotic function not well studied	Long; No DED nor CARD, but Ser/Thr-rich domain at its N-terminus with unknown function
Dcp-1	Null allele -third instar larval lethal—no imaginal discs or gonads, melanotic tumors GMR transgene—promotes cell death Germ line clones—nurse cell dumping defect	Short
drICE	Effector caspase	Short
Decay	Effector caspase	Short
Damm	Genetically interacts with hid	Short

1.62 Inhibitor of Apoptosis proteins (IAPs): the central negative regulators of apoptosis

The mechanism of activation of Caspases in *Drosophila* is mediated by an exclusively important antagonist molecule: the IAPs (Lisi et al., 2000; Wang SL et al., 2000). This conserved family of inhibitors was first identified in baculovirus La Casse et al., 1998, by means of their ability to suppress viral infection. Subsequently homologs were identified in *Drosophila melanogaster, Caenorhabditis elegans*, mammals, and yeast (Uren et al. 1998).The structural characteristic of IAPs is the presence of one or more N-terminal repeats of a 70 residue long motif called baculovirus IAP repeat(BIR) (thus also called BIR- repeat containing proteins or BIRPs)and a C- terminal RING finger domain, usually possessing E3 ubiquitin ligase activity (Wang et al., 1999).

The four major BIRPs identified in *Drosophila* are *DIAP1* or *thread* , *DIAP2 Inhibitor of apoptosis 2* (Hay et al., 1995), *deterin*, a homolog of Survivin (Jones, 2000), and *Bruce*, a homolog of BRUCE. Out of these, DIAP1 is considered the most important executioner in regulation of downstream caspases and keeping uncontrolled apoptosis in control. Loss of function mutations of diap1 are embryonic lethal while ectopic expression can inhibit apoptosis (Hay et al., 1995).The primary mode of regulation occurs through its targeted ubiquitination and proteosomal degradation of initiator caspase DrONC (Palaga et al ., 2002). Genetic studies show that ectopic expression of pro DRONC in the *Drosophila* eye generates a severe eye ablation phenotype, which can be completely rescued by the co expression of DIAP1.However such reversion fails when the ablation phenotype is induced by pro domain lacking DRONC, thus confirming the specific interaction between DIAP1 and prodomain of Dronc. In addition, a heterozygous locus at endogenous locus promotes proDRONC mediated eye phenotype (MeierP et al., 2000). Biochemical analysis shows that the RING finger domain of DIAP1 functioning as a E3ubiquitin ligase ,is able to promote ubiquitination of Dronc and thus marks it for degradation (Wilson R et al.,2002).

Partly, DIAP1 also binds through its BIR1domain to the effector caspase, drICE, thus directly inhibiting its catalytic activity and suppressing apoptosis (Kaiser WJ et al., 1998; Yan et al., 2004). Both DIAP1 and its mouse homolog, MIHA are able to physically interact with activated drice ,but not the proform of drICE ,as revealed by transient transfection and coimmunoprecipitation experiments carried out

in SF-21 cell lines.Diap1 also inhibits caspase-3 and Sf-caspase1 mediated apoptosis(Kaiser WJ., et al.,1998). A third caspase, directly inhibited by DIAP1 interaction is DCP-1 (Hawkins CJ., 1999).

1.63 Pro apoptotic proteins: Hid, Reaper and Grim

Three regulatory pro-apoptotic proteins Reaper, Hid and Grim (RHG),that act as antagonists of Baculovirus, *Drosophila* and mammalian IAPs and can predominantly induce apoptosis when ectopically expressed in transgenic animals and cultured insect and mammalian cells (Vucic et al. 1997; McCarthy et al., 1998) These are a set of closely linked genes encoded by the 75C1,2 region of the *Drosophila* third chromosome ,also known as the H99 region ,which when deleted, suppresses apoptosis post γ-irradiation and during embryogenesis (White et al., 1994; Grether et al., 1995; Chen et al., 1996). The baculovirus caspase inhibitor protein, p35 and chemical caspase inhibitors can inhibit RHG induced ectopic cell death ,indicative of their role in caspase mediated cell death (Bump et al., 1995 ;Hay et al., 1994, Chen et al., 1996). Studies from an yeast expression system later confirmed that these proteins act by disrupting DIAP1-caspase interaction (Wang SL et al.,1999).Several signalling pathways transducing death signals can trancriptionally activate the RHG proteins, thus activating apoptosis (Nordstrom et al., 1996). For example: the RAS-MAP kinase pathway post transcriptionally regulates HID via MAPK mediated phosphorylation (Bergmann et al., 1998a,b). It is believed that a competition exists between Dronc and RHG proteins for binding to the BIR2 domain of DIAP1.Auto ubiquitination and degradation of DIAP1 occurs through recruitment of a ubiquitin –conjugating E2 enzyme, UBCD1(Ryoo et al., 2002) or E2-like protein Morgue (Wing et al., 2002) by RHG proteins. Another mechanism of DIAP1 ubiquitination and anti apoptisis that exists in cells operates downstream of caspases and doesn't require the RING domain of DIAP1.It is thought this second mechanism is operative to keep existing basal level of caspases in check to prevent any unwanted apoptosis in absence of death signals(Danial N.,N et al., 2004).

1.7 Multiple signalling pathways exist for transduction of apoptotic signals in *Drosophila*

Several upstream pathways transducing death signals may serve to activate and regulate the RHG proteins in *Drosophila*, thus controlling caspase mediated cell

death. In particular the pro apoptotic gene hid is subject to activation or repression by factors as varied as developmental cues, hormonal changes during metamorphosis and intercellular communications.

1.71 Ionizing radiation and p53 mediated signaling

In humans, p53 tumor suppressor protein is hailed as the guardian of the genome, either arresting the cell cycle allowing for repair, or promoting cellular apoptosis on irreparable damage. A *Drosophila* homolog of human p53, bearing structural and functional domains similar to that of humans, was identified by Brodsky et al., Ollman et al., and Jin et al (2000) through homology searches of the expressed sequence tag database of the Berkeley *Drosophila* Genome Project.

Ectopic expression of a dominant negative form of p53 in posterior halves of *Drosophila* wing discs through an *engrailed* GAL4 driver, and checking for radiation induced apoptosis in cells in that region, confirms the requirement of dmp53 for the process. Wild type p53,when overexpressed in the adult eye, causes apoptosis and small ablated eye phenotype (Brodsky et al., 2000).Such cell death, unlike that caused by p53 mediated radiation induced apoptosis, cannot be rescued by co expression of viral caspase-inhibitor p35;this clearly indicates an existing difference in the mechanism of apoptosis induction in the given situations.

Exposure to DNA damaging signals like IR or UV upregulates hid transcription in *Drosophila* embryos and larvae. Loss-of-function hid heterozygote mutants are specially sensitive to such stresses, with impaired ability to undergo apoptosis (Brodsky MH., 2004). *Rpr* promoter has been shown to contain a radiation - inducible, consensus p53 binding sites. Upon IR radiation, p53 can bind here and activate transcription, both in yeast cells and *in vivo* in *Drosophila*. (Brodsky MH et al., 2000).Subsequently reaper can promote activation of apical caspases either through direct inhibition of IAPs and/or the Apaf-1/CED-4 homolog, Dark, leading to apoptosis. Though p53 has not been shown to bind directly to hid promoter, p53 and its activator kinase are required to induce increase in levels of hid transcripts on IR exposure.

Surprisingly, despite the conservation of cell cycle regulators from flies to humans, role of p53 in mediating cell cycle arrest in response to genotoxic stress is not that apparent, as observed in studies from functional studies of DP53 in wing and eye discs.P53 targets –p21 and Bax, so well characterised in humans, have not been

identified so far in *Drosphila*. In mammals, an activated p53, on receiving DNA damaging signals, can, activate transcription of p21,which leads to G1 arrest and also inhibition of the activity of cyclin –dependent kinases(Cdks) and phosphorylation of retinoblastoma(Rb) protein (Hengstschlager M et al.,1999).

1.72 Signaling through JNK pathway

On UV exposure, studied in developing *Drosophila* retina, signalling through the Jun N-terminal Kinase (JNK) signaling and transcription factors, Foxo and AP-1 (*Drosophila* Fos) acts to induce hid transcription (Jassim OW et al., 2003, Luo X et al., 2007). This effect is completely absent in retinal cells of JNK mutants and drastically reduced with lowered FOXO levels, post UV irradiation (Luo X et al., 2007).

1.73 Signaling through EGFR/Ras/MAPK pathway

Repressive signaling by the EGFR/Ras/MAPK pathway through RTKs or receptor tyrosine kinases (EGFR and IGFR) serves to inhibit hid at both transcriptional and post transcriptional levels and prevent apoptosis during embryogenesis and development in *Drosophila* pupal retinal cells, larval eye imaginal disc cells and embryonic midline glia (Miller DT et al., 1998; Baker NE ., et al., 2001). Signaling through EGFR can activate Ras, which transmits hid suppressor signals via several downstream effectors like RAF/MAPK and PI3 Kinase/Akt, but not Rel. Hid transcripts are present at reduced levels in embryos that are gain -of - function mutants of Ras or Raf or overexpression mutants of their target protein, Pointed (Kurada P. Et al., 1998) .Inhibitory phosphorylation at consensus MAPK sites on the Hid protein, can block apoptosis. Site directed mutagenesis at these residues, abolishes such inhibition and enhances hid induced apoptosis in S2 cells (Bergmann A et al., 1998a).The eye ablation phenotype characteristic of hid ectopic expression cannot be rescued by constitutively active Ras or MAPK. It is believed that UV induced Ras signaling through PI3-Kinase and Akt acts to inhibit Foxo and thus leads to hid transcriptional inhibition (Luo X., et al., 2007).This is suggestive of MAPK/Ras pathway acting antagonistically to JNK signalling to regulate hid levels and apoptosis.

1.74 Signaling through Hippo pathway

Genetic evidence suggests that the Hippo tumor suppressor pathway is operative in inducing hid expression and cause apoptosis of inter ommatidial cells in the developing eye (Udan RS et al., 2003). Such cells are generated initially by excessive mitotic proliferation but are destined to die during pupal stages. Their ability to undergo apoptosis is impaired in Hpo –mutants , which also register decreased hid MRNA levels.The mechanism by which Hpo activates hid still remains elusive; it is believed to be mediated by hid suppressor microRNA bantam, which itself is repressed by Hpo signalling (Thompson BJ., et al 2006).

1.75 E2F factor signalling

Transcription factor E2F inhibits hid transcription in *Drosophila*, with E2F1 binding to consensus sequences in the hid enhancer and blocking apoptosis in S2 cells (Moon NS et al., 2005, 2006). The dorsal/ventral boundary of wing imaginal discs also accumulate hid RNA in Dp mutants; Dp is binding partner to form active complexes with E2F1 and E2F2.Another level of hid repression is offered by RB proteins, which are known to bind to E2F/Dp transcription complexes. Extracts prepared from mutant eye discs or clones of RbF1 have increased *hid* transcripts (Tanaka-Matakatsu M et al., 2009). Morever isolation of a hid mutant in a forward genetic screen aimed at identifying suppressors of RBF1 mediated apoptosis provide evidence of an Rb-E2F2-Dp mediated regulation of hid expression in eye and wing discs (Bilak et al., 2009).

1.76 Role of Apaf1-like, apoptosome forming, protein -Dark

RHG proteins may also control caspase activation though a mechanism involving formation of an apoptosome like complex containing Dark (*Apaf-1-related-killer* or *Ark*)-the *Drosophila* homolog of mammalian Apaf-1 and *Caenorhabditis elegans* CED-4, cell-death proteins. Although DIAP1 is supposed to be the central molecule regulating caspase induced cell death, dark functions as co activator of cell death in parallel or downstream to DIAP1 in the apoptotic pathway. The action of dark is to activate apical procaspases in response to apoptotic stimuli; this coupled with derepression of caspases by RHG proteins serves to induce PCD *in vivo*. Thus a delicate balance between positive (Apaf-1/CED-4/Dark) and negative regulators (IAPs) determines cellular caspase activity and the ultimate fate of the cell.

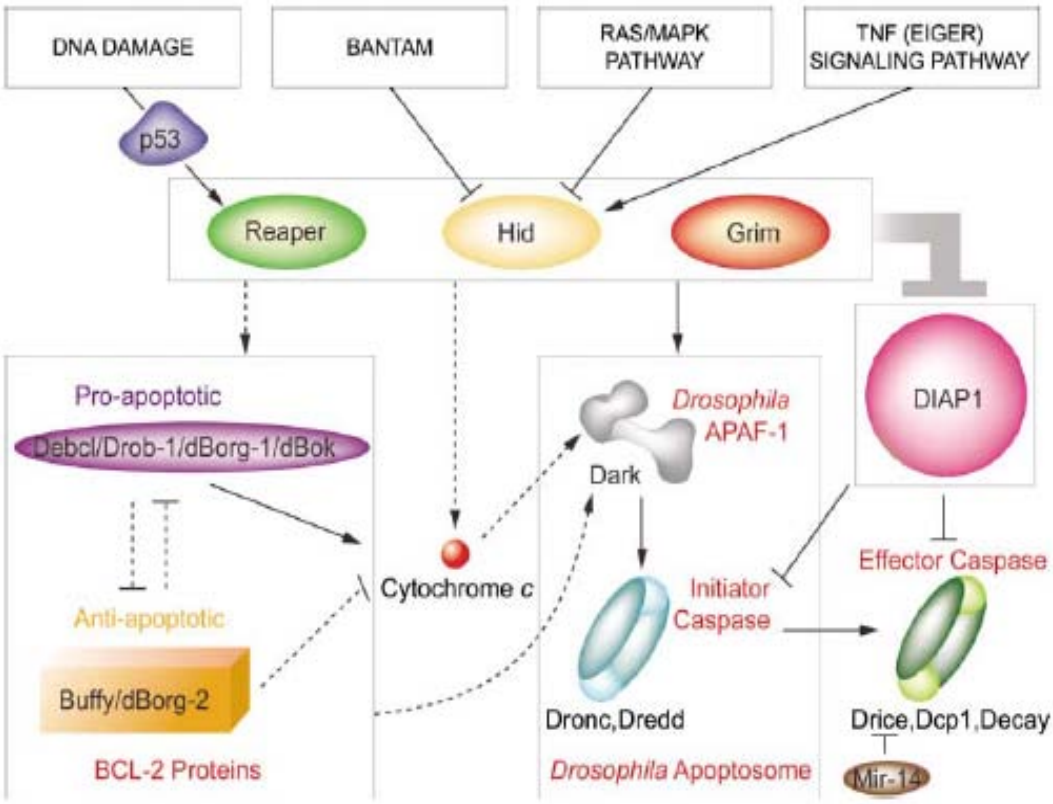


Fig 1.3. Apoptotic pathways in *Drosophila* (Daniel N et al., 2004)

1.8 Epigenetic regulation of *Drosophila* apoptosis mediated through chromatin remodelers

An interesting mechanism to epigentically regulate Hid and Rpr induced apoptosis exists in *Drosophila* embryos. Hid and Rpr induced cell death is inducible in young embryos, with undifferentiated, rapidly dividing cells but not in later stages. It was observed that post-stage 12 embryos, which contain mostly differentiated cells are resistant to IR doses and do not undergo apoptosis. These 2 pro apoptotic genes harbour an irradiation responsive enhancer region (IRER), immediately upstream of *rpr* and about 250 kb away from hid ORF (Zhang Y et al., 2008). The IRER confers these genes with sensitivity to IR stimuli, so that they are radidly upregulated following irradiation. Post stage 12, the IRER accumulates repressive histone modifications: trimethylated H3K27/H3K9, acquiring heterochromatinised, condensed conformation and lowering greatly the responsiveness of these genes to radiation stimuli. Thus critical developmental chromatin marks impinged by specific chromatin remodelers seem to confer stage-specific apoptotic ability in *Drosophila*. The histone modifying enzymes Polycomb Repressive Complex2 or PRC2(consisting

of Suppressor of zeste 12 [Su(z)12], Extra sexcombs (ESC), and Enhancer of zeste [E(z)]) trimethylates H3K27 while that of H3K27 is brought about by Su(var)3-9.For both modifications, histone deacetylase (Hdac1/rpd3) is also involved (Zhang Y et al., 2008). There is also evidence of the presence of 2 putative non coding RNA transcripts flanking the ends of a small region within the IRER, that remains open and accessible till embrogenesis ends. The exact mechanism of how these participate in hid/rpr transcriptional regulation remains to be elucidated. Larval imaginal disc cells are also subject to such epigenetic regulation .High levels of hid transcripts and an elevated level of spontaneous apoptosis have been reported in discs of HDAC3 (but notHDAC1 mutants(Zhang Y et al., 2008, Zhu CC et al., 2008).

1.81 MicroRNAs in *Drosophila* apoptosis

An interesting role of microRNAs regulating expression of apoptotic factors is beginning to emerge. Bantam microRNA regulates hid by binding to its 3' UTR site, independent of the Ras/MAPK pathway (Brennecke et al., 2003). miR-6 and mIR—2/13are also implicated in regulating hid transcripts as nullifying them by injecting antisense strands increases Hid levels and causes large scale apoptosis in embryos (Leaman Det al., 2005). In addition to harboring bantam binding sites, the 3'UTR of hid also carries a nanos responsive element (NRE) which recruits Nanos to hid mRNA and promotes its degradation. Thus ,embryos obtained from Nanos depleted mothers undergo major hid mediated apoptotic cell death (Sato K., et al.2007). Caspase Drice has been shown to be regulated by miR-14 (Xu et al., 2003).

1.82 Comparative description of apoptotic mechanisms in flies, nematodes and mammals

Genetic studies to identify prominent componenets of apoptotic machinery were initiated in *C.elegans* in which 3 major genes were described: *ced-3, ced-4 and ced-9*. Evolutionary conserved homologs corresponding to these were later found in flies and mammals,including humans.CED-3's mammalian homolog is the protein interleukin 1_ converting enzyme (ICE) involved in inflammation (Yuan et al., 1993) and both can induce apoptosis in mammalian cells. These are the founding members of the apoptotic family of proteases or caspases(caspase-1). Subsequently a dozen or so caspases have been identified in humans, with majority of them involved in apoptosis. Caspases enzumes sequences are conserved from *C.elegans* to humans.

Identified by a gain of function mutation *n1950*, CED-9 bears homology to the Bcl-2 family of apoptotic proteins in *Drosophila* and vertebrates (Gross et al., 1999;Chen and Abrams,2000).CED-4 bears homology to the *Drosophila* Dark/Hac1/Dapaf-1(Rodriguez et al., 1999).These operate through the apoptosomal pathway of apical caspase activation (Zou et al.,1999).

In vitro experiments in mammalian cells designed to reconstitute caspase activity led to identification of 3 intermediate but important proteins –Apafs (apoptotic protease activating factors). Apaf-1, 2 and 3 were described as homologs of ced-4, cytochrome c and caspase-9, respectively (Li et al., 1997). A well established model exists for apaf-1 mediated Caspase-9, an initiator caspase activation in humans.Apaf-1 molecule is essentially an adapter/amplifier molecule characterised by the presence of WD40 domains, 2 of which bind its caspase recruitment domains(CARD).Binding to cytochrome c exposes CARD domain ,allowing Apaf-1 to interact with and activate the initiator caspase-9 in an ATP dependent manner (Li et al., 1997). ATP binding to Apaf-1 results in the formation of a wheel like apoptosomal complex, with CARD and CED-4 homology domains form the hub, while the spokes consist of WD40 domains, and procaspase-9 binds the hub (Acehan et al., 2002).WD40 domains are absent on *C.elegans* CED-4; thus it doesn't bind cytochrome c. Instead CED-4 localizes to mitochondria and is dislodged into the perinuclear region; an action requiring CED-9 and its interaction with EGL-1, a proapoptotic BH3-only BCL-2 family homolog (Conradt et al., 1998). Oligomerization of CED-4 and autocatalytic activation of CED-3 caspase then follows (Yang et al., 1998). Three major apoptotic pathways have been identified in humans: extrinsic or death receptor pathway , the intrinsic or mitochondrial pathway and the T cell mediated perforin-granzyme-dependent pathway (Igney and Krammer, 2002; Martinvalet et al.,2005). There is evidence for considerable interaction between the members of the first two pathways and all the three of them converge at the terminal or execution pathway.

1.83 The intrinsic and extrinsic pathways

This pathway is initiated at the mitochondrial membrane by the receipt of cell death signals from a number of non receptor mediated stimuli and the discharge of 2 classes of proapoptotic proteins from the intermembrane space into the cytosol . Both positive and negative signals may act through the intrinsic pathway to suppress or

activate apoptosis. Negative signals involve the absence of certain growth factors, hormones and cytokines while positive signals include radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. The first class of molecules thus released includes the cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Du et al., 2000; van Loo et al., 2002; Garrido et al., 2005). Cyt c binds to and activates Apaf-1 and procaspase-9 ,forming apoptosome and leading to active caspase -9 mediated apoptosis (Li et al., 1997). Smac/DIABLO and HtrA2/Omi induce apoptosis by inhibiting IAP (inhibitors of apoptosis proteins) activity (van Loo et al., 2002).The second class of pro apoptotic proteins include AIF, endonuclease G and CAD. Release of these factors occurs in the later stages of apoptosis.AIF cause genomic DNA fragmentation in the nucleus and condensation of peripheral nuclear chromatin (Joza et al., 2001), named the "stage I" condensation (Susin et al., 2000). Endonuclease G, like AIF, also translocates to the nucleus and fragments chromatin,in a caspase independent manner .Cleavage of DNA by CAD occurs through cleavage of caspase-3, leading to 'stage 2" condensation (Enari et al., 1998).

The BCl-2 family of checkpoint proteins, are important checkpoint proteins, regulating the intrinsic pathway of apoptosis (Cory and Adams, 2002). Identified first as a proto-oncogene operative in human follicular B cell lymphoma, overexpression of BCL-2, localized in mitochondria, can inhibit apoptosis. The Bcl-2 members control mitochondrial membrane permeability and hence the release of cytochrome c from the mitochondria. Their regulatory action can be both pro- as well as anti apoptotic. Anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. Mice deficient in Bcl2 gene show display impaired cellular homeostasis, and gross apoptotic cell death in lymphocutes, developing renal cells and melanocytes (Veis et al., 1993). BAX, coded by an important proapoptotic gene in mammals interacts with BCL-2 (Oltvai et al., 1993). The members of the Bcl2 family can be subgrouped into 3 major classes ,depending on the homology they possess to the 4 conserved Bcl2 Homology (BH)domains. The first class demonstates complete homology to all four BH domains and includes the antiapoptotic members - BCL-2, BCL-XL (Boise et al., 1993), MCL-1 (Kozopas et al., 1993), A1 (Choi et al., 1995), and BCL-W (Gibson et al., 1996). BAX and BAK constitute the second class of proapoptotic members, possessing only 1-3 BH1 domains and are required for

entry through the intrinsic pathway at the mitochondrion and endoplasmic reticulum (Wei et al., 2001) on receipt of apoptotic stimuli. Normal cells consist of monomers of BAX and BAK. Death signals causes inactive BAX monomers present in the cytosol to insert themselves into the outer mitochondrial membrane as homo-oligomers. Besides, existing inactive BAK molecules at the mitochondria also undergo an allosteric conformational change,to form oligomers (Wei et al., 2001). Such oligomers possess similarity with the pore forming helices of bacterial toxins and possibly form pores in the mitochondrial outer membrane ,leading to release of cytochrome c (Elmore S., 2007).

Several other mechanisms of Bcl-2 regulation of caspase mediated cell death have been proposed. The Bcl-2 family member, Bid may undergo caspase-8 mediated cleavage (Li et al., 1998). Alternatively the pro apoptotic protein Bad, might undergo dephosphorylation and get released from its capture in the cytosol by 14-3-3 upon receiving death signals. This allows it to migrate to mitochondria and release cyt c (Zha, et al., 1996).There is also possibility of Bad interacting and capturing anti apoptotic proteins, Bcl-Xl or Bcl-2, and leading to cell death. The latter are supposed to control caspase activation and thus inhibit cell death under normal conditions (Newmeyer et al., 2000).The tumor suppressor p53 is known to play a crucial role in regulating the Bcl-2 family of proteins. Notably, the pro apoptotic member Puma can be induced by p53, after receipt of genotoxic stress stimuli or oncogene activation. Increase in expression of Puma, is accompanied by simultaneous increase in BAX levels. This leads to a change in BAX conformation , changing the mitochondrial membrane potential and releasing Cyt c (Liu et al., 2003). Noxa, another member of the Bcl2 family is also known to mediate p53 dependent apoptosis (Elmore, 2007).

The homologs of Bcl-2 family proteins include proapoptotic protein Debcl/Drob-1/dBorg-1/dBok and an antiapoptotic protein Buffy/dBorg-2 (Brachmann et al., 2000; Quinn et al., 2003). Like their counterparts in humans, these proteins regulate caspase activation through the mitochondrial pathway. Both consist of BH1-3 domains and a hydrophobic membrane segment for localization to the mitochondrion. Their interaction with RHG proteins is still being studied.

Apoptotic signalling through the extrinsic pathway is initiated through receipt of death signals by several transmembrane receptors including members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). The role of these signal transduction pathways came extensively from studies in the immune

system, where they serve to regulate apoptosis to maintain cellular homeostasis. These receptors are characterised by the presence of cyteine-rich extracellular domains and an 80 residue cytoplasmic domain called the ‘death domain’ (Ashkenazi et al., 1998). A prominent member of this family, is Fas, a cell surface receptor, discovered through its loss of function mutation in the lymphoproliferative disorder, *lpr* (Itoh et al., 1991). Its ligand is Fas ligand (FasL), generally mutated in the lymphoproliferative, autoimmune disorder *gld c* (Takahasi et al., 1994).

CHAPTER-2

MATERIALS AND METHODS

2.1 *Drosophila* culture and maintenance

Canton S was used as wild type strain. All crosses were maintained at 25°C on standard culture media of corn meal, molasses, yeast extract, agar and anti-bacterial and anti-fungal preservatives. Common balancer stocks used were obtained from the Bloomington Stock Collection. The genetic markers and phenotypes of each mutation, are described in Flybase (http://flystocks.bio.indiana.edu/). Ectopic expression of transgenes was done using the UAS –GAL4 system, as described by Brand and Perrimon (1993).The glass multiple reporter GMR-GAL4 line (Kohler and Milstein, 1975) was used to drive expression of UAS transgenes in the larval eye discs and adult eyes.

Table 2. Fly stocks used.

S.No	Genotype	Source/Contributer
1.	Canton S	Bloomington
2.	Mutiple balancer *yw; In(2LR)Gla/CyO;TM3Ser/MKRS*	Bloomington
3.	Basc	Bloomington
4.	*mof1*/ Basc	John C. Lucchesi
5.	*y w mof 3; [mof t6.8]18H1*	Joel C. Eissenberg
6.	*y w mof 6; [mof t6.8]18H1*	Joel C. Eissenberg
7.	*FM7,P{w[+mC]=ActGFP}JMR3/C(1)DX, y[1] f[1]*	Bloomington
8.	*yw67c23*	Bloomington
9.	*mnkp6/ mnkp6*	William E. Theurkauf
10.	GMR-Gal4	Bloomington
11.	UAS-*p35*/ UAS-*p35*	Hermann Stellar
12.	GMR-*hid*/GMR-*hid*	Andreas Bergmann
13.	GMR-*rpr*/CyO	Andreas Bergmann
14.	GMR-*grim*/ GMR-*grim*	Andreas Bergmann
15.	EP-*mof*	Bloomington
16.	Sco/CyO; *H99*/ Tb	Hermann Stellar
17.	diap1 $^{33\text{-}1s}$	Hermann Stellar
18	diap1 $^{22\text{-}8s}$/Sb	Hermann Stellar
19	Hid 109/Tb	Hermann Stellar
20.	th^4/Tb	Hermann Stellar
21.	*diap1-lacZ*	Andreas Bergmann
22.	$bsk^2 cn^1 bw^1 sp^1$/CyO	Bloomington
23.	w^{1118}P{UAS-bsk.DN}2	Bloomington

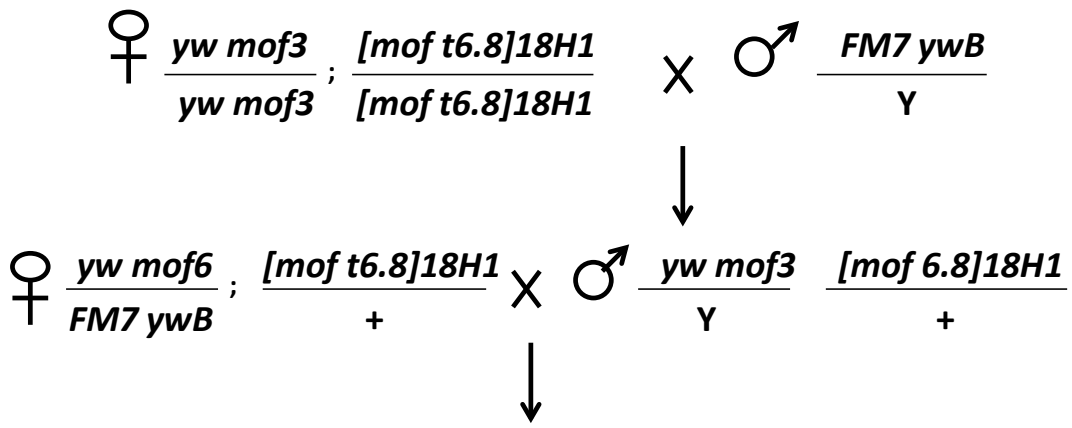
Table 3. RNAi lines.

All stocks carrying constructs designed to knock down gene expression using the RNAi pathway were obtained from Bloomington Stock Centre. These were generated by the Transgenic RNAi Project (TRiP) (http://www.flyrnai.org/TRiP-HOME.html).

S.No	Gene	Genotype
1.	*mof*	y[1] sc[*] v[1];P{y[+t7.7]v[+t1.8]=TRiP.HMS00573}attP2/TM3, Sb[1]
2.	*Ark*	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00870}attP2
3.	*Dredd*	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00063}attP2
4.	*Ice*	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00398}attP2
5.	*Dronc*	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00758}attP2

2.2 **Genetic Crosses.** Schemes of genetic crosses are given below.

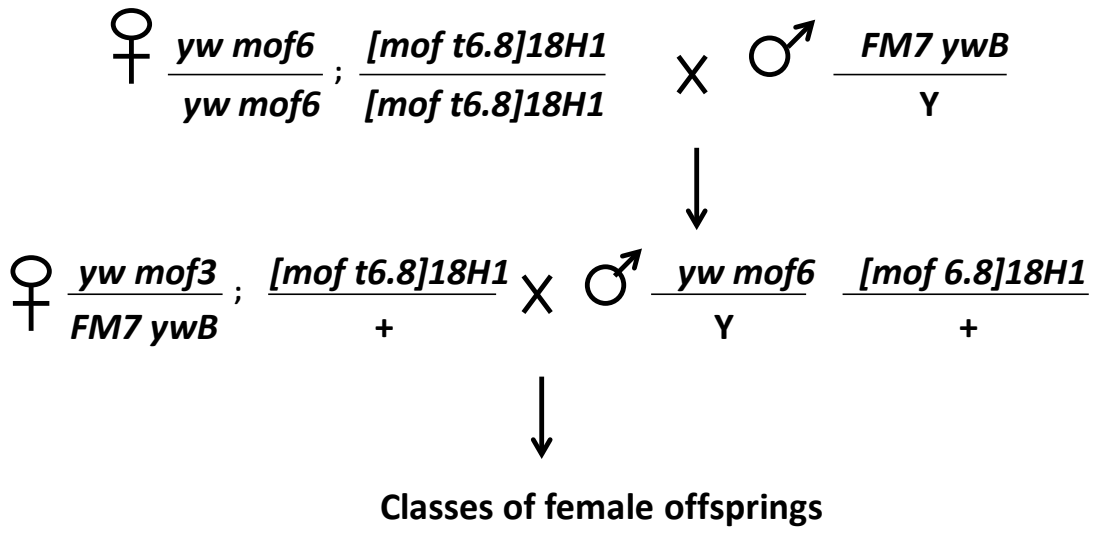
A



Classes of female offsprings

With doses of wild type 18H1 transgene	*mof mutants*
yw mof3 / yw mof6 ; 18H1 / +	*yw mof3 / yw mof6*
yw mof3 / yw mof6 ; 18H1 / 18H1	
yw mof3 / FM7 ywB ; 18H1 / +	*yw mof3 / FM7 ywB*
yw mof3 / FM7 ywB ; 18H1 / 18H1	

B



With doses of wild type 18H1 transgene	*mof mutants*
yw mof3 / yw mof6 ; 18H1 / 18H1	*yw mof3 / yw mof6*
yw mof3 / yw mof6 ; 18H1 / 18H1	
yw mof6 / FM7 ywB ; 18H1 / +	*yw mof6 / FM7 ywB*
yw mof6 / FM7 ywB ; 18H1 / 18H1	

Fig. 2.1. Heteroallelic *mof3/mof6* females were generated by crossing **A.** *yw mof3 [mof t6.8]18H1* females and **B.** *yw mof6; [mof6 t6.8]* with *FM7 y w B* males.

C

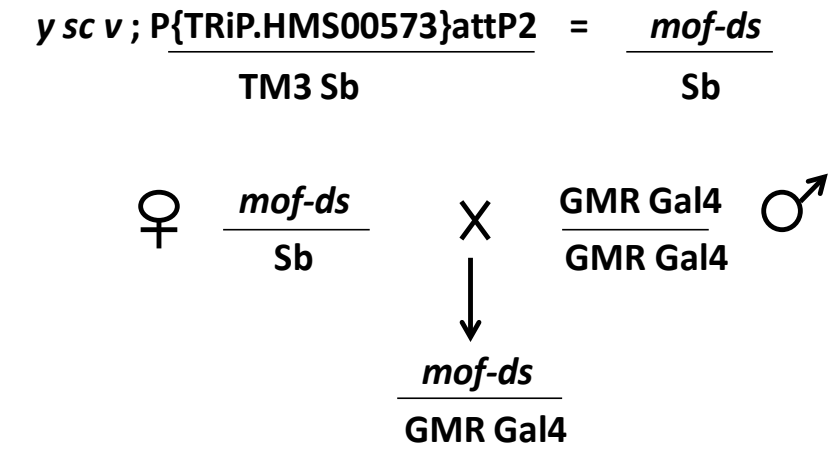


Fig. 2.2 RNAi-mediated silencing of *mof* in eye. Similar scheme is followed for all crosses containing UAS element and driven by GMR Gal4 driver.

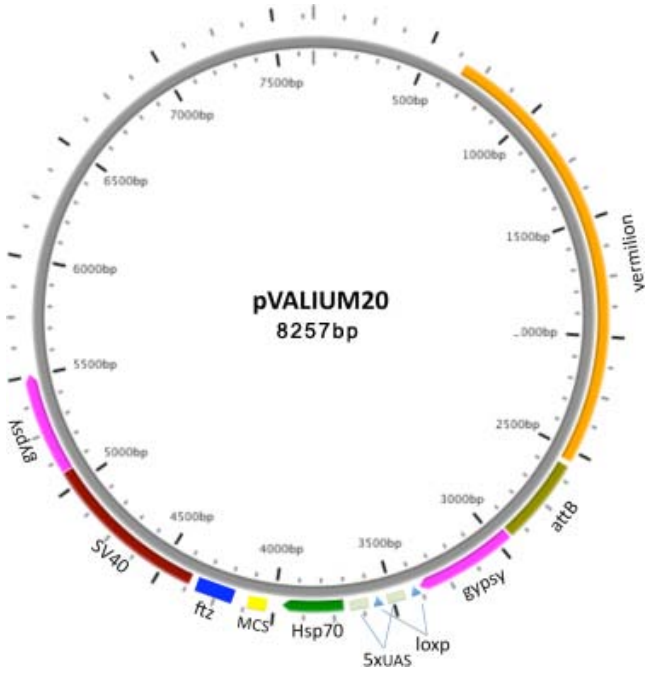


Fig.2.3. Transgenic stock for *mof* knockdown constructed using 'Valium20'.TRiP vector relies on endogenous microRNA pathway to deliver siRNAs (Perrimon et al., 2010).

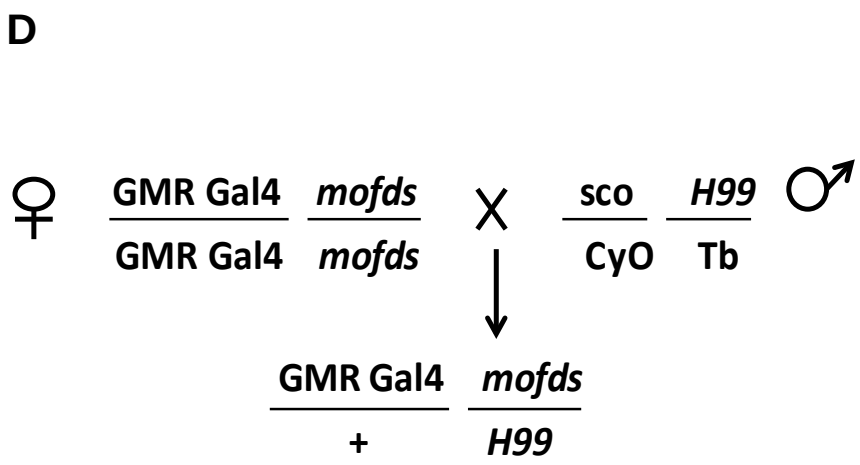


Fig. 2.4. Genetic interactions of *mof* with components of the apoptotic pathways. Similar scheme is followed for all crosses aiming to bring together GMR Gal4, UAS-*mof*^{ds} and a 3rd allele under investigation.

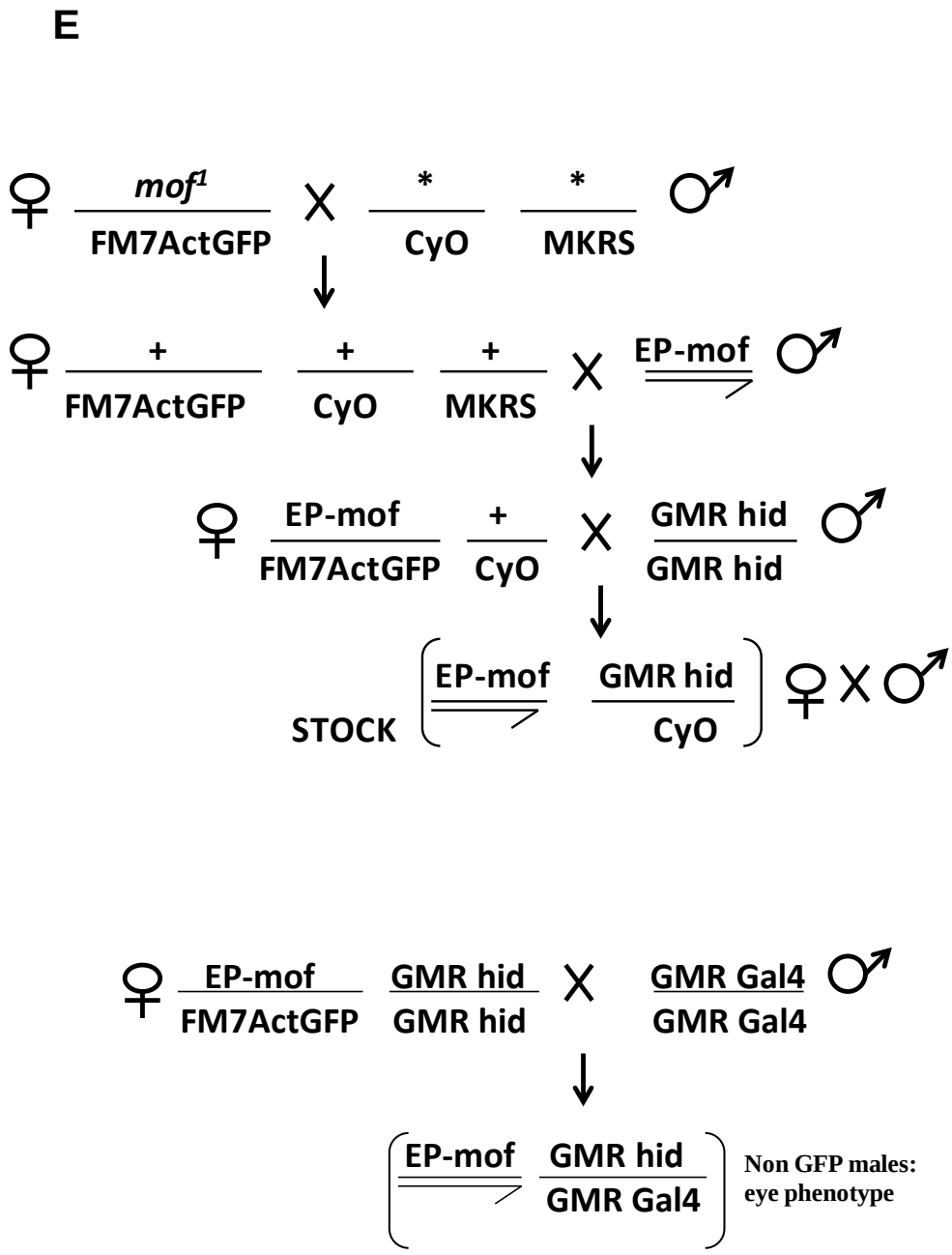


Fig. 2.5. Genetic interaction between *mof* and proapoptotic genes –*hid, rpr* and *grim*. S Similar schemes were followed for *rpr* and *grim*. * denotes a non related mutation.

2.3. Embryo Staining

Chemicals and Solutions required

1. Commercial Bleach
2. TritonX-100
3. 0.5M Hepes pH(6.9)
4. 1M Magnesium Sulphate
5. 0.5M EGTA [ethylene- glycol-bis(2-aminoethylether)-N,N-tetra acetic acid, pH8]
6. 20% Paraformaldehyde
7. Heptane
8. Phosphate Buffer Saline(PBS)
9. PBT (PBS + 0.1% Tween20)
10. 10mg/ml RNase
11. Animal Serum(Goat and donkey)
12. Primary Antibody
13. Fluorescence Conjugated Secondary Antibody
14. Mounting Media-DAPI (4',6-diamidino-2-phenylindole) or PI (Propidium Iodide)

Methodology

Collection and Preparation of Embryos

Drosophila embryos were harvested from yeast-agar plates. The stages of embryos are referred to in hours after egg laying (AEL) at 25°C.They were collected in small baskets, washed thoroughly with water and dechorionated in 50% commercial bleach (5% sodium hypochlorite) for 2-3 mins. They were then washed in 0.1% Triton X-100 and fixed with paraformaldehyde.

Paraformaldehyde Fixation

The embryos were transferred to glass scintillation vials containing 1.6 ml of 0.1 M Hepes pH 6.9, 2 mM magnesium sulphate, 1 mM EGTA. 0.4ml of 20% paraformaldehyde and 8ml of heptanes was added. The vial was placed in a shaker for 20mins to maintain an effective emulsion of organic and water phase. The lower phase was removed, 10ml of methanol was added and embryos sink to the bottom of the tube.The embryos may now be stored in methanol for several weeks at 4°C.If not

stored, the embryos are transferred to a solution of 90% methanol + 10% 0.5M EGTA in 1.5ml tube. The embryos were refixed and rehydrated by passage through a series of steps containing 0.5M EGTA-Methanol (ME) and PP(4% paraformaldehyde in PBS).

7 parts of ME: 3PP-5mins
1ME:1PP-5 mins
3ME:7PP-5 mins
PP alone-20 mins
Embryos were further washed in PBS for 10 mins.

Pretreatment

All pretreatment steps were performed in 1.5 ml Eppendorf tubes in 1 ml solution at RT on a revolving wheel.Embryos were washed thrice in PBT, each for 5 mins and incubated in 10mg/ml RNaseA (in PBS) at 37°C for 15 mins.The embryos were further washed twice each for 5mins in PBT and refixed for 20 mins with PP (4% PP in PBS) alone and washed thrice each for 10 mins in PBT.

Hybridization

The blocking solution contains 2% goat or donkey serum in PBT. Embryos were incubated in it for 30 mins and then hybridization with primary antibodies in appropriate dilution was performed overnight. After this, embryos were washed in PBT(5 mins) and twice wuth PBS(5 mins).They were incubated with fluorescence tagged secondary antibody for 4 hrs, followed by washing with PBT(5 mins) and PBS(twice for 5 mins) and finally mounted in DAPI or PI.

Microscopy and Imaging

Specimens were viewed on a confocal microscope (Olympus) using 20X or 100X oil immersion objectives.3-D images were generated by projections or z-stack projections with FluoView software.

2.4 Polytene chromosome preparation

Chemicals and Solutions required

Solution1: 0.1% Triton-X 100 in PBS pH7.5

Solution 2: 3.7% paraformaldehyde, 1% Triton X-100 in PBS pH7.5

Solution 3: 3.7% paraformaldehyde, 50% acetic acid

Block Solution: 3%BSA, 0.2% Bovine Serum Albumin (BSA),0.2%(w/v)Nonidet P-40, 0.2%(w/v) Tween 20,10% non fat dry milk.

PBS: 300mM NaCl, 0.2%Tween 20, 0.2% NP-40

Methodology

For polytene chromosome preparation, salivary glands were dissected from well fed 3rd instar larvae in Solution1and the gland associated fat bodies were removed subsequently. The glands were transferred to a drop of Solution2 on a siliconized cover slip.

2.5 Acridine Orange Staining

Acridine orange stain is commonly used to detect apoptotic cells in live tissues. Eye discs from third instar larvae were dissected in PBS and stained with freshly diluted acridine orange solution (1 mg/ml). The discs were then washed, mounted in PBS, and immediately viewed in a confocal microscope with the Rhodamine Red channel.

2.6 Immunostaining of Imaginal Discs

Eye discs were dissected from well fed 3rd instar larvae, fixed for 20 mins in 4% paraformaldehyde in PBT and then washed in PBT for 10 mins. The discs were treated with RNase A(10mg/ml) at 37°C for 15 mins ,washed in PBS, blocked in 2% animal serum for 1hr and incubated with primary antibody overnight at 4°C.They were then washed again with PBT and PBS(5 mins each) and stained with fluorescence conjugated secondary antibody for 4 hrs. The discs were mounted in Vectashield mounting media (Vector Laboratories, USA) containing DAPI or propidium iodide and viewed in confocal microscope.

2.7 Quantitative analysis of stained discs.

Stained imaginal discs were processed digitally and fluorescent spots quantified with Image J software (NIH, Bethesda, USA).The region posterior to the morphogenetic furrow was chosen for analysis in eye discs. Threshold intensity was set for the spots and region of interest (ROI) was selected. The Area Fraction tab

enabled calculation of points with fluorescence intensity greater than the set threshold. Approximately 20 discs for each genotype were analyzed in each experiment.

2.8 Plasmid DNA isolation using Alkaline Lysis Method

Chemicals and Solutions required

1. Solution 1[50mM Glucose, 25mM Tris-HCl(pH8.0),10mM EDTA(pH8.0)]
2. Solution 2[Freshly prepared 0.2N NaOH, 1% SDS]
3. Solution 3[5M potassium acetate pH(5.2)]
4. 100% chilled Ethanol
5. 3M Sodium Acetate (pH5.2)
6. Tris saturated phenol (pH8.0)
7. Chloroform: Isoamyl alcohol[24:1]
8. TE (pH8.0) [10mM Tris and 1mM EDTA]
9. RNase A [10mg/ml]

Methodology

A single bacterial colony was inoculated in 3ml LB (Lural Bertani) minimal media containing appropriate antibiotic selector. The bacterium was inoculated overnight at 37°C in the shaking incubator. The culture was centrifuged at 5000 rpm for 3 mins to pellet the bacterial cells. The pellet was dissolved in 150 µl of Solution1 containing 2 µl of 10mg/ml RNase A.200µl of freshly prepared Solution 2 was added, mixed gently by inverting the tube and incubated at RT for 5 mins.150µl of Solution 3 was further added to it, mixed gently and incubated on ice for 10 mins. The contents were centrifuged at 12,000 rpm for 15 mins at 4°C.The supernatant was discarded and the pellet dissolved in 60 µl TE.Phenol: chloroform(1:1) and chloroform : Isoamyl alcohol (24:1) extraction was carried out at room temperature to remove the proteins. The final supernatant was precipitated using 2.5 vol of chilled ethanol and 1/10 vol of sodium acetate (pH 5.2) at -20°C for overnight. The DNA was precipitated by centrifugation at 12,000 rpm for 20 mins at 4°C, followed by a 70% ethanol wash to remove the salts. The pellet containing plasmid DNA was air-dried at room temperature and dissolved in 50 µl of TE. The quality of plasmid DNA was estimated on 0.8% agarose gel and the concentration of DNA was determined using Nanodrop

Spectrophotometer. Three discrete bands show up in the agarose gel: supercoiled form is the lowermost and brightest band, linearized in the middle and nicked circular form is at the top of the gel.

2.9 Fly Genomic DNA preparation.

Genomic DNA was prepared from adult flies as per protocol described by BDGP (Berkeley *Drosophila* Genome Project).

Chemicals and Solutions required

1. Buffer A [100mM Tris-HCl (pH7.5), 100Mm EDTA,100Mm NaCl , 0.5%SDS]
2. Lithium Chloride/Potassium Acetate solution [1 part 5M KAc: 2.5 parts 6M LiCl]
3. Isopropanol
4. TE (pH8.0) [10mM Tris and 1mM EDTA]
5. 70% Ethanol

Methodology

Nearly 30 adult flies were collected and crushed thoroughly to fine powder in liquid nitrogen with mortar and pestle.200 µl of Buffer A was added and incubated at 65°C for 30 mins. Further, 800 µl of LiCl/KAc was added to the tube and incubated on ice for 10 mins.The contents were then centrifuged at 12,000 rpm for 15 mins at room temperature.The supernatant was transferred to a new tube, 600 µl of isopropanol was added ,mixed thoroughly by inverting the tubes and centrifuged at RT for 15 mins at 12,000 rpm.The genomic DNA pellet was further washed with 70% ethanol, air dried and dissolved in 150 µl of TE. The DNA was stored at -20°C for long term storage and 4°C for immediate use.

2.10 Polymerase Chain Reaction (PCR)

PCR is an effective tool for selective amplification of a desired sequence from template DNA.

Chemicals and Solutions required

1. Template DNA (Plasmid, Genomic or cDNA)
2. 10X PCR Buffer
3. 25mM $MgCl_2$
4. 2.5mM dNTPs
5. 2.5pmol primers
6. Taq DNA polymerase

Methodology

The following components were added to set up a PCR reaction.

	Initial Concentration	**Final Concentration**
a.	10X PCR Buffer	1X
b.	2.5mM dNTPs	250µM
c.	25mM $MgCl_2$	1.5mM
d.	2.5 pmol	0.25pmol
e.	Taq DNA polymerase (5U/ µl)	1U
f.	Template DNA (Plasmid/cDNA-10ng, Genomic-50 ng for 20 µl Reaction volume)	
		20 µl

After mixing all the components, reaction was carried out in a PCR machine (Eppendorf) according to the following conditions:

Step I. Initial denaturation at 95°C for 5 mins(genomic DNA) or 2 mins(plasmid DNA).

Step II. Denaturation at 94°C for 1 min (genomic DNA) or 30s(plasmid DNA).

Step III. Primer annealing for 30s at a temperature that varies with primer length and $T_{m.}$

Step IV. Primer extension at 72°C(nucleotide extension is 1kb/min)

Step V. Steps II-IV repeated for 29 or 35 cycles.

Step VI. Final extension for 4mins for small fragments and 7mins for large fragments.

Step VII. Reaction maintained at 4°C.

Size of the PCR product was verified by running an agarose gel with appropriate DNA ladder.

Table 4. Primers used for PCR reactions

Name	Sequence
18S rRNA	Fwd: CCTTATGGGACGTGTGCTTT Rev: CCTGCTGCCTTCCTTAGATG
Mof(RT)	Fwd: CTGGGTAGGCTGAGCTATCG Rev: CCAGACGAGGTAATCGGTGT
Diap1(RT)	Fwd: CCGAGGAACCTGAAACAGAA Rev: GAATCGGCACTGACTTAGCC
Diap1(chip1)	Fwd: TTGAGGGAAGCCACAATTAGA Rev: AATGCGTTCTTTTTGCATCC
Diap1(chip2)	Fwd: ACCAGGCGAAAAGAGTGCTA Rev: ATATTTTCGGTGGCGTTCAA
Diap1(chip3)	Fwd: AAGCCCAGAGAGCACTGAAA Rev: GCGGTATTGCACAAAATCCT
Diap1(chip4)	Fwd: GTCGCGGCTGTTGAATTTAT Rev: TTTCGCGCTCTCTCTCTCTC
Diap1(chip5)	Fwd: AGTTTTTGCCCCATCCTCTT Rev: TGTGCGTTTGCTTTATCAGC
Diap1(chip6)	Fwd: ACACACATCTGGAGCAGTCG Rev: TCTTGGCAGATCCTTCTTGG
Diap1(chip7)	Fwd: ACAAGTCGAATGGCCAGAAC Rev: CCACAAAATCGAGTCAAGCA
pUC	Fwd: ATCGAAGATGCACGGAAAAC Rev: GGGTGCTTAATCCCACAGAA
hid(RT)	Fwd: ACCTACTACGCGGGCTACAC Rev: CATGATCGCTCTGGTACTCG
rpr(RT)	Fwd: CGGGAGTCACAGTGGAGATT Rev: CGATATTTGCCGGACTTTCT
Chk2	Fwd: CAAGCTGCTGATCAACCAAA Rev: GCCTCGACCCTCACGTATTA
ATM	Fwd: ATCATAGCTTGGGCATACGG Rev: TTTGTTCTCCTTCGCGATCT
p53	Fwd: AATGCCCATCCAACCACTTA Rev: AAGGCTCAACGCTAAGGTGA
Chk1/grp	Fwd: CCGGACTCAATTACCTGCAT Rev: GTTTGCTCCAAGGAGTCTGC
ATR/Mei-41	Fwd: TCAGGAGACGCTAGCCATTT Rev: TGCAGAACTGCCATGAACTC

2.11 Purification of DNA from agarose gel

DNA fragments from agarose gels were extracted using Nucleospin Gel Extraction Kit (Macharey-Nagel). After electrophoresis on an agarose gel, DNA was excised out with a clean scalpel. The gel slices were weighed and dissolved in twice volumes of Buffer NT. The agarose was melted at 50°C for 10 mins with intermittent shaking. Then sample was loaded onto the silica membrane of a Nucleospin Column and centrifuged at 11000*g* for 30sec.Flowthrough was discarded and 700µl of Buffer NT3 was added to wash the membrane bound DNA. Column was spun at 11000*g* for 30sec and membrane dried by further spinning at 11000*g* for 1 min. Finally the DNA was eluted in 15-30 µl of elution buffer NE and quantified.

2.12 RNA extraction from flies-Trizol Method

Chemicals and Solutions required

1. Trizol reagent (Invitrogen)
2. Chloroform
3 Isopropanol
4. 70% ethanol

Methodology

About 50-100mg of flies was used as starting material. The flies were homogenized in 1ml of Trizol reagent. The samples were incubated at 55°C for 5mins to allow dissociation of nuclear proteins.250µl of chloroform was added to the tubes and mixed vigorousy for 15 sec followed by incubation at 55°C for 2-3 mins. To allow for aqueous phase separation, the tubes were centrifuged at 12,000 rpm at 4°C for 20 mins(a lower red phenol-chloroform phase, an interphase and a colorless upper aqueous phase was observed). The upper aqueous phase contains RNA that was precipitated by addition of equal volume of isopropanol and centrifuged at 12000 rpm for 15 mins at 4°C.The pellet was washed twice with 70% ethanol to remove salts and finally dissolved in 50µl of RNase free double autoclaved water and stored in -80°C.

2.13 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out using SuperscriptII Reverse Transcription System (Invitrogen).

Chemicals and Solutions required

1. SuperScript™ II Reverse Transcriptase
2. 5X First-Strand Buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl)
3. 0.1 M DTT
4. 25mM $MgCl_2$
5. RNAase inhibitor
6. 10mM dNTPs
7. Template RNA
8. Gene specific primers (10pm) or $(oligo\ dT)_{12\text{-}18}$ primers(500 µg/mL) and 18S rRNA primers(10pm) for internal control.

Methodology

1 ng to 5 µg of pure RNA was used for each reaction. The following components were added into a nuclease free microcentrifuge tube.

Oligo(dT)12-18 or gene-specific primer (GSP)	1 µL
Template total RNA	x µL
dNTP Mix (10 mM each)	1 µL
Sterile, distilled water to 12 µL	
	20 µL

Mixture was heated to 65°C for 5 min and quick chilled on ice. The contents of the tube were collected by brief centrifugation and following components were added:

5X First-Strand Buffer	4 µL
0.1 M DTT	2 µL
RNaseOUT (40 units/µL)	1 µL
25mM $MgCl_2$	2 µL

Contents of the tube were mixed gently and incubated at 42°C for 2 mins. 1 µL (200 units) of Super Script™ II RT was added, mixed gently and mixture incubated at 42°C for 50 mins. Finally reaction is inactivated by heating at 70°C for 15 min. 1 µL of RNaseH was added and incubated at 37°C for 20 min to digest RNA

strands of RNA-cDNA hybrids. 0.5 µl of RT product (cDNA) was used as a template for subsequent PCR reaction.

2.14 Total Protein Extraction from flies

Chemicals and Solutions required

Lysis buffer- 6% SDS, 1mM EDTA, 2 mM PMSF,10 µg/ml Aprotinin,10 µg/ml Leupeptin, 10 µg/ml Pepstatin.

Methodology

Nearly 50 flies were collected in 1.5 ml centrifuge tube and homogenized in sample buffer thoroughly. Sample was boiled at 95°C for 5 mins and centrifuged at 12,000 rpm at 4°C for 10 mins. The supernatant was collected into a new centrifuge tube and stored at -20°C.

2.15 Preparation of Histones (Acid extraction of Protein)

Chemicals and Solutions required

1. **Lysis Buffer**	**Final Concentration**
a) 1M Hepes	20mM
b) 0.5M EDTA	1 mM
c) 1M NaCl	10mM
d) 1M $MgCl_2$	2mM
e) 1M Sodium β-Glycerophosphate	20mM
f) 100% NP40	2.5%
Protease inhibitors (added just before use)	
g) 0.1 Na_3VO_4	1mM
h) 0.25M PMSF	1mM
i) 10mg/ml Aprotinin	10 µg/ml
j) 10mg/ml Pepstatin	10 µg/ml
k) 10mg/ml Leupeptin	10 µg/ml
l) 1M DTT	1mM

2. 0.4N HCl/ 10% Glycerol
3. Trichloroacetic Acid

4. Acetone/ 0.02N HCl
5. 1X Sample Buffer(50 mM Tris-HCl pH6.8, 2% SDS, 0.2% Bromophenol Blue, 10% Glycerol and 100mM DTT)

Methodology

Approximately 500 flies were collected and crushed into fine powder in liquid nitrogen. The material was transferred to Down's homogenizer and thoroughly homogenized (20 strokes in ice) in 5 ml of lysis buffer. The solution was pass through 26G syringe 10 times for separating nuclei from the cell debris and was centrifuged at 12,000 rpm for 20sec at 4°C.The supernatant was discarded completely, nuclei were dissolved in 0.4N/10% glycerol and incubated in cold room for 30 mins by moderate rotation on a vertical rotor. After centrifugation at 12,000 rpm for 10 mins at 4°C, the supernatant was transferred to a new tube and 120µl of TCA was added and incubated on ice. The contents were then centrifuged at 12,000 rpm for 10 mins at 4°Cand the pellet was washed with acetone/ 0.02N HCl(500 µl) and centrifuged for 5 mins at 4°C.After complete removal of supernatant, the pellet was air-dried and dissolved in 1X sample buffer. The nuclear extracts were divided into several aliquots and stored in -70°C.

2.16 Total Protein Estimation using Bradford Assay

Bradford Assay utilises Coomassie Blue G250 dye which has a λ =595 nm absorption peak when bound to side chains of lysine and arginine. A standard calibration curve was first generated using mixture of Coomassie Bradford reagent and known concentrations of Bovine Serum Albumin (BSA).100µl of BSA (Sigma) aliquots (ranging from 0.05mg/ml to 1.0mg/ml) were mixed with 5ml of Bradford Reagent (Invitrogen) and incubated at 25°C for 5 mins. Absorbance at λ =595 nm was measured for each standard on a multimode reader (Varoskan Flash, Thermo Scientific) and a calibration curve was plotted using Absorbance as a function of concentration (µg/µl). The concentration of our unknown sample could be then estimated from the standard curve, based on its absorbance.

2.17 SDS-PAGE (Sodium dodecyl -polyacrylamide gel electrophoresis)

Chemicals and Solutions required

1. 30% acrylamide [29g acrylamide+ 1g bis- acrylamide in 100ml water]

2. 1.5 M Tris-HCl pH 8.8

3. 1M Tris-HCl pH 6.8

4. 10% SDS

5. 10% Ammonium persulphate

6. TEMED

7. 10X Running Buffer [30g Tris base,144g Glycine,10g SDS in 1000ml water]

8. 10X TBST pH7.2[30g Tris base,80g NaCl, 10ml Tween20 in 1000ml water]

9. 10X Western Blot Buffer[30g Tris base,144g Glycine in 1000ml water.1X buffer was made by addition of 100ml 10X buffer,200ml methanol in 1lit water]

10. 2X sample buffer [100 mM Tris Cl pH6.8, 4% SDS, 0.2% Bromophenol Blue, 20% Glycerol, 200mM DTT]

11. Hybond-P membrane

12. Methanol

13. Ponceau stain 10X (Ponceau S-2g,Trichloroacetic acid-30g, Sulfosalicyalic acid-30g in 100ml of water).

14. Blotto (non fat dry milk)

15. Bovine Serum Albumin (BSA)

16. Primary and HRP conjugated secondary antibodies

17. ECL plus (Western Blot detection Reagent)

18. X –ray film

Methodology

A 12% resolving gel and 5% stacking gel of the following components were prepared:

12% resolving gel composition: 10 ml

a) 30% Acrylamide mix 4 ml

b) 1.5mM Tris-Cl[pH8.8] 2.5 ml

c) 10% SDS 100µl

d) 10% APS 100µl

e) TEMED 4µl

f) Sterile water 3.3 ml

5% stacking gel composition: 5 ml

a) 30% Acrylamide mix 0.83 ml

b) 1.5mM Tris-Cl[pH6.8] 0.63 ml

c) 10% SDS 100µl

d) 10% APS 100µl

e) TEMED 10µl

f) Sterile water 3.3 ml

20-75µg of protein was mixed with 5 µl of 2X sample buffer, heated at 95°C for 5 mins and loaded onto the assembled polyacrylamide gel. The gel was electrophoresed at a constant current of 20mA for stacking the proteins and later increased to 40mA for resolving. When not proceeding for blotting, gel was stained with Coomassie blue for viewing the proteins.

2.18 Western Blotting

After running the proteins on the gel, they were transferred on to a Hybond-P membrane using semi-dry transfer apparatus (GE Healthcare) for 1hr 10 mins. At this stage, proteins could be visualized on the blot by staining with Ponceau. Further, the blot was destained with 1XTBST and incubated in 5% non fat dry milk blocking solution (0.5g Blotto in 10ml of 1XTBST) with slow shaking for 1.5 h. After blocking, the blot was incubated in 2 ml primary antibody (appropriately diluted in blocking solution) at 4°C overnight with gentle shaking. The blot was washed extensively thrice 10 mins each in 1XTBST and incubated in 2ml HRP conjugated secondary antibody (appropriately diluted in blocking solution) for 1h at room

temperature on a shaking platform. After final washes (thrice 10 mins each) with 1XTBST, the signals were detected using ECL Plus Kit (Amersham Pharmacia) according to manufacturer's instructions. The amount of protein hybridized in each lane was estimated by densitometric calculation using Gel Documentation System. The ratio between the specific protein signals to the loading control determines the increase or decrease in the protein expression.

2.19 DNA damage assay

In order to assess DNA damage, genomic DNA was isolated from 0–2 h methanol-fixed control and mutant embryos as described earlier with slight modifications (Sibon OC et al., 2000). NaOH lysis is followed by incubation at 65°C for 40 min. The embryos were gently homogenised and treated with proteinase K for 8 h at 55°C, followed by RNAse A treatment and phenol-chloroform extraction. Each sample of DNA (100 ng) was incubated at 37°C for 20 min in 25 µl of a reaction mixture containing 50 mM imidazole pH 6.4, 12 mM $MgCl_2$, 1 mM β-mercaptoethanol,100 µM ADP, 20 units T4 DNA Kinase (Gibco BRL) and 2.5 µl of 3000 Ci / mmol [32P]ATP (Amersham). Column purification was carried out using G-50 spin columns to remove unincorporated labeled nucleotides. The level of p32 incorporated into the genomic DNA was measured by liquid scintillation counter. Over 300 embryos were used to generate the DNA for each experiment and the experiments were repeated thrice.

2.20 Knock down studies

MOF knockdown by RNAi was conducted in S2 cells using Schnieder's insect medium supplemented with 10% fetal bovine serum at 25°C. For effective knock down, we transfected 50 µg dsRNA in S2 cells and were incubated for 72 h as described earlier (Sibon OC et al., 2000).Control RNAi experiments using GFP was also performed.

2. 21 Chromatin Immunoprecipitation (ChIP)

Isolation of chromatin from *Drosophila* larvae

Chemicals and Solutions required

1. Buffer A1:

a) 60 mM KCl

b) 15 mM NaCl

c) 4 mM $MgCl_2$

d) 15 mM HEPES (pH 7.6)

e) 0.5% Triton X-100

f) 0.5 mM DTT

g) 10 mM Sodium butyrate

h) Protease Inhibitor, Roche (25X)

2. Lysis buffer with SDS:

a) 140 mM NaCl

b) 15 mM HEPES (pH 7.6)

c) 1mM EDTA

d) 1% Triton X-100

e) 0.5mM EGTA

f) 0.5 mM DTT

g) 0.1% sodium deoxycholate

h) 1% SDS

i) 10 mM Sodium butyrate

j) 0.1% N-lauroylsarcosine

k) Protease Inhibitor, Roche (25X)

3. Lysis buffer without SDS:

a)140 mM NaCl

b) 15 mM HEPES (pH 7.6)

c) 1mM EDTA

d) 1% Triton X-100

e) 0.5mM EGTA

f) 0.5 mM DTT

g) 0.1% sodium deoxycholate

h) 10 mM Sodium butyrate

i) 0.1% N-lauroylsarcosine

j) Protease Inhibitor, Roche (25X)

Methodology

Approximately 200–300 mg of well-fed third instar larvae was used for each reaction. All larvae were resuspended in 5 ml of buffer A1 and crosslinked with 1.8% formaldehyde at room temperature. To stop the crosslinking reaction, 2.5 M glycine was added to a final concentration of 225 mM, mixed thoroughly and further incubated for 5 min on ice. The homogenized samples were centrifuged for 5mins at 4000g at 4°C. Fresh 3 ml of buffer A1 was added, pellet was resuspended and centrifuged as before. This step was repeated thrice. The cross-linked larvae was then resuspended in 2.5 ml of lysis buffer without SDS, spun at 4000g, 4^0 C for 5min and supernatant was discarded. The pellet was dissolved now in 2.5ml of lysis buffer with SDS and incubated for 10 min in rotating wheel at 4^0 C. Freezing (liquid N_2) and thawing (37°C water bath) of the sample was done for 6-7 times. Chromatin was sheared in a Bioruptor (Diagenode) (15 cycles-30 s on – 30 s off per cycle, at high amplitude) to achieve chromatin fragments of an average length from 300–500 bp. Samples were rotated for 10 min in cold room and then transferred to 2 ml Eppendorf tube; centrifuged for 5 min at 4^0 C at 12,000 rpm. Supernatant was transferred to new 2 ml tube and to the samples 100 – 200ul (depending upon pellet size) of lysis buffer was added, vortexed and rotated for 10 min at 4^0C. Further, samples were centrifuged at 12,000 rpm for 5 min at 4^0C and the supernatants were combined. The isolated chromatin was quantified on a Nanodrop Spectrophotometer after diluting 10 times to nullify the buffer effect and then was stored at -80^0 C.

Immunoprecipitation

Chemicals and Solutions required

1. **ChIP dilution buffer**

   a)0.01% SDS,

   b) 1.1% Triton-X100,

   c) 1.2mM EDTA,

   d) 16.7mM Tris-HCl (pH 8),

   e) 167mM NaCl,

f) 1x Roche protease inhibitor cocktail

2. **Low salt wash buffer**

   a) 0.1% SDS

   b) 1.0% Triton – X100

   c) 2mM EDTA

   d) 20mM Tris-HCl (pH 8)

   e) 150mM NaCl

3. **High-salt wash buffer**

   a) 0.1% SDS

   b) 1.0% Triton – X100

   c) 2mM EDTA

   d) 20mM Tris-HCl (pH 8)

   e) 500mM NaCl

4. **LiCl Wash buffer**

   a) 0.25M LiCl

   b) 1% NP-40

   c) 1% Deoxycholate

   d) 1mM EDTA

   e) 10mM Tris-HCl (pH8)

5. **TE Buffer**

   a) 10mM Tris-HCl (pH8)

   b) 1mMEDTA

6. **Elution buffer**

   a) 1% SDS

   b) 0.1M $NaHCO_3$

Methodology

The sonicated chromatin was diluted 2-fold with ChIP dilution buffer and pre-cleared with Protein A agarose beads (Millipore) for 1 hour at 4°C. About 200 µg of precleared chromatin was incubated with 2.5 – 5.0µg antibody overnight on a rotating wheel at 4°C.Next day, sample was precipitated with protein A Sepharose beads for

2h at 4°C, pelleted down (3000xg for 1min) and immunoprecipitated complexes were washed sequentially with the following buffers (3mins each):

1. Low-salt wash buffer
2. High-salt wash buffer
3. LiCl wash buffer
4. TE, pH8.0
5. TE, pH8.0

The bound DNA was eluted with elution buffer Protein A-sepharose was pelleted down at 3000xg for 2min. Supernatants were combined, NaCl added to a final concentration of 200mM(200µl elution buffer and NaCl added to input samples also) and cross links were removed by incubating for 6 hrs at 65°C. After treatment with RNase A (10mg/ml, Sigma)(37°C for 30 min) and Proteinase K (Sigma) (42°C for 2h), ChIP DNAs were purified using Nucleospin Extract II DNA purification columns according to manufacturer's instructions (Macharey Nagel). DNA from triplicate samples were pooled together and lyophilized. Lyophilized samples were resuspended in 20 µl of distilled water and quantified. The ChIP DNA was PCR amplified with appropriate primers following a standard PCR protocol. The ratios of amplified immunoprecipitated DNA and DNA amplified from 5% of input were calculated from triplicate gels by densitometry.

2.22 Quantitative PCR (Q-PCR)

Using oligo-d(T) primers and ultra pure RNA (extracted by Trizol method and column purified with Macharey-Nagel kit), first strand cDNA was reverse trasncribed. Then using Takara SYBR Green Master Mix on Real time PCR (Applied Biosystems ,7900HT), relative quantification was performed. Each experiment was repeated at least twice independently. Using the $2^{-\Delta\Delta CT}$ method, fold change in product levels for each sample was determined, as per protocol described in Livak KJ et al., 2001. 18S rRNA was used for the normalization.

2.23 Statistical Analysis

Statistical analysis was performed using the graph pad software to evaluate the significant difference between the control and treated samples. The results obtained were expressed as mean ± SD. All the experiments were conducted

in triplicates. Statistical significance was assessed using student t-test. *** indicates P<0.001, ** indicates P<0.01, * indicates p<0.05.

Table 5. **List of Primary Antibodies**

S.No	Primary Antibody	Host	Dilution	
			Western Blot	**Immunostaining**
1	anti-β-actin	mouse	1:500	1:30
2	anti-Phospho Histone H3Ser10	rabbit		1:30
3	anti-Mei-41	rabbit	1:500	
4	anti-Chk1	rabbit	1:500	
5	anti-p53	rabbit	1:500	
6	anti-MOF	rabbit	1:300	
7	anti-H4K16Ac	rabbit	1:300	
8	anti-Histone4	rabbit	1:300	
9	anti-α-tubulin	rat		1:20
10	anti-vasa	goat		1:20
11	anti-H2Av-PO_4	rabbit	1:500	
12	anti-diap1	rabbit	1: 200	
13	anti -cleaved caspase3	rabbit	1: 1000	1:100
14	anti-JNK		1: 300	
15	anti-Phospho JNK		1: 300	

Table 6. **List of Secondary Antibodies**

S.No	Secondary Antibody	Dilution	
		Western Blot	**Immunostaining**
1	anti –mouse FITC		1:50
2	anti –rabbit FITC		1:50
3	anti- rabbit Cy5		1:50
4	anti –goat FITC		1:50
5	anti –mouse HRP	1:2000	
6	anti –rabbit HRP	1:2000	

CHAPTER-3

RESULTS-1

3. Role of *Drosophila mof* in response to ionizing radiation (IR) mediated DNA damage

3.1 Introduction

Drosophila mof (*dmof*) and its human ortholog (*hmof*), share a number of similarities (both have the same substrate specificity and enzymatic activity; both reside in multiprotein complexes and interact with evolutionary conserved protein partners therein. Despite this, the two share a number of differences as well, notably in their functions within the cell. The dosage compensation role of MOF in *Drosophila* has not yet been observed in elsewhere, notably mammals (Rea et al., 2007).

Loss of function analyses and interaction studies has uncovered a number of diverse and novel functions of hMOF in a variety of cellular processes. Some of these roles may be mediated through acetylation of histone H4 Lays 16 and some may be through modifying other substrates. Immunoaffinity purification from a human cell line reveals hMOF to be a part of a complex containing H3K4 methyltransferase MLL1 (Mixed-Lineage-Leukemia-1) and both activities are required for optimal transcription activation on a chromatin target *in vitro* and *in vivo*. (Dou et al., 2005). hMOF has been shown to be a key regulator of the embryonic stem cell(ESC) core transcriptional network, maintaining the self renewal and pluripotency of ESCs (Li et al., 2012).

Elevated MOF levels and H4K16 acetylation in all immortalized human normal and tumor cell lines and primary tumors are an epigenetic signature of increased cellular proliferation and oncogenesis (Gupta et al., 2008). In addition, mammalian MOF has a critical role at multiple points in the cellular DDR and double strand break (DSB) repair pathways (Sharma et al., 2010).

In the last decade, there has been growing evidence of the role of human MOF in the DNA damage response. RNAi mediated MOF knockdown in mammalian cell lines (HeLa and 293T) led to a cell cycle arrest at the G2-M checkpoint (Taipale et al., 2005; Smith et al., 2005). Such an observation can be explained by several, yet unexamined, possibilities. Microarray data and real time analysis in such cells reveal a modest reduction in the transcription of a number of genes involved in cell cycle progression (Smith et al., 2005).This may account for the observed G2-M arrest. Alternatively, an S phase arrest may be responsible for the observed number of cells

in the G2-M checkpoint. Knockdown of *hmof* leads to a drastic decrease in the global acetylation status of a cell's epigenome. This may hinder DNA replication, as this modification peaks in the S phase (Rice et al.,2002) and consequently stall the progression to cell division. Yet another possibility is that loss of MOF could indirectly impair the DNA repair process that results in the G2-M phenotype.

In mammalian cells, MOF activates ATM in response to DNA damage (Gupta et al., 2005). The ATM protein kinase, mutations of which are associated with the human disease ataxia-telangiectasia, transduces DNA damage signals, from sensor molecules to downstream effectors, to bring about cell cycle arrest, DNA repair or apoptosis. The ATM mediated signalling pathway is well characterised and molecular data suggests hMOF may play a role in the activation of this pathway. However the mechanism by which this activation is brought about still remains to be elucidated. Elevated doses of IR bring about a global increase in H4K16 acetylation, which does not depend on the presence of ATM (Gupta el., 2005). This may loosen up the chromatin and make it accessible to the action of DNA repair enzymes. Alternatively MOF could directly activate ATM or an activator of ATM, as yeast two-hybrid assays and Co-IP experiments show that hMOF directly interacts with ATM through its chromo domain. hMOF inactivation leads to reduction of both ATM autophosphorylation and its kinase activity following IR (Gupta *et al.*, 2005).Independent studies in *Drosophila* and mammals suggest that DDR (DNA damage responses) is conserved in both. Here we discuss DDR majorly in the context of adult flies and compare it to that in mammals, including humans. Similar to mammals, signalling pathways elicited in response to DDR in *Drosophila* include intra-S-phase checkpoint, G2/M arrest, metaphase arrest, centrosome inactivation, and apoptosis.

S phase checkpoint: DNA replication checkpoints function to put S phase progression on hold until the damaged DNA is repaired. In eukaryotes, signals to activate this checkpoint comes from the Replication Protein A (RPA), coating long strands of single stranded DNA .The signal attracts the evolutionary conserved kinase ATR (ataxia telangiectasia and Rad3 related kinase) to associate with RPA and possibly phosphorylates Mcm2-7 proteins, thus preventing the CMG helicase complex from further unwinding DNA (Cortez et al.,2004). This temporarily halts replication forks. In *Drosophila,* the protein kinase Grapes (the homolog of Chk1 in fission yeast) has been shown to be operative at the DNA replication checkpoint

during midblastula transition (Sibon et al.,1997). mei-41,the ATM/ATRR homolog in *Drosophila* has been implicated in the DNA replication checkpoint in the embryo (Brodsky et al., 2000; Garner et al., 2001).

G2-M arrest: The G2-M checkpoint causes cells containing damaged DNA to be arrested prior to progression to division. In mammalian cells, cyclin dependent kinase Cdc2/CyclinB1 cell cycle regulator complexes are required to drive entry into mitosis (Paulovich et al., 1997). Inhibitory phosphorylation on tyrosine 15 and threonine 14 by the Wee1 and Myt1 kinases helps maintaining inactive complexes of Cdc2/CyclinB throughout G2 (Booher et al., 1997). These residues are dephosphorylated by phosphatase CDC25 (Draettaet al.,1997) at mitotic entry, producing active Cyclin B–Cdk complexes.On DNA damage, these complexes can be inhibited by a number of evolutionary conserved regulatory factors. Damage can stimulate kinases ATM and ATR, which in turn activate the Chk1 and Chk2 kinases to phosphorylate Cdc25.14-3-3 proteins can then bind to Cdc25 preventing it from activating CyclinB-Cdc2 complexes (Taylor et al., 2001). Also, IR can inhibit Cdc2 activity by inhibiting the accumulation of CyclinB1 mRNA and protein .Another regulation of G2-M transition is regulated by p53.This transcription factor can mediate G2 arrest by activating p21, 14-3-3 sigma and Gadd45 transcription.p21 binds to Cyclin B/cdc2 complex, keeping it in inactive form while Gadd45 inhibits the formation of the complex.P53 also inhibits G2-M transition by repressing the transcription of cdc2 and Cyclin B (Taylor et al., 1999).

Drosophila bears structural and functional homologues of Chk1 and ATR proteins, namely grapes (grp) and mei-41 respectively. *Grp* as well as *mei-41* have a well established role in regulating a checkpoint that enables proper completion of S phase before commencement of mitosis during mid blastula transition during *Drosophila* embryogenesis (Sibon,1997; Brodsky,2000) *mei-41* mutants have been shown to highly sensitive to DNA damaging agents like X-rays(Sibon,1999) and the protein is required for DNA damage checkpoint in larval imaginal discs and neuroblasts (Brodsky,2000). Besides, it is also said to be operative at a meiotic checkpoint that senses double strand DNA breaks and enforces both delay of prophase1 progression and repair of the damage (Ghabrial, 1999).

Centrosome inactivation: Exposure to genotoxic stress activates a centrosome inactivation checkpoint in both *Drosophila* embryos and somatic mammalian (Chinese hamster ovary- CHO) cells (Sibon et al., 2000; Hut et al., 2003)

This organelle, in normal cells, is required for organizing the bipolar mitotic spindle, ensuring correct segregation of chromosomes during cell division. DNA damage provokes centrosomal fragmentation or amplification, causing a 'mitotic catastrophe' to eliminate damaged cells. Involvement of checkpoint kinase 2(Chk2) as a potential mediator of this checkpoint has been implicated both in *Drosophila* and human HCT116 cancer cells (Loffler et al., 2006).

Apoptosis: Studies in a number of genetic model organisms have revealed that DNA damage signals can induce a number of signalling pathways into action, all of which finally lead to activation of death proteases: the caspases. Between *Drosophila* and humans, despite some differences in the signalling pathway leading to apoptosis, a number of apoptotic regulators like caspases,Bcl-2 family members, inhibitor of apoptosis proteins (IAPs),IAP antagonists and caspase activators remain conserved (Kornbluth et al., 2005).

The caspases or effector proteases of apoptosis were first identified in *C. elegans*, where mutants for the caspase Ced3 were unable to undergo developmental programmed cell death (Miura M et al., 1993). The fly genome codes for seven caspases: Dronc, Dredd and Strica (the initiator caspases) and drICE, Dcp-1,Decay and Damm (the effector caspases) (Song et al., 1997;Harvey et al., 2001; Kornbluth et al., 2005). Dronc shares considerable structural homology with its human ortholog caspase-9, with a conserved N-terminal CARD domain (Dorstyn et al., 1999) meant for docking site for adaptor proteins (Dark in *Drosophila* and Apaf-1 in humans) (Li et al., 1997). Structural and functional homology also exist between Dredd and its mammalian counterpart, caspase-8 (Leulier et al., 2000).

In mammals, various apoptotic stimuli promote release of Cytochrome c from mitochondria and its binding to Apaf-1, thus activating the apoptosome. This release is positively regulated by the pro-apoptotic Bcl-2 family members, Bax and Bak while Bcl-2 and Bcl-xL act as antagonists of the process (Kluck et al., 1997; Yang et al., 1998). Similarly in flies, Dark/Dronc apoptosome activation in response to apoptotic stimuli is mediated by the release of cyt c or some other mitochondrial factor. The *Drosophila* Bcl-2 family members include Debc1 (which also promotes apoptosome activation, by possibly dimerizing with the apoptosome and removing the antagonist Buffyfrom the complex) and Buffy/dBorg2. Evidences from cultured S2 cells suggest Debcl can induce both caspase dependent and independent modes of apoptosis (Kornbluth et al., 2005).

Both flies and mammals contain endogenous caspase inhibitors or IAPs (Inhibitor of apoptosis proteins) possessing conserved baculoviral IAP repeat (BIR) domains trequired for binding to caspase active sites. Inhibition mechanisms also include degradation of active caspases or sequestering caspases away from their substrates (Tenev et al., 2004). A crucial member of the IAP protein family,DIAP1 was first identified in insect viruses (Crook et al., 1993) and its knockdown leads to rapid apoptosis in *Drosophila* cells (Yoo et al., 2002).Though knockdown of its mammalian counterpart XIAP has little effect on apoptosis (Harlin et al., 2001), it does make the cells more sensitive to apoptosis (Potts et al., 2003).The pro apoptotic proteins, Reaper, Grim, Hid and Sickle promote apoptosis in flies by binding to the BIR domains of IAPs through their IAP-binding motif(IBM)domains (Vucic et al., 1998) and promoting their degradation (Holley et al., 2002). Mammals do contain IBM proteins: SMAC/DIABLO and OMI/HTRA2, though they do not play as significant a role in induction of apoptosis as in flies (Kornbluth et al., 2005).

Overall, mutations in *Drosophila mei-41* (*Drosophila* ATR ortholog), *grp* (grapes: *Drosophila Chk1* ortholog), *mus304* (*Drosophila* ATR ortholog), *mei-41*, *mre11*, *rad50*, *Chk2*, *p53*, and *14-3-3* show defects in DNA damage checkpoint responses similar to those caused by the mammalian gene mutations (Brodsky et al. 2004; Jaklevic and Su 2004; Song et al., 2004). Although a role for MOF in the IR response has been established in a variety of cell-based models, it is unknown whether MOF contributes to the IR response in whole animals. MOF knockout mice die during embryogenesis, so we turned to *Drosophila* to test whether MOF contributes to IR during development. *mof* mutant males can only survive to the end of third instar, and MOF is not required at all for female development. In the following study, we exploit the genetic opportunities in *Drosophila* to show that post-irradiation DDR in *Drosophila*, as in mammalian cells is MOF dependent, determined by both *in vitro* and *in vivo* assays.

3.2 *Mof* mutants in *Drosophila* have preferential sensitivity towards IR exposure.

In order to get a first insight whether *Drosophila mof* has a role in IR response in the whole animal, EMS induced, loss-of-function *mof* mutations - *mof3* and *mof 6* were chosen. Both these alleles are non sense mutations, introduced at residues

151(Q151X) and 28(Q28X) respectively, and produce severe truncations before the conserved catalytic domain of the 827 residue MOF protein. The [*mof* $^{t6.8}$]18H1 transgene on the third chromosome rescues the male lethality in *mof* mutations by providing 6.88 kb of *mof* gene (Kayegama et al 2001).

3.21 Study of the survivability of *mof* mutant females to IR.

To study the survivability of mutant females after radiation exposure, heteroallelic *mof3 mof6* female mutants were analyzed. *yw mof3; [mof t6.8]18H1* and *yw mof6; [mof6 t6.8]* females were crossed to *FM7 y w B* males. From the F1 progeny, *yw mof3; [mof t6.8]18H1/+* were crossed to *yw mof6/FM7; [mof t6.8]18H1/+* females and *yw mof6; [mof t6.8]18H1/+* males were crossed to *yw mof3/FM7; [mof t6.8]18H1/+* females. The embryos from these crosses were collected over a 4 hour interval from apple juice-agar plates and aged 24 hours before irradiation to deplete maternally loaded MOF. After irradiation, the first instar larvae were transferred to standard cornmeal-agar-molasses food and all allowed to develop to adulthood. Daughters inheriting *yw mof3/yw mof6* had a substantial developmental delay compared to their heteroallelic siblings carrying *mof* transgene *moft6.8]18H1*(control).

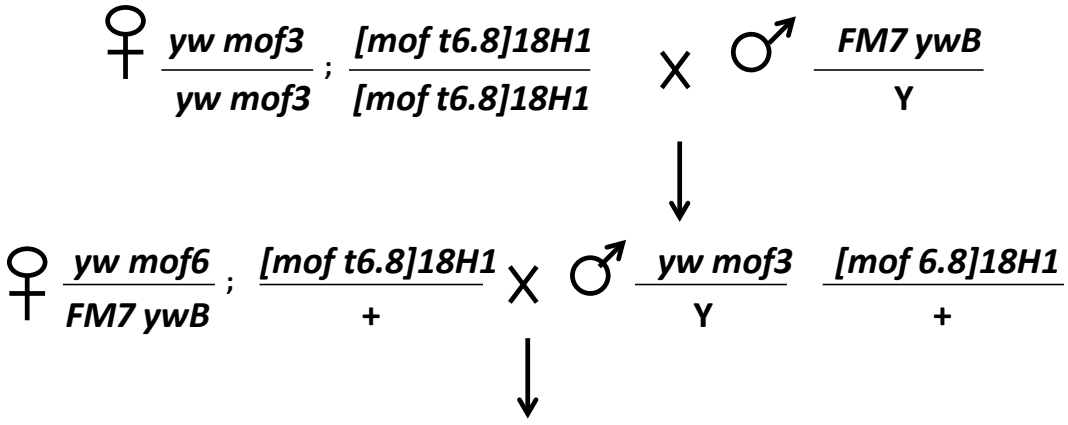
Table 7 . Preferential sensitivity to IR in *mof3/mof6* females

Dose (Gy)	*mof3/mof6; 18H1*[a]	*mof3/mof6*	Significance[b]
0	366	97	
5	383	75	No(P=0.0765)
10	396	95	No(P=0.572)
15	50	0	Yes(P<0.0001)

Table 7. The progeny were scored and statistical test of significance and t-test were done. At the highest dose of 15 Gy, no *mof3/mof6* progeny survived. The experiment was repeated 3 times and the mean number of offsprings of each genotype were considered in each case.

[a] Combination of flies with one or two doses of the *18H1 mof*$^{+}$ transgene
[b] Compared with 0 Gy control ,using Fishers Exact Test, two tailed

A

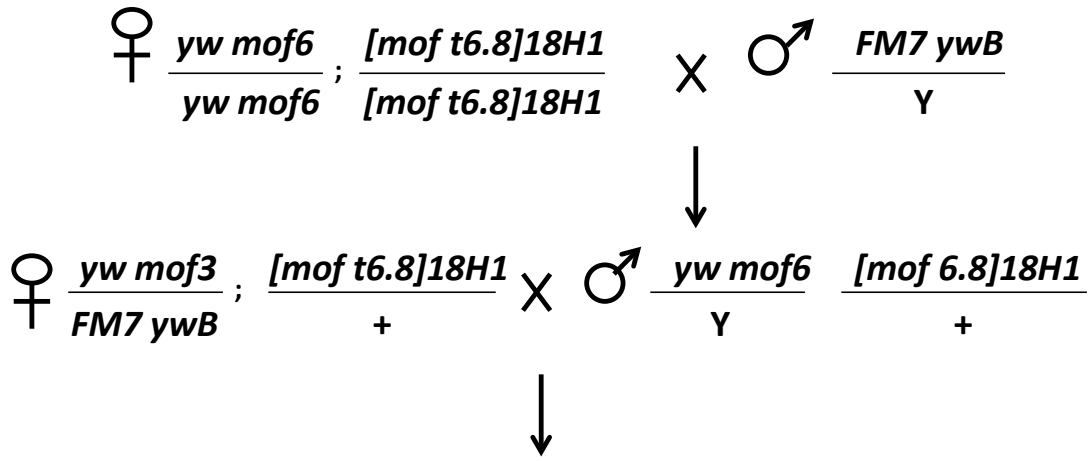


Classes of female offsprings

With doses of wild type 18H1 transgene	*mof mutants*
yw mof3 / yw mof6 ; 18H1 / +	*yw mof3 / yw mof6*
yw mof3 / yw mof6 ; 18H1 / 18H1	
yw mof3 / FM7 ywB ; 18H1 / +	*yw mof3 / FM7 ywB*
yw mof3 / FM7 ywB ; 18H1 / 18H1	

Fig. 3.1. A. Genetic crosses to determine the survival of mutant *mof* females in response to ionizing radiation. Heteroallelic *mof3/mof6* females were generated by crossing *yw mof3; [mof t6.8]18H1* females with *FM7 y w B* males.

B



With doses of wild type 18H1 transgene	*mof mutants*
yw mof3 / yw mof6 ; 18H1 / 18H1	*yw mof3 / yw mof6*
yw mof3 / yw mof6 ; 18H1 / 18H1	
yw mof6 / FM7 ywB ; 18H1 / +	*yw mof6 / FM7 ywB*
yw mof6 / FM7 ywB ; 18H1 / 18H1	

Fig.3.2. B. Genetic crosses to determine the survival of mutant *mof* females in response to ionizing radiation. Heteroallelic *mof3/mof6* females were generated by crossing *yw mof6; [mof6 t6.8]* females with *FM7 y w B* males.

3.22 Study of the survivability of *mof* mutant males to IR.

To examine the survivability in *mof* mutant males post IR exposure, embryos were collected on apple juice agar plates from y^1 w^{67c23} $mof^1/FM7$ stock. mof^1 is a EMS mutation having a single amino acid substitution –Glycine replaced by a Glutamic acid at position 691 in the acetyl co-enzyme motif(Hilfiker et al., 1997). Males carrying loss of function *mof* mutation do not survive past third instar larval stage since they lack the H4K16Ac enrichment on the X-chromosome for transcription of the X-linked genes (Smith et al., 2000; Akhtar et al., 2000). In this stock, male larvae can be distinguished based on mouth hook pigmentation; mof^4 male larvae have yellow-brown mouth hooks, due to the liked y^1 allele, while *mof*+ ones have black mouth hooks due to the linked y^{31d} allele. The embryos were irradiated at various doses while still on the plates, then allowed to hatch and develop at 25°C until third instar larvae were visible, but before any pupae had formed. Third instar larvae were collected and sexed. Multiple irradiations were conducted, together with controls, and survival numbers for each experiment were pooled for each genotype at a given treatment for several experiments.

As seen in Fig.3.3 the trendline for survival of wild type male larvae post IR shows a steeper slope, indicating lesser survivability of *mof* mutants. Since significantly lower number of *mof* mutants (in both males and females) survived to adulthood than wild types following exposure to IR, it indicates that loss of dMOF results in hypersensitivity to DNA damage. *Drosophila* SL-2 cultured cells with knocked down *mof* gene have a reduced cell number post IR irradiation.This is consistent with studies in mammalian cells, which when depleted of MOF or expressing a catalytically dead MOF protein show higher cell killing following exposure to IR (Gupta et al., 2005). All this data suggests that MOF contributes to survival of irradiated cells (Gupta et al.,2005, 2008; Li et al.,2010).Our results show that in whole animals too, MOF is essential for survival after irradiation.

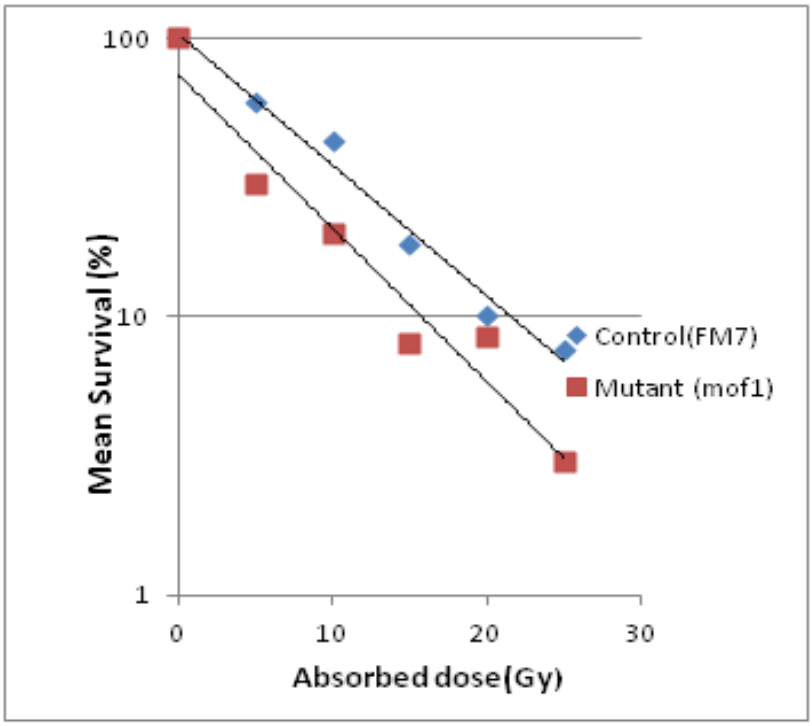


Fig. 3.3. Comparison of wild-type and *mof* mutant male *Drosophila* survival post-irradiation. Male third instar larvae, irradiated as embryos, were scored as *mof* ^{1} or *mof* + *(FM7)* at the third instar larval stage. Mean survival from three to four experiments is presented for each dose point. Mutant embryos displayed significantly increased sensitivity to IR compared with their irradiated *mof* + male sibs.

3.3 *Drosophila* MOF is required for IR induced apoptosis

In *Drosophila melanogaster*, exposure to IR such as X-rays or Y-rays, induces apoptosis in the imaginal discs of wild type larvae (Su et al., 2005). The response is typically assayed at 4–6 hr after exposure to a LD_{50} dose (4000R),by staining with the vital dye acridine orange (A.O) that preferentially stains apoptotic cells (Spreij 1971). 96-120 hr old third instar larvae from both wild type and *mof* mutants were irradiated with 25 Gy (2500 R) of X rays. Imaginal discs were dissected 4 hrs after irradiation and stained with A.O. to detect apoptotic cells. Among unirradiated controls, wild type discs had very few stained cells while mof^1 mutants already showed a substantial basal level of apoptotic cells. Post irradiation, considerable increase in AO staining was detected in wild type discs, while mof^1 mutant discs had an obvious but small increase, due to high basal level of apoptotic cells. It can be concluded that functional MOF is required to embark a normal DNA damage triggered apoptosis.

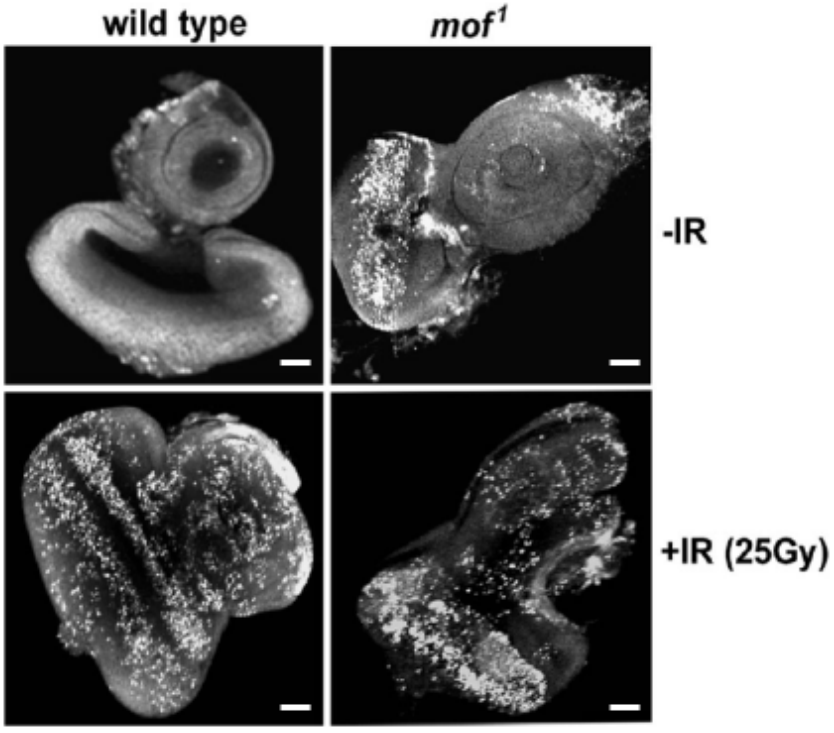


Fig. 3.4 Acridine orange staining in wild type and mof^1 mutant male larval eye imaginal discs. 4hrs after irradiation with 25Gy, wild type showed large increase in apoptotic cells, while *mof* mutants had no significant increase, owing to excessive spontaneous apoptosis in unirradiated cells. Scale bar represents 50 µm.

3.4 *Mof* mutants in *Drosophila* have a defective checkpoint response to IR

Cell cycle checkpoints monitor genomic DNA and modulate cell cycle progression, stalling it in the event of DNA damage to allow for repair .This ensures critical events like DNA replication and chromosome segregation to be completed with high fidelity and hence faithful transmission of the genetic information transmission from one generation to another (Weinert et al., 1989).

Normal cells in all multicellular eukaryotes progress through 3 crucial checkpoints on the way to completion of cell division- the DNA replication and DNA damage checkpoints (collectively known as DNA structure checkpoints) and the kinetochore attachment checkpoint. The core components of the DNA damage response (DDR) are conserved in *Drosophila*. A functional G2-M checkpoint delays entry of cells into mitosis when DNA is damaged and sets of a multitude of DNA repair pathways into action (Su 2006).

Mammalian cells deficient in MOF have a defective checkpoint response to radiation (Gupta 2005). RNAi mediated hMOF-depleted HeLa cells undergo a G2-M arrest, shown by comparing the DNA content of h*mof* knockdown cells to control and determining the distribution of cells various stages of the cell cycle (Smith et al., 2005). To test for a similar response in *Drosophila mof* mutants, wing imaginal discs were dissected from both wild type and mof^{1} mutant 3rd instar larvae, 1hr post irradiation with 25Gy, fixed and immunostained with the mitotic cell marker-phosphorylated histone H3(H3S10ph) antibody.

Typically, in irradiated wild type imaginal discs, an activated checkpoint response arrests the cell cycle at the G2-M transition, allowing cells time to repair damaged DNA before allowing progression to mitosis. PH3 staining at 1 hr after irradiation helps detect mitotic cells that escape past the checkpoint. Unirradiated discs from wild type and mof^{1} 3rd instar larvae had fairly large and equal number of PH3 stained cells. Since post irradiation, this number decreased drastically in wild types but with no appreciable change in mof^{1} discs, is indicative of an inactive DNA damage checkpoint in *mof* mutants.

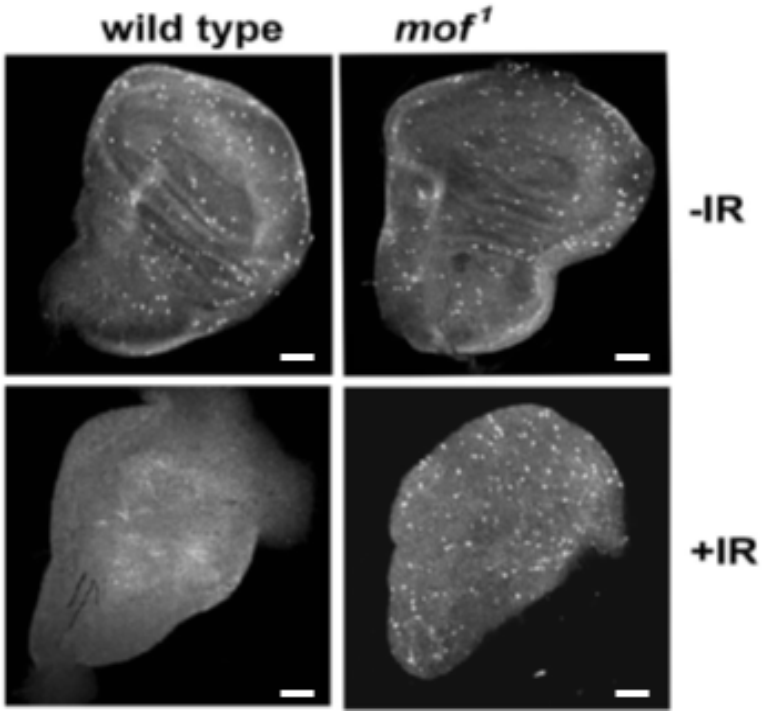


Fig. 3.5 DNA damage checkpoint response in wing imaginal discs of *Drosophila* wild type and *mof* mutants. Larval wing discs from wild-type and *mof*^{1} mutant male were immunostained with anti-phospho histone3 (PH3) antibody before irradiation and 1 h after ionizing radiation dose of 25Gy.

3.5 DNA damage response proteins levels in irradiated *mof* mutants indicate that *mof* mediated response is conserved in mammals and *Drosophila*

In mammalian cells, *ATM* (*ataxia telangiectasia mutated*) protein kinase is the central mediator of cellular DNA damage response pathways following IR. Double strand breaks can lead to intermolecular autophosphorylation and activation of ATM ,which in turn can phosphorylate downstream targets like Mdm2 and p53(contributing to G1 arrest)and Chk2 (apoptosis) (Kastan et al., 2008). Cells deficient in ATM have a defective p53 response to IR (Kastan et al., 2008; Pandita et al., 2002, 2003). A decrease in ATM autophophorylation can be brought about by the expression of a dominant negative hMOF or by RNAi mediated *mof* knockdown in human 293 cell lines. Besides, hMOF directly interacts with ATM. (Gupta et al., 2005).We were therefore interested to determine the levels of crucial proteins in the ATM/ATR pathway in wild type and *mof* mutant *Drosophila* embryos, by Western analysis, before and 2 h post irradiation.

In wild type *Drosophila*, MOF levels remained unchanged upon irradiation - consistent with results obtained from mammals (Gupta et al., 2005) and indicating that DNA damage did not affect the transcription of *mof*. p53 protein had a low basal level, that markedly rose upon irradiation. In contrast, mutant *mof Drosophila* had a higher basal level p53 and showed little obvious change following IR exposure.

The levels of *mei-41* and *chk1* before and after IR exposure were similar between wild type and mo^{f1} mutant flies, a response seen in mammals as well. *Drosophila mei-41* (homolog of ATM/ATR) mutants have been shown to be sensitive to DNA damaging agents like X-rays (Sibon,1999). *mof* mutants harbour higher or equal basal levels of mei-41 and chk1 and proteins respectively. IR exposure brings on a 50% decrease in chk1 levels in wild type larvae. For *mof* mutants the decrease is considerably lesser (20%).A similar trend is observed for mei-41 levels as well. These patterns led us to conclude that MOF-mediated DDR is conserved in *Drosophila*.

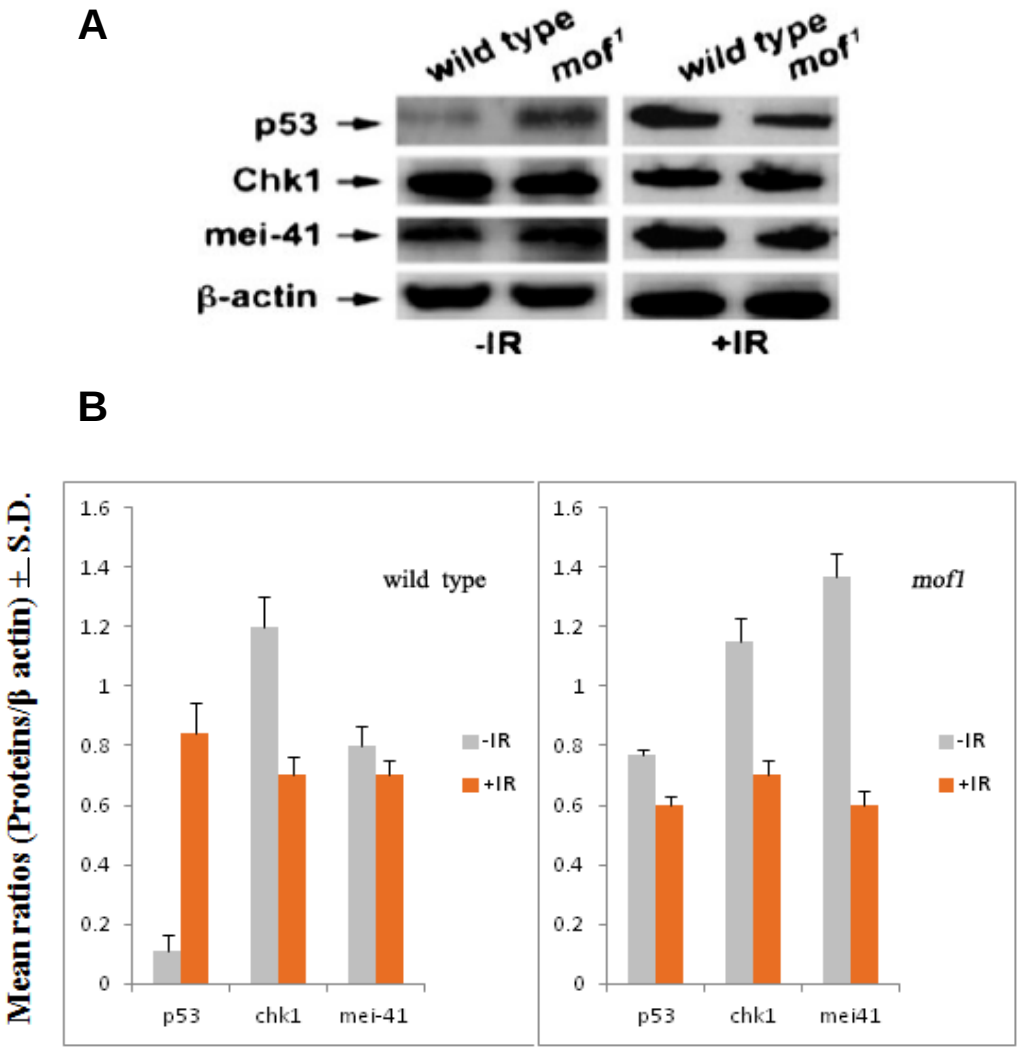


Fig. 3.6 Determination of Chk1, p53, and mei-41 protein levels in larvae extracts of wild-type and *mof*^{1} mutant *Drosophila* male larvae with and without irradiation. Larvae were irradiated with 25 Gy of gamma rays and the proteins extracted within 1 h post-irradiation. **A**. Western blots and **B.** histogram representing the relative amount of proteins estimated from triplicate blots.

3.6 Effect of IR on levels of *mof* mediated acetylation of histone H4K16.

Drosophila MOF, like its human ortholog, has been shown to acetylate histone H4 uniquely at Lys16 (Gupta et al., 2005). Human cell lines expressing mutant hMOF or with hMOF knockdown, displayed decreased levels of H4K16 acetylation post irradiation compared to control cells. Also, cells overexpressing full length hMOF had higher levels of IR induced H4K16 acetylation. These changes are independent of ATM function as cells with or without functional ATM showed no difference in acetylation levels (Gupta et al., 2005). Similar observations have been made in mouse cells (Li et al., 2010). In parallel collaborative studies conducted in *Drosophila* SL-2 cells, H4K16Ac levels were seen to rise post gamma irradiation (without any change in MOF).

To study whether IR exposure enhances MOF dependent acetylation of histone H4K16 in *Drosophila in vivo*, 3rd instar larvae (male and female) were irradiated(25 Gy) and histones extracted for analysis. Consistent with the *in vitro* SL-2 cell results, IR exposure of larvae increased the levels of H4K16 acetylation (H4K16Ac) in wild type larvae of both the sexes-(about 3 folds in males and 4 folds in females-lanes **1** and **2** in Fig 3.7). Mutant *mof* larvae showed minimal increase in the levels of IR induced H4K16Ac (lanes **3** and **4**).

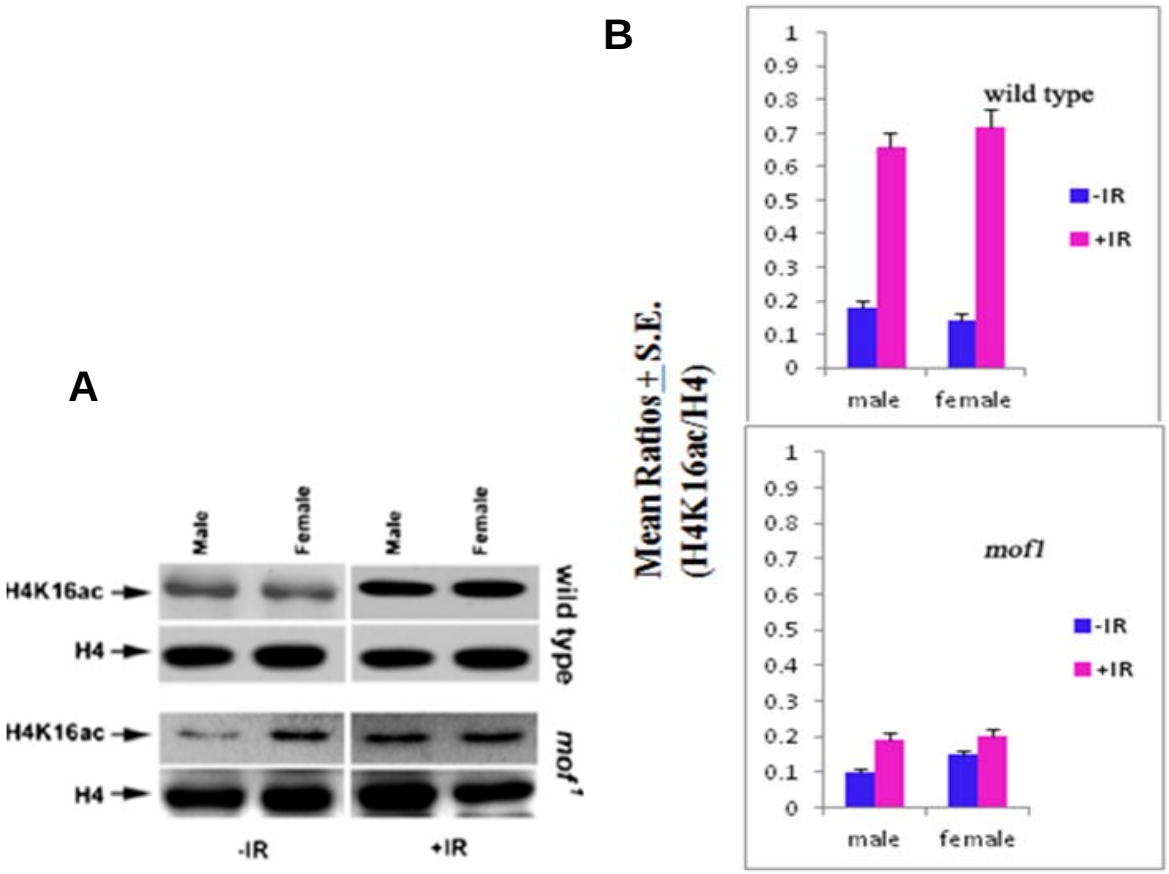


Fig. 3.7 Effect of IR on MOF and H4K16ac levels in *Drosophila* larvae. A.Wild-type and *mof* ^{1} mutant third instar larvae were irradiated (25 Gy), and the histones extracted within 1 h of irradiation. Quantitative Western blot analysis was carried out with H4K16ac antibody. The same blots were re-probed with antibody to total histone H4 as a gel loading control. **B.** Histogram representing the relative amount of 4K16ac/total histone H4 (H4K16ac/H4) as estimated from triplicate blot analysis.

3.7 Effect of IR on distribution of *mof* mediated acetylation of histone H4 at K16.

The male X chromosome in *Drosophila* males has a high enrichment of MOF mediated H4K16 acetylation and dMOF is targeted to chromatin entry sites on the male X as a part of the dosage compensation complex (Akhtar et al., 2000; Smith et al., 2000; Hilfiker et al., 1997).We therefore examined whether IR exposure induced any specific relocalization or delocalization of MOF or changed the pattern of H4K16ac modifications on the male X chromosome.

Male larvae were exposed to irradiation dose of 25Gy and polytene chromosomes were fixed and immunostained with MOF and H4K16ac antibodies at various time points post irradiation. There was minimal change in the intensity of MOF or H4K16ac immunostaining on the X chromosome. These results are consistent with previous observations (Sharma et al., 2010) that MOF does not show any major change in preferential localization on the *Drosophila* male X chromosome immediately post irradiation.

Our results were corroborated by parallel experiments conducted on *Drosophila* SL-2 cells. Chromatin bridges observed at anaphase and early telophase in irradiated cells but not in the control sample were indicative of DNA damage. But no detectable change in MOF or H4K16 ac signals was observed on the X chromosome.

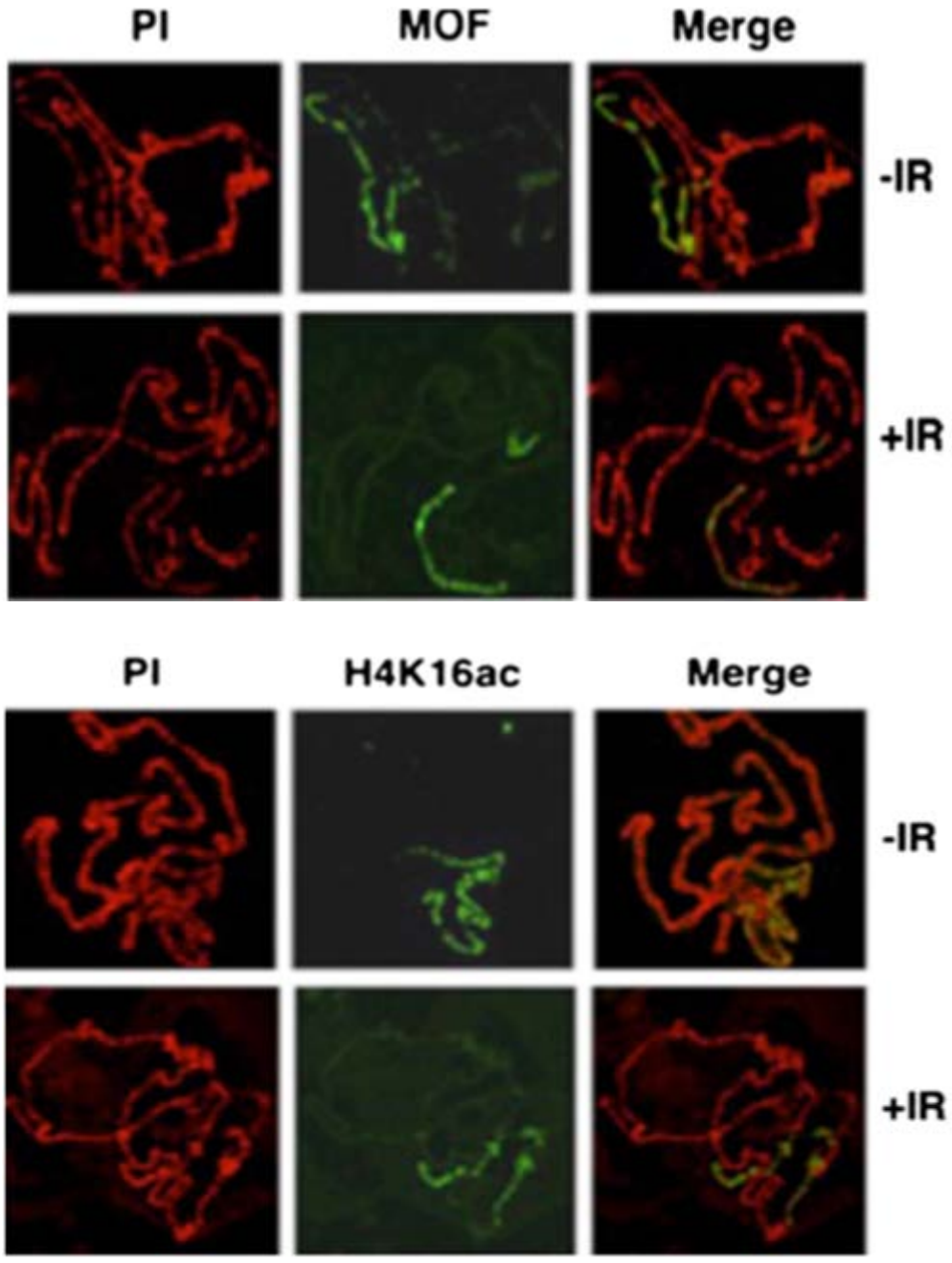


Fig. 3.8 Wild-type male larval polytene X-chromosomes stained with anti- MOF and antiH4K16Ac antibodies. For irradiation, *Drosophila* larvae were treated with 25 Gy IR and the salivary gland chromosomes, fixed and immunostained.

3.8 Discussion

Outside the purview of its role in dosage compensation in *Drosophila*, other cellular roles of dMOF have been not explored extensively. Cytological studies show DCC can localize on several loci on euchromatin and transcriptionally active "puffs" on polytene chromosomes (Kotlikova I.V. et al., 2006). Recently, genome wide analysis has shown that dMOF binds to promoters of autosomal genes in both males and female flies, acting as a transcriptional inducer (Kind, J. *et al.*, 2008). On such loci dMOF occurs as part of a multiprotein NSL (non-specific lethal) complex (Raja, S. J. *et al*., 2010).

Few reports from recent years, point towards role of human MOF or MOF-like proteins in DNA damage response. The yeast ESA1, a MYST histone H4 acetyltransferase is required for repair of damaged DNA at double strand breaks (Bird et al., 2002). Ionizing radiation treatment to HeLa cells causes increased H4K16Ac by hMOF, which also interacts with ATM, the central kinase for initiating DNA damage and repair signals (Gupta et al., 2005). hMOF also interacts with DNA-dependent protein kinase catalytic subunit (DNA-PKcs), that functions in non-homologous end-joining (NHEJ) repair. Both NHEJ and homologous recombination (HR) mediated DNA damage repair are compromised severely in absence of hMOF (Sharma GG et al., 2010). Despite being few, all these reports unanimously point towards the requirement of this protein in damage response and repair mechanism that are so essential for maintaining genome stability.

The goal of this study was to test whether MOF is similarly required for the response to ionizing radiation (IR) in *Drosophila*. We observed that *Drosophila mof* mutations in males and females, like *mof* knockdown in SL-2 cells,reduce post-irradiation survival. MOF depletion in SL-2 cells results in an elevated frequency of metaphases with chromosomal aberrations, suggesting that MOF is involved in DDR. In human cells, MOF depletion results in breakdown of cell cycle checkpoint response to DNA damage at double strand breaks (Gupta et al., 2005). We obtained similar responses in *mof* mutant whole tissues as well. Mutation in *Drosophila mof* also resulted in a defective mitotic checkpoint, enhanced apoptosis, and a defective p53 response post-irradiation. In addition, IR exposure enhanced H4K16ac levels in *Drosophila* as it also does in mammals. Thus our results are the first to demonstrate a requirement for MOF in the whole animal IR response.

CHAPTER-4

RESULTS-2

4. *Drosophila* MOF controls Checkpoint protein2 and regulates genomic stability during early embryogenesis

4.1. Introduction

Faithful transmission of genetic information in cellular organisms is carried out by two basic processes: DNA replication and cell division. Cell cycle checkpoints help preserve the fidelity of these two processes by regulating the progression of the cell cycle and inducing apoptosis in response to DNA damage to eliminate deleterious mutations from the genome. (Abraham,2001). In *Drosophila* embryos too, checkpoints maintain genome stability by delaying cell cycle progression that allows time for damage repair or to complete DNA synthesis, thus maintaining genome integrity and stability. Disruption of checkpoint function plays an important role in carcinogenesis and embryonic lethality (Zhou B.B.et al., 2000; Jaklevic B.R. et al., 2004).

The first 13 syncytial nuclear divisions during *Drosophila* embryogenesis proceed very rapidly, without cytokinesis. These are maternally controlled and consist mainly of S and M phases with short or undetectable gap phases (Tram U et al., 2002). The syncytial cycles from 1–7 occur inside the embryos and nuclear migration to the cortex occurs during cycles 8 and 9 where further synchronous divisions take place before the onset of cellularisation at 14th nuclear cycle. During cycle 9 few nuclei migrate to the poles to form the pole cells that become the germ cells of the embryo (Foe V.E et al.,1993). After completion of 13 syncytial cycles, the embryo undergoes cellularization. Defects in cell cycle checkpoints cause a wide variety of defects such as aging, genetic diseases, oncogenesis and neurodegeneration. Though DNA damage and replication checkpoint induced apoptosis has been extensively studied, less is known about the cellular responses to stress during mitosis. Checkpoint failures lead to progression of mitosis without damage repair leading to ‘mitotic catastrophe’. Embryos exhibiting mitotic catastrophe have giant and fragmented nuclei lacking a regular pattern and 2N ploidy (Dor E et al., 2006).

The early syncytial nuclear divisions of *Drosophila* embryos are very rapid, with no proper cell cycle checkpoints (Weinert et al., 1989). However lack of checkpoints does not severely compromise fidelity of replication and division in these stages. Embryonic surveillance mechanisms exist that serve to effectively cull abnormal nuclei from the

dividing population of cortical nuclei and thus preventing these to be incorporated into adult structures (Sullivan W et al., 1993). Thus, unlike the cell cycle delays that occur to repair the damaged or incompletely replicated DNA, *Drosophila* embryonic system utilizes the delay to identify and discard those abnormal nuclei. These aberrant nuclei may result from external stresses like X rays or chemical treatment or may develop spontaneously as in Recq5 DNA helicase (Nakayama M et al., 2009) or PcG (Dor E et al., 2006) mutants. These abnormal nuclei stain negatively for Phospho histone 3(a mitotic marker), are asynchronous and are seen during nuclear cycles 11–13. Following mitotic failure the defective nuclei drop into the interior of embryos and free centrosomes are seen in the cortex (Sakurai H et al., 2011).

The duration of S phase increases progressively during the last 4 syncytial blastoderm stage divisions and a DNA replication checkpoint comes into play, delaying mitosis in the event of damaged or unreplicated DNA. Checkpoint mutant embryos enter mitosis prematurely (Sibon et al., 1997, 1999) and display spontaneous mitotic defects such as centrosome inactivation and failure in anaphase segregation (Sibon et al., 2000). Centrosome inactivation results from the loss of three components of the γ-tubulin ring complex (TuRC), a critical microtubule-nucleating factor from a core centrosome structure (Sibon et al., 2000).

Drosophila chk2 (*Dmchk2*) plays an essential role in this mitotic response to genotoxic stress. Upon DNA damage, Chk2 localizes to centrosomes and spindle fibres and delinks the chromosomes from their centrosomes, which ultimately results in loss of the nuclei (Sakurai H et al., 2011). Mutation in *mnk* gene (*Drosophila* homolog of *Chk2*) prevents centrosome inactivation and suppresses chromosomal segregation defects. In a variety of systems, cell cycle checkpoint defects lead to progression into mitosis with damaged DNA or incompletely replicated DNA leading to “mitotic catastrophe”, characterized by mitotic division failure and cell death, and a process distinct from apoptosis(Roninson IB et al., 2001). Essentially DmChk2 is crucial for the “mitotic catastrophe” signal during embryogenesis.

It has been proposed that Chk2 functions at two points during early embryogenesis in response to genotoxic stress. At the onset of mitosis DNA lesions leads to activation of Chk2 that target proteins involved in centrosomal spindle activity and in

maintaining γTURC localisation. This causes failure in anaphase chromosome segregation. Once failure of mitotic division occurs, Chk2 causes centrosomal inactivation and disrupts the link between centrosomes and nuclei. Since centrosomes anchor nuclei to the cortex, Chk2 response to DNA damage results in loss of nuclei from the cortex (Raff JW et al., 1989). DNA damage induces the activation of chromatin bound Chk2 by a chromatin derived signal resulting in the dissociation of the activated Chk2 from the chromatin. Chk2 is phosphorylated at T68 by ataxia telangiectasia mutated (ATM) and transmits the DNA damage signals from the upstream phosphatidylinositol 3'-kinase like kinases to the effector substrates including p53, Braca1, Cdc25A and Cdc25C (Li J et al., 2005). Chk2 has also been reported to phosphorylate p53, thereby enhancing the transcriptional activity of p53 responsive genes (Takai H et al., 2002). Further the functional link between p53 and Chk2 during DNA damage occurs through the phosphorylation and acceleration of degradation of Hdmx, a negative regulator of p53 (Chen LD et al., 2005).

4.2 *mof* heterozygote embryos are haplo-insufficient for maternal MOF gene product

mof^{1} is a EMS mutation having a single amino acid substitution in the acetyl co-enzyme motif (Hilfiker A et al., 1997). Sequence analyses revealed that *mof3* results from a nonsense mutation at aminoacid 151 (Q151X). The nature of the *mof* alleles has been studied by quantifying the amount of maternal MOF gene product. For this purpose total protein was isolated from control (*yw67c23*) *mof*^{1} (Ethyl Methane Sulphonate mutation) and *mof3* (non-sense mutation) embryos (1–2 h) and western blot analysis was carried out using MOF antibody. A drastic decrease in MOF expression in *mof* heterozygote embryos compared to wild type controls indicated that *mof* mutation is haplo-insufficient for maternal gene product.

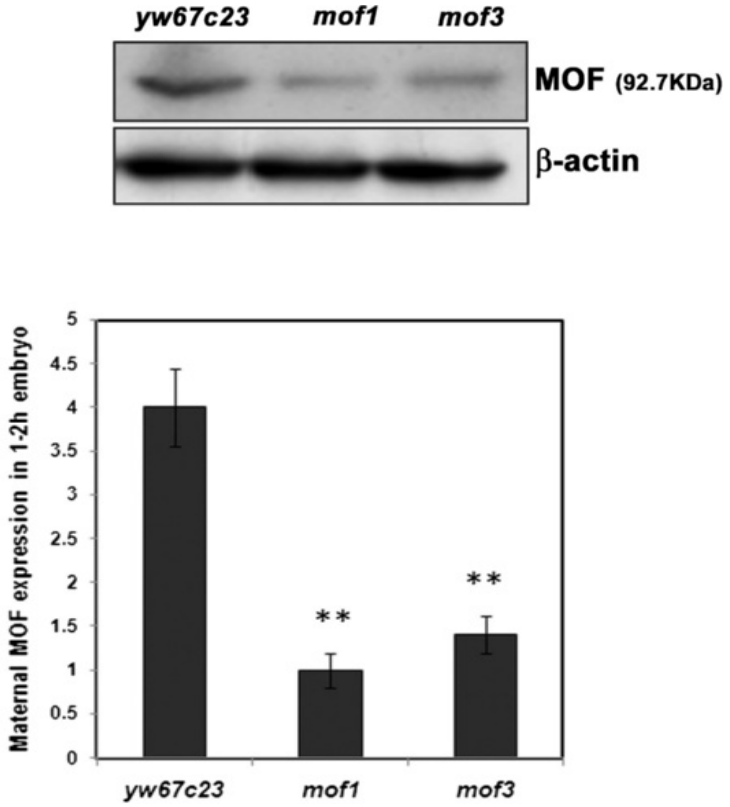
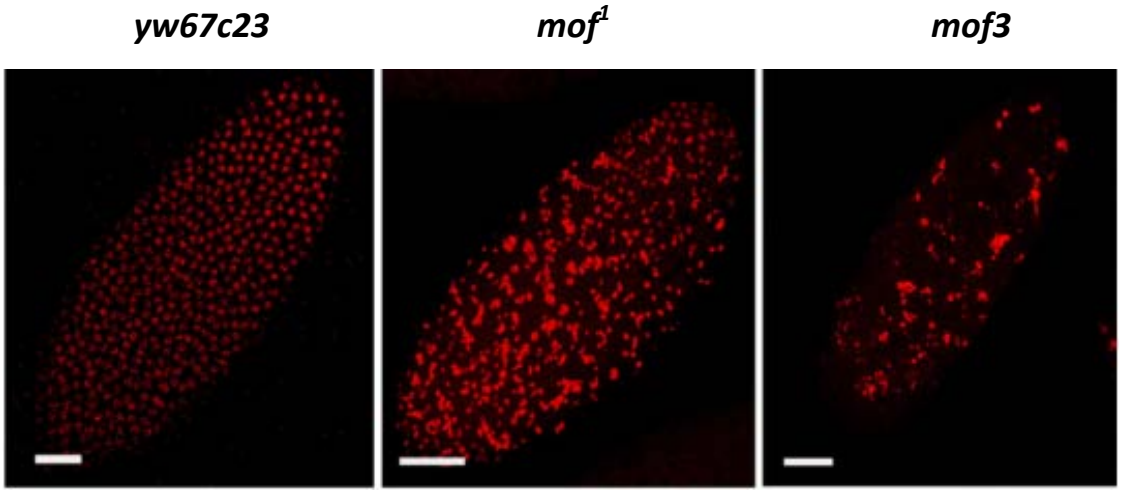


Fig 4.1. Haplo-insufficiency of *mof*^{1} heterozygotes. Total protein was isolated from early embryos of control (*yw67c23), mof 1*and *mof 3* (1–2 h) and Western blot analysis carried out using anti-MOF antibody has shown 3-fold reduction in the maternal MOF protein. β-actin is used as an internal loading control. Statistical significance was assessed using Student t-test. *** indicates P<0.001, ** indicates P<0.01, * indicates p<0.05.

4.3 Asynchronous cell cycle and Mitotic catastrophe in *mof*^{1} embryos

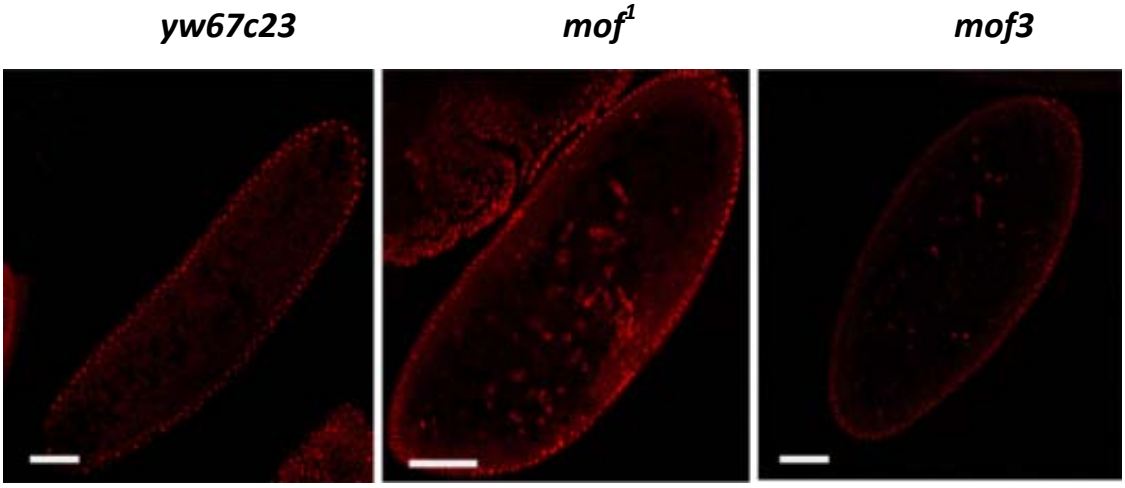
Studies of *mof* null mice have shown delayed development with massive abnormal chromosomal aggregation, leading to death at an early stage (Gupta et al., 2002). *In vitro* and as well as *in vivo* studies in *Drosophila* reveal that MOF is required for efficient repair of DNA damage induced by ionizing radiation. Since *mof*^{1} mutants are haplo-insufficient for the maternal gene product, we were interested to study the role of MOF during early syncytial mitotic divisions. Embryos derived from heterozygous mothers (haplo-insufficiency of maternal gene product) of *mof*^{1}*/FM7* (EMS mutagenesis), *mof3/FM7* (non-sense mutation) as well as *yw67c23* (control) were collected. Early embryos (0–2 h) were fixed and mounted in propidium iodide (PI) to visualise the nuclei. *mof* heterozygote embryos exhibited mitotic catastrophe with fragmented nuclei that appear as large masses of chromatin compared to wild type control where the nuclei appeared normal (Figure 4.2A). During early embryogenesis the initial seven syncytial divisions occur at the interior of the embryo. During cycles 8 and 9 the nuclei migrate to the cortex leaving only few yolk nuclei. We observed that abnormal nuclei in the *mof* heterozygote embryos are eliminated by nuclear fallout mechanism wherein they are digested inside the yolk tissues. Nuclear fallout mechanism protects the organism by eliminating the abnormal nuclei from forming adult structures that might be deleterious. Nearly 70% of the *mof* heterozygotes exhibited a large number of fall out nuclei (high severity fall out nuclei=more than 5 fall out nuclei/embryo) (Figure 4.2B, 4.2C) compared to control nuclei (*yw67c23*) where the number of fall out nuclei is negligible. Hence a decreased number of nuclei are present in the *mof* embryos compared to control (*yw67c23*). The fall out nuclei in embryos were scored when they are 2-20μm below the cortex in the syncytial blastoderm stage as they are misinterpreted in later stages where fall out co-exists with normal nuclear migration.

A



Mitotic Catastrophe

B



Nuclear Fall out

C

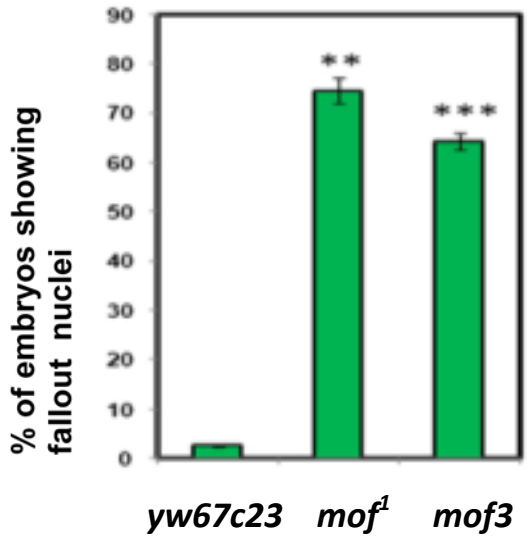


Fig. 4.2 Loss of MOF causes asynchronous cell cycle, mitotic catastrophe and nuclear fallout. Early embryos (0–2 h) of *yw67c23*, *mof*^{1} and *mof3* were collected, fixed with DNA dye PI and visualized using confocal microscopy (60X objective). **A**. Large fragmented nuclei indicating occurrence of mitotic catastrophe, seen in the early embryos of *mof1*and *mof3* mutants when compared to control embryos. **B.** Increased number of fall out nuclei is observed in the *mof*^{1} and *mof3* mutants compared to control *yw67c23* embryos. Scale bar indicates 10 μm. **C**. The data is represented in the form of bar diagram. Statistical significance was assessed using student t-test. *** indicates P<0.001, ** indicates P<0.01,* indicates p<0.05.

4.4 Abnormal mitosis in *mof* heterozygotes

Mof is a maternal effect gene and homozygotes for *mof* mutation do not survive (late larval lethal) till adult stage. To study the role of MOF in early mitosis, we collected embryos derived from heterozygous mothers (haplo-insufficiency of maternal gene product) of mof^{1}*/FM7* and *yw67c23* (control). During the early syncytial nuclear divisions, mof^{1} mutant embryos exhibited several mitotic defects such as chromatid bridges resulting in lagging chromosomes (Figure 4.3A), defects in sister chromatid separation (Figure 4.3B); telophase defects (Figure 4.3C) indicating that *mof* heterozygous embryos may be entering mitosis with damaged or incompletely replicated DNA. The lethality associated with *mof* homozygotes was fully rescued with the addition of *mof* transgene. Although the transgenic line expressing *mof* transgene was viable and fertile, it did not completely restore the chromosomal defects (only 60% of the defects were rescued) (Figure 4.4). The embryos from mof^{1}/+ display similar mitotic defects as that of mof^{1}*/FM7* while the embryos from *FM7*/+ females do not show any mitotic defects indicating that FM7 balancer has no role in causing the mitotic defects observed in the case of mof^{1}*/FM7* embryos.

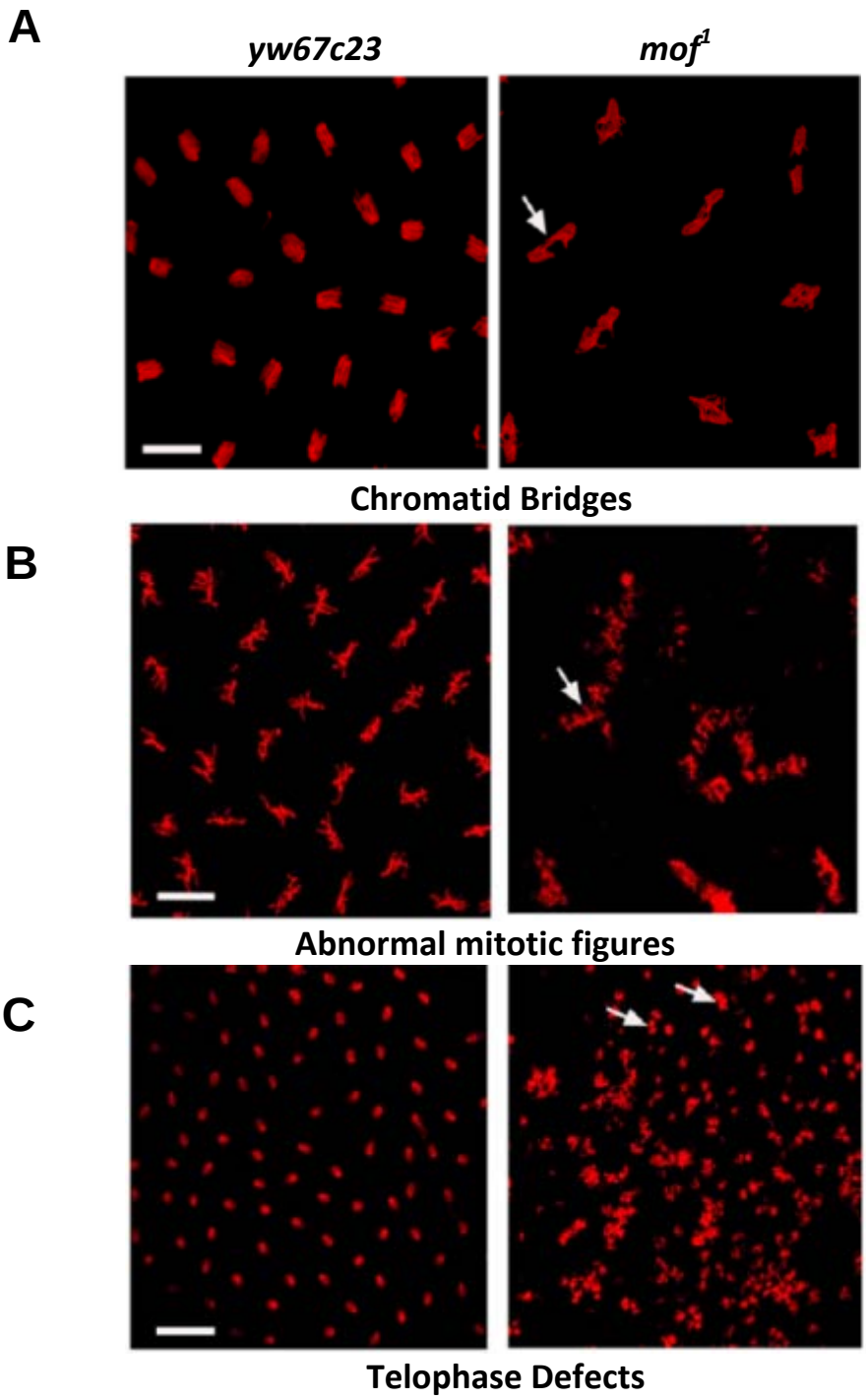


Fig. 4.3. Loss of maternal MOF causes chromosomal defects in early embryos. Early embryos (0–2 h) from *mof*^{1} and *yw*^{67c23} were collected, fixed with DNA dye PI and visualised with 100X objective on a confocal microscope. The *mof*^{1} mutants displayed a wide variety of chromosomal defects like **A**. chromatid bridges which indicates the presence of lagging chromosomes **B**.sister chromatid separation and **C.** telophase defects. Bar indicates 10 μm scale.

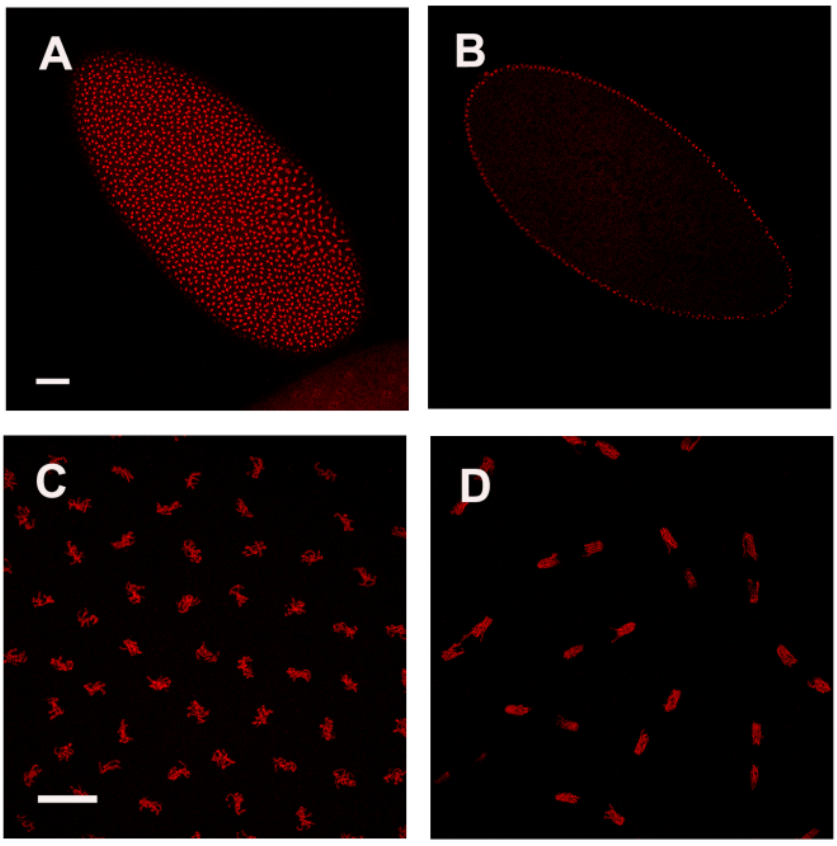


Fig.4.4 Rescue of mitotic defects in *mof* embryos by *mof* transgene. A. Early embryos from females of *mof3+ mof* transgene were collected, processed and stained with DNA dye PI. The mitotic defects were rescued by the *mof* transgene and the embryos appear normal with **A.** mitotic synchrony and no mitotic catastrophe **B.** without nuclear fall- out **C.** sister chromatid separation and **D.** chromatid bridges.

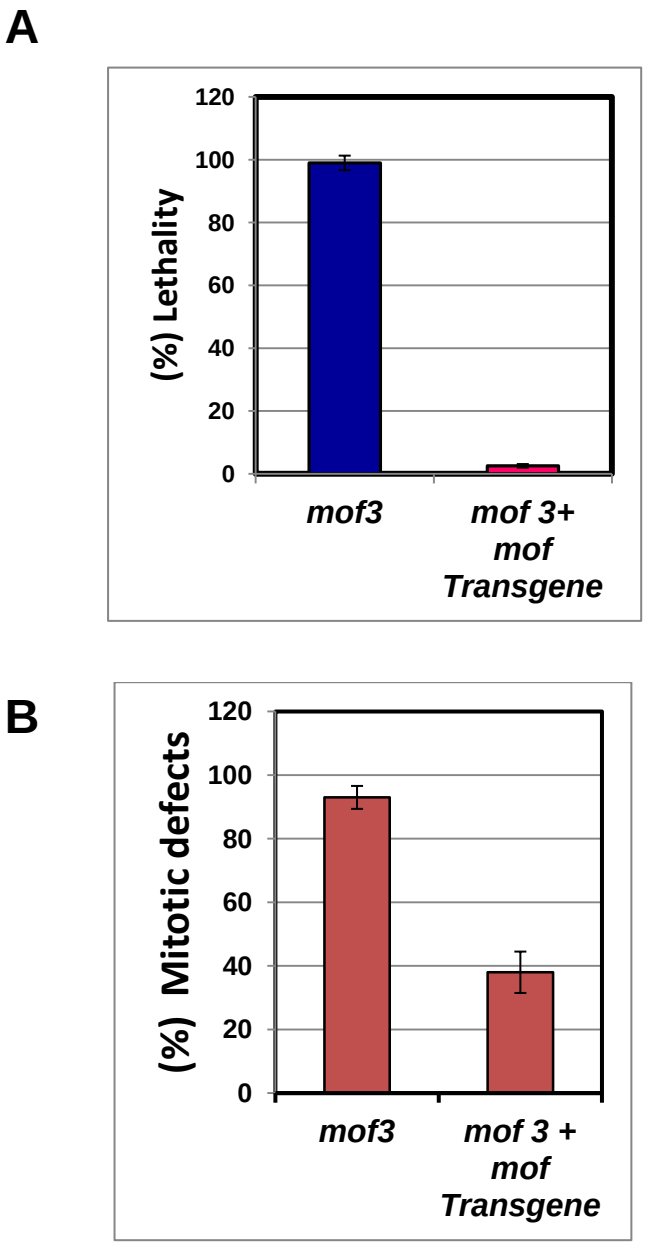


Fig. 4.5 A. *mof3* mutant females were crossed with *mof* transgene males and survival of genotype *mof3+mof* transgene were assayed. The resulting data was quantified and represented as bar diagram. Addition of *mof* transgene in the background of *mof3* mutant could completely rescue the lethality associated with the *mof* mutation. **B.** *mof3* mutant females were crossed with *mof* transgene males. The resulting embryos from mothers of *mof3 +mof* transgene were studied for mitotic defects and the data is represented in the form of histogram. The mitotic defects in the *mof3* mutants were rescued upto 60% with the addition of *mof* transgene.

4.5 Mitotic asynchrony during early nuclear divisions in *mof* heterozygyous embryos

In early wild type embryos, mitosis occurs synchronously and proceeds in the form of waves starting from the poles. Mitotic synchrony during pre-syncytial and syncytial divisons in mof^{1} and control embryos was studied by staining with antibody against Histone H3 Ser10 Phosphorylation (PH3) (the mitosis marker). Control embryos showed PH3 staining on all the chromosomes while in the case of mof^{1} heterozygotes both PH3 positive (dividing) and PH3 negative (non-dividing) chromosomes were observed. Thus the PH3 negative chromosomes in mof^{1} heterozygotes indicate the existence of abnormal nuclei. Our data indicates that maternal supply of MOF is required for mitotic synchrony in pre-syncytial and syncytial blastoderm embryos (Figure 4.6A-A', B-B'). These abnormal nuclei which lose association with cortex (fall out nuclei) are unlikely to divide since they do not have centrosomes attached to them.

To further confirm nuclear fallout early embryos of mof^{1} heterozygotes and *yw67c23* were immunostained with anti-centrosomin antibody. A number of free centrosomes lacking the chromosomes were present in the mof^{1} heterozygous early embryos compared to control embryos (*yw67c23*). The free centrosomes in the embryo indicated the presence of abnormal nuclei that are eliminated by the nuclear fallout mechanism (Figure 4.7). In addition to free centrosomes, we also observed chromosomes lacking centrosomes or with only one centrosome. These findings strongly suggest the involvement of centrosome inactivation in the mof^{1} early embryos.

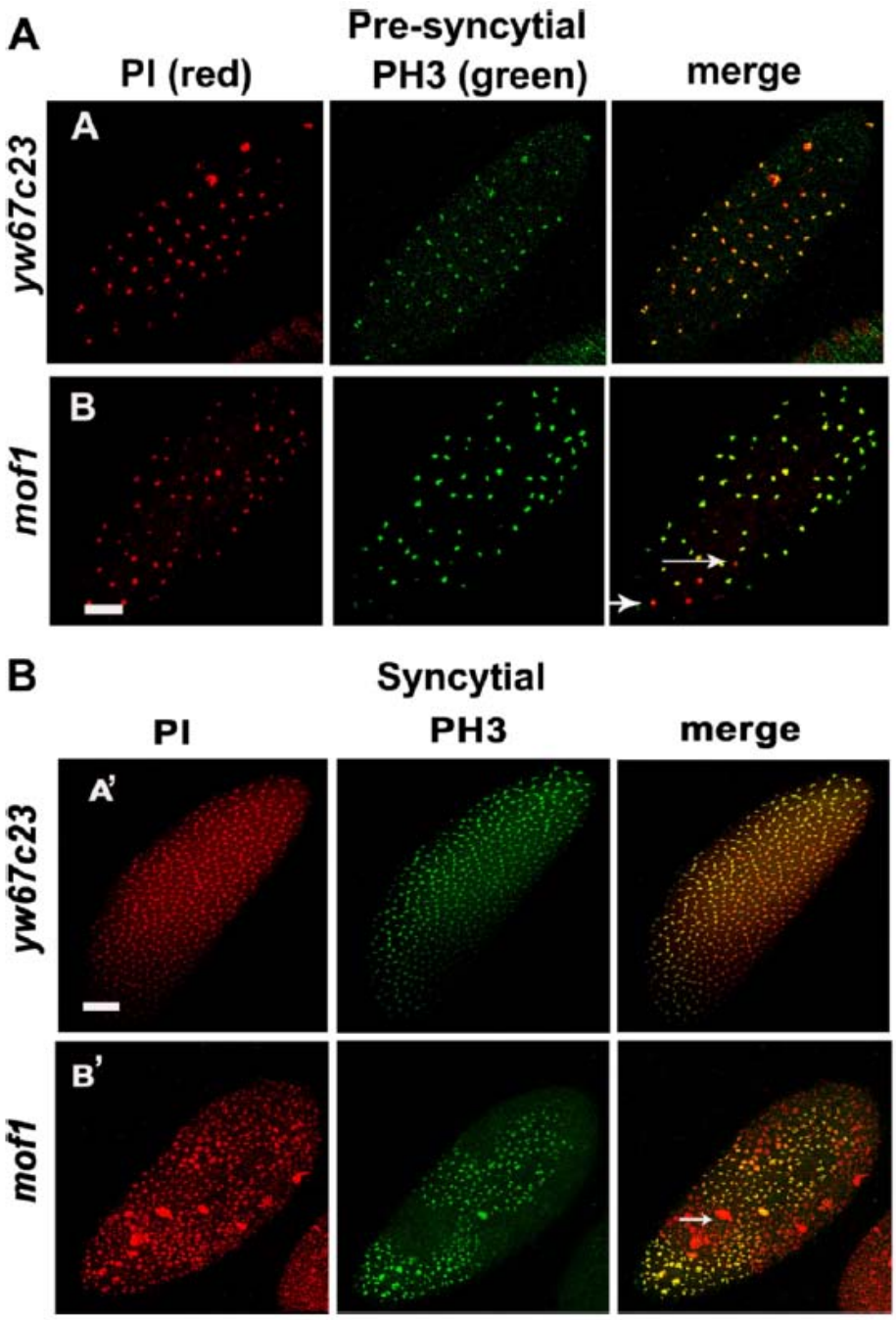


Fig. 4.6. Mitotic asynchrony in *mof*^{1} heterozygotes. Early embryos from 0–2 h were collected from control *yw67c23* and *mof*^{1} mutants and immunostaining was carried out using PH3 antibody (green) which is mitotic marker. DNA was stained with PI (red). **A** and **A'**: control *yw67c23*, and **B** and **B'**: *mof*^{1} embryos, during pre-syncytial and syncytial blastoderm stages respectively. In the case of *mof*^{1} embryos the nuclei are unevenly spaced and not all chromsomes are stained with PH3 indicating mitotic asynchrony during pre-syncytial blastoderm stage. Bar indicates 10μm scale.

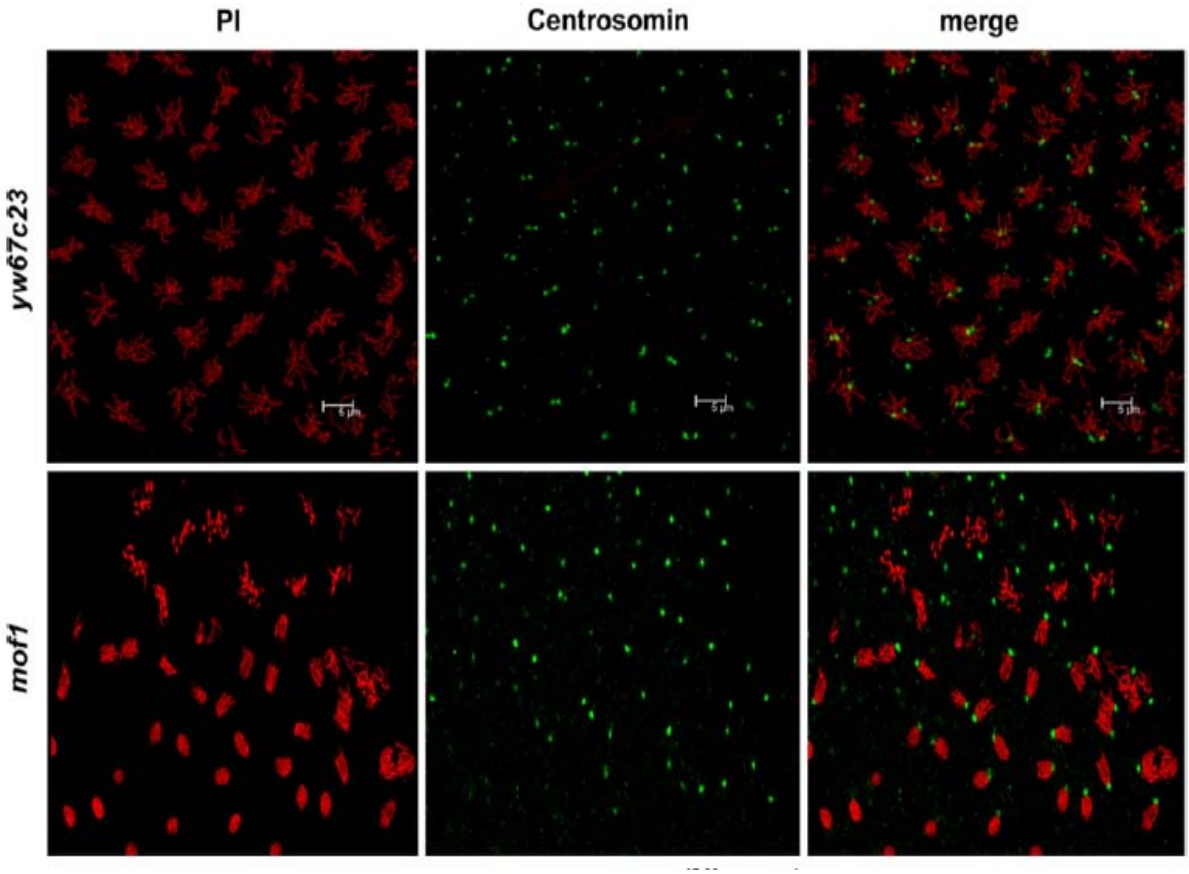


Fig. 4.7. Loss of MOF results in free centrosomes. Embryos from 0–2 h from *yw67c23* and *mof*^{1} mutants were immunostained with anti-centrosomin antibody (green) and DNA was stained using PI (red). Free centrosomes without the chromosomes were present in the *mof*^{1} embryos indicating the presence of abnormal nuclei which have been removed by nuclear fallout mechanism. Bar indicates 5μm scale.

4.6 Disruption of cytoskeleton in the *mof* heterozygous embryos

Cytoplasmic organization, nuclear division and nuclear migration in the syncytial embryos are modulated by the cytoskeletal proteins. Following the syncytial divisions, individual cells are produced by a process called cellularization that occurs during interphase of nuclear cycle 14. We were interested to study the changes in the organization of actin cytoskeleton and hence control *yw67c23* and mof^1 heterozygous embryos were immunostained with ß-actin antibody. The typical honeycomb like structure of actin cytoskeleton observed in the control was lacking in the case of mof^1 embryos. Moreover in the mof^1 embryos chromosomes were incompletely surrounded by the actin filaments along with few small cells that lack nuclei. These empty cells indicate the presence of abnormal nuclei which have been eliminated by nuclear fallout mechanism (Figure 4.8).

In addition to the actin filaments, the polymerization and depolymerization of microtubule network helps in mediating the coordinated nuclear movement (chromosomes) during syncytial stage of embryogenesis. Since polymerization and depolymerization of the microtubules is required for proper chromosome movement, we stained the *yw67c23* and mof^1 embryos with alpha-tubulin antibody to visualize the organization of spindle fibres. Around 66% of mof^1 embryos as opposed to only 7% of *yw67c23* embryos exhibited attachment of spindle fibres all over the chromsomes instead of the kinetochore, indicating disruption of the spindle fibre assembly and therefore leading to improper movement of chromosomes during anaphase resulting in lagging chromosomes. Number of embryos counted in the present study is 100 (Figure 4.9A, 4.9B).

The integrity of cell's cytoskeleton is crucial for the first occasion of vasa localization in the preplasmic cytoplasm as well as second occasion in the pole plasm. Proper function of the cytoskeleton is important for nuclear migration leading to formation of pole cells. Here *yw67c23* and mof^1 embryos were immunostained with antibody against vasa which selectively stains the pole cells. As anticipated, we observed drastic reduction in the number of pole cells in the mof^1 embryos compared to control (*yw67c23*) indicating that MOF is required for proper nuclear migration and formation of pole cells (Figure 4.91A, 4.91B).

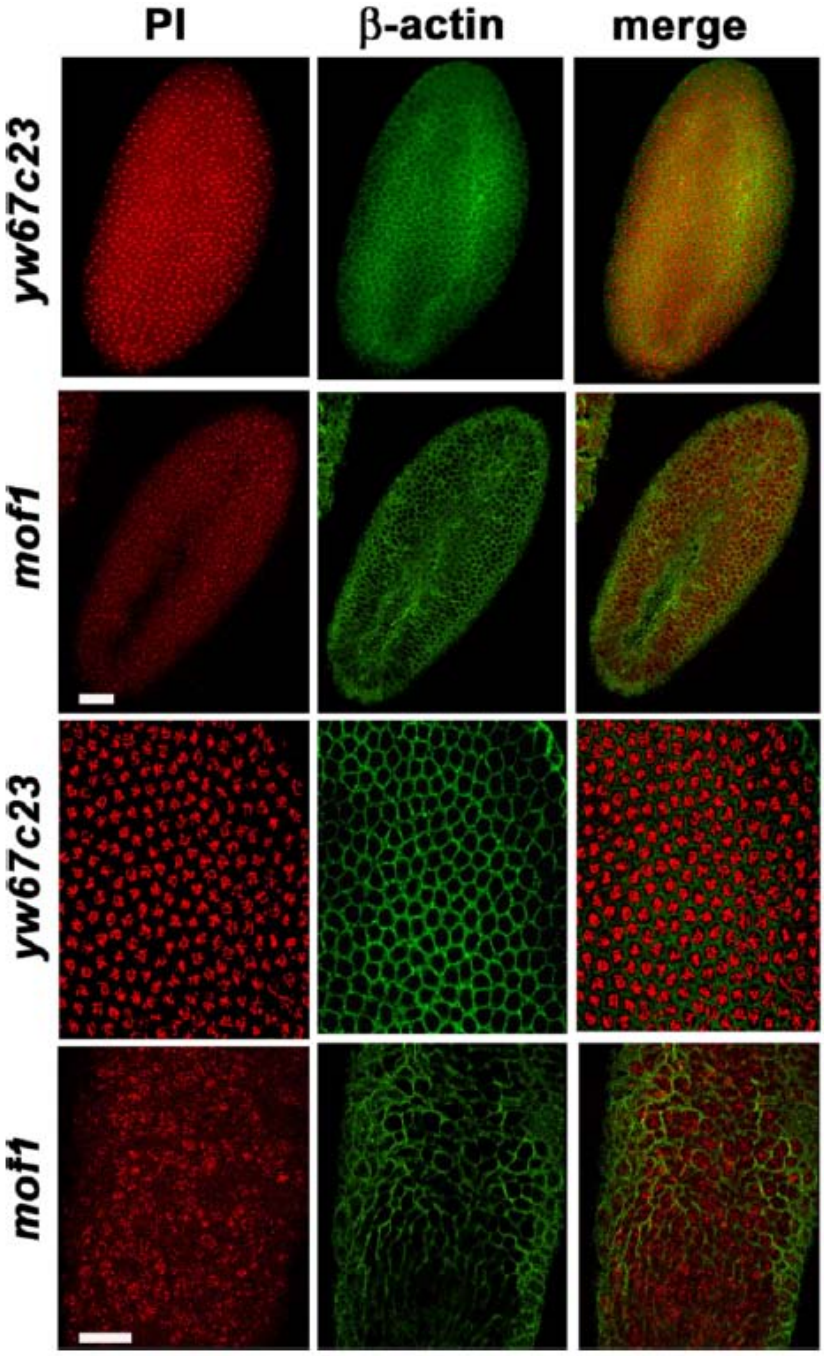


Fig. 4.8. Defects in actin cytoskeleton in the *mof*^{1} heterozygotes. Early embryos (0–2 h) were stained with β-actin (green) antibody in both *yw*$_{67c23}$ and *mof*^{1} embryos. PI is used to stain the DNA. The honeycomb like structure which is characteristic of actin cytoskeleton is largely disrupted by the *mof*^{1} mutation. There are few cells which do not have chromsomes in them indicating abnormal nuclei which have been dropped into the cortex and digested by the yolk nuclei. Bar indicates 10 μm scale.

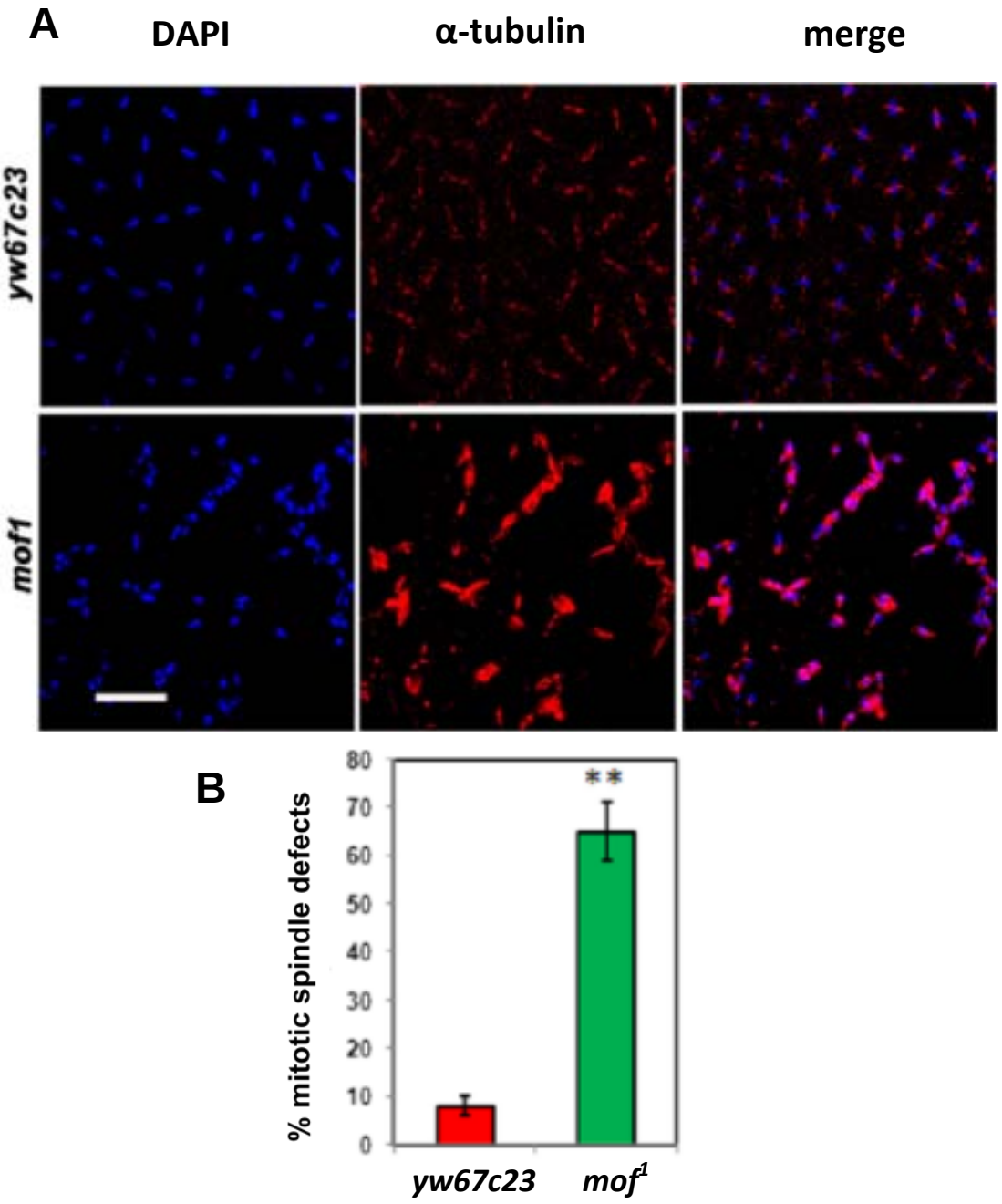


Fig. 4.9 Loss of MOF causes defects in spindle fibre organisation and nuclear migration. A.0–2 h *mof*^{1} and *yw67c23* embryos were stained with alpha-tubulin antibody (red) and DAPI is used as DNA dye. In mutants, chromosomes were not properly aligned in the metaphase plane with tubulin fibres attached all over the chromosomes. **B**. The percentage of embryos exhibiting mitotic spindle defects represented in the form of bar diagram. Bar indicates 10 μm scale. Statistical significance was assessed using student t-test. ** indicates P<0.01.

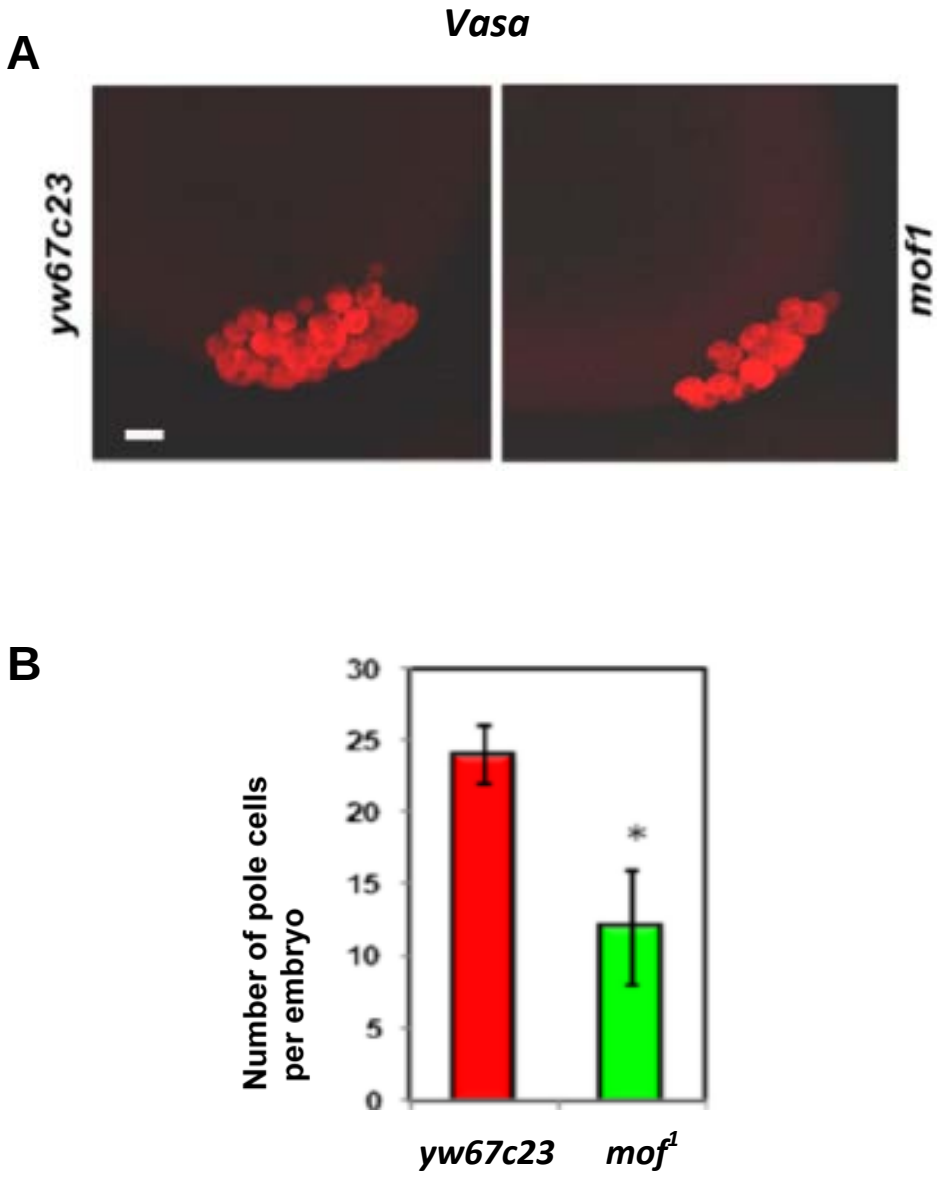


Fig. 4.91 A. Early embryos of *mof*[1] and *yw67c23* were stained with vasa antibody to visualise the pole cells. There was drastic decrease in the number of pole cells in the *mof*[1] embryos indicating that nuclear migration is affected. **B**. The number of pole cells in control and *mof*[1] mutant per embryo is represented in the form of bar diagram. Bar indicates 10 μm scale. Statistical significance was assessed using student t-test. *** indicates P<0.001, ** indicates P<0.01, *indicates p<0.05.

4.7 Elevated levels of DNA damage in *mof* heterozygous embryos

We were interested to study more specific roles of MOF in DNA damage. *mof*^{1} heterozygote embryos as well as control embryos were used in an assay that determines the extent of DNA damage (single, double stranded DNA breaks) (Fogarty P. Et al,1997). Genomic DNA was isolated from the embryos (0–2 h) and incubated with T4 DNA kinase and 32P ATP. The amount of incorporation of 32PATP determined the number of exposed 5' phosphate groups in the DNA, indicating the number of single and double stranded lesions. Genomic DNA from *mof*^{1} embryos showed a highly elevated level of P32 incorporation of 6500 ± 370 cpm/ng compared to the controls which is 1011 ± 200 cpm/ng suggesting that the increase in lesions in *mof*^{1} embryos is due to progression through mitosis with damaged DNA or incompletely replicated DNA. To further confirm the double strand breaks observed in the *mof*^{1} heterozygotes we carried out western blot studies using phospho H2Av antibody. As expected, in *mof*^{1} heterozygotes we observed an increase in the levels of H2Av phosphorylation when compared to control (Figure 4.92).

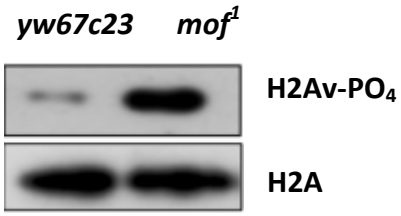


Fig. 4.92 *mof*^{1} mutation causes double strand breaks in DNA. Total protein was isolated from *yw67c23* and *mof*^{1} heterozygyous embryos and western blot was carried out using H2Av-PO4 antibody. Here H2A antibody was used as loading control. An increase in the level of phosphorylation of H2Av was observed in the *mof*^{1} heterozygotes.

4.8 Abnormal nuclei are eliminated by Chk2 activation

Late syncytial embryos of *Drosophila* exhibit two-stage response to DNA damage or replication defects (Sibon O.C.et al., 2000). Two different kinase pathways, ATM/Chk2 pathway and ATR/Chk1 pathway play a major role in response to DNA damage that is evolutionarily conserved. The DNA checkpoint mediated by *mei-41* and *grp*, the *Drosophila* orthologs of *ATR* and *Chk1* kinases, respectively, delay entry into mitosis via inhibitory phosphorylation of Cdk1, which allows repair of DNA damage or completion of DNA replication (Sibon O.C.et al,1997;1999). When this checkpoint fails, a second control operating during mitosis is activated, that results in changes in spindle structure and chromsome segregation to stop propagation of defective or damaged nuclei. This second step of control is mediated by activation of *Chk2* by centrosomal inactivation (Oishi et al., 1998). The increased number of fall out nuclei and defects during early mitosis in the *mof* heterozygous embryos led us to speculate the possible involvement of a DNA replication dependent or DNA damage dependent cell cycle checkpoint defect. *Drosophila* embryos that lack *Chk1* homologue (*grp)* and *ATR* homologue (*mei-41*) show inactivation of centrosome during the late stages of syncytial division proving that both the homologues are not required for centrosome inactivation (Sibon O.C et al., 2000).

To show that DNA replication checkpoint is intact in the mof^{1} embryos, we studied the levels of *grp (Chk1)* and *mei-41* (*ATR*) and we found that their levels remained the same in syncytial cycles 10–13 (Figure 4.93A). Also the levels of cyclins remained the same in the wild type and mof^{1} embryos in syncytial cycles 10–13 indicating that our data do not support a role of *Drosophila* MOF in the control of cell cycle in the syncytial embryos through regulation of cyclins and *grp*. Thus our data do not support a role for *Drosophila* MOF in control of cell cycle timing in syncytial embryos via regulation of cyclins or *grp* levels.

The defective mitotic spindles that are short, anastral and associated with poorly aligned chromosomes in the *mof* embryos exhibited key features reminiscent of *Chk2* mediated centrosomal inactivation. This led us to investigate the possible role of checkpoint gene *Chk2* in this event. Total RNA was isolated from syncytial cycles 10–13 of control *yw67c23,* mof^{1} heterozygotes and RT-PCR was carried out using *Chk2* specific primers. We observed increase in the transcript level of *Chk2* by 4-folds in the mof^{1}

embryos compared to *yw67c23* control indicating *Chk2* mediated centrosome inactivation (Figure 4.93B). To further confirm MOF mediated *Chk2* regulation we have knocked down *mof* experiment using dsRNA in S2 cells. The expression of *Chk2* was found to be enhanced upon *mof* depletion.*GFP* dsRNA did not cause any significant change in levels of mof and thus used as control in the RNAi study (Figure 4.93B').

Since *Chk2* is a major target of ataxia telangiectasia-mutated (ATM), the expression pattern of ATM in mof^{1} heterozygous embryos was also studied. We observed that there was pronounced increase in levels of ATM in mof_{1} heterozygotes. We also observed increased expression of *p53* in mof^{1} heterozygous embryos indicating that *mof* mutation causes spontaneous DNA damage leading to the activation of ATM-Chk2 pathway (Figure 4.93A).

4.9 Centrosome inactivation in asynchronous nuclei of syncytial mof^{1} heterozygyous embryos

Drosophila Chk2 is encoded by *mnk* (*maternal nuclear kinase*) gene (Oishi I et al., 1998) and *mnkp6* homozygous null mutation flies produce DNA damage induced apoptosis (Xu J et al., 2001). To further confirm *ATM/Chk2* mediated centrosomal inactivation, we crossed mof^{1}*/FM7* virgin females with *mnkp6* males to produce heterozygous mof^{1}*/+; mnkp6/+* flies. The mof^{1}*/+; mnkp6/+*females were further mated with wild type males and 0–2 h embryos were collected. These embryos were stained with PI to check for nuclear fallout. The mof^{1} embryos exhibited high severity fall out nuclei (more than 5 fall out nuclei/embryo) while mof^{1}*/+; mnkp6/+* embryos had only low severity fall out nuclei (less than 5) (Figure 4.94). Thus Chk2 activation contributes significantly to the mof^{1}phenotype in syncytial embryos.

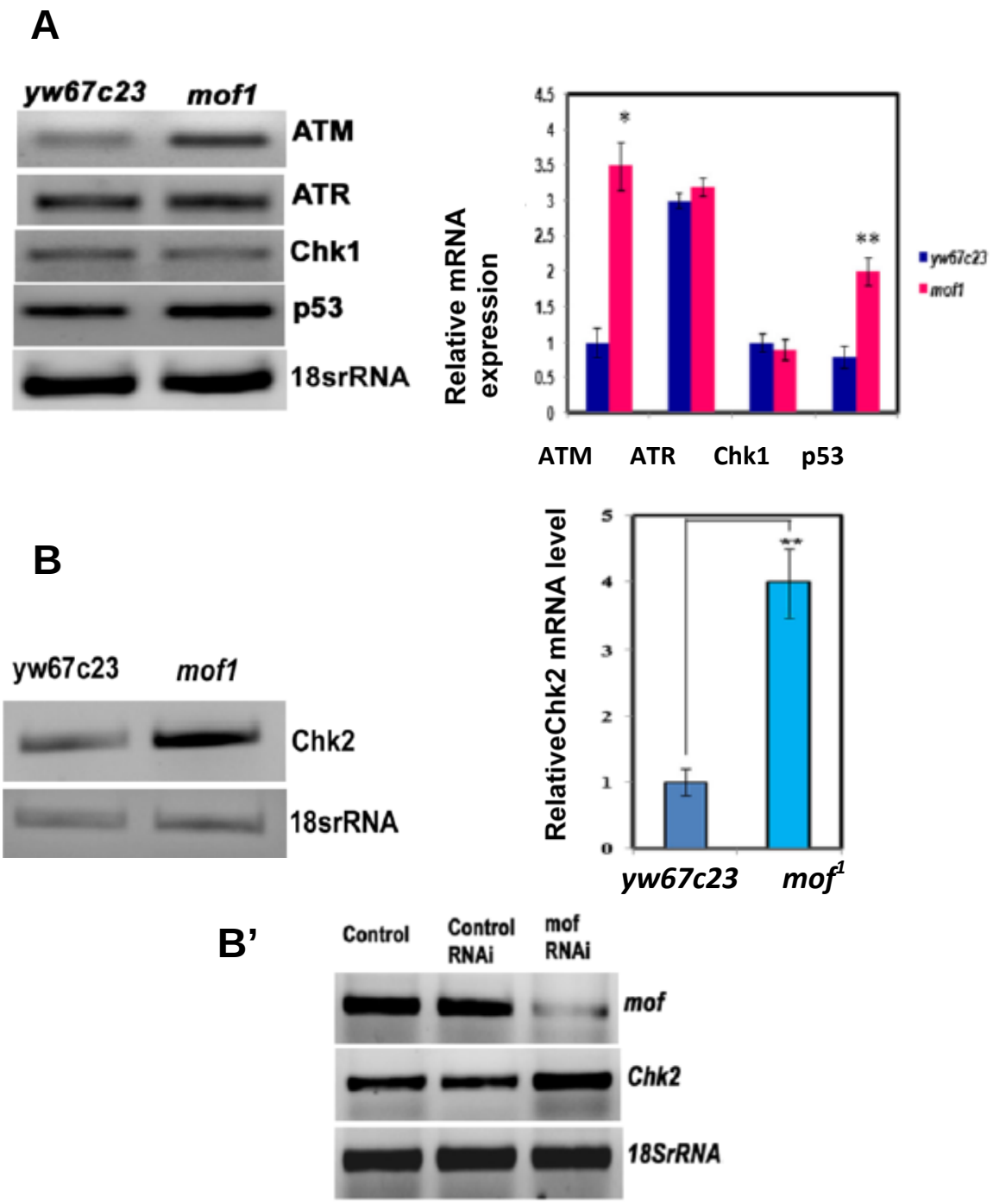


Fig. 4.93 Activation of *Chk2* in *mof* heterozygyous embryos. A. Total RNA was isolated from embryos of control *yw67c23* and *mof*^{1} (syncytial cycles of 10–13) & semi-quantitative RT-PCR was carried out to study their expression levels of ATM, ATR, Chk1, p53 and **B**. *chk2*. Levels of *Chk2* increased 4-fold in *mof*^{1} indicating an activation of this gene. Adjacent histograms quantify the fold change. Statistical significance was assessed using student t-test. ** indicates P<0.01, *indicates p<0.05. **B'**. Depletion of MOF by RNAi was performed in S2 (*Drosophila* Schneider) cells by incubation with dsRNA against *mof*. Transfection was conducted for 72 h time period. Total RNA isolated was subjected to RT-PCR analysis against *Chk2* and *mof*. Here dsRNA against GFP was used as control (control RNAi). The depletion of *mof* leads to an increase in the levels of *Chk2* mRNA.

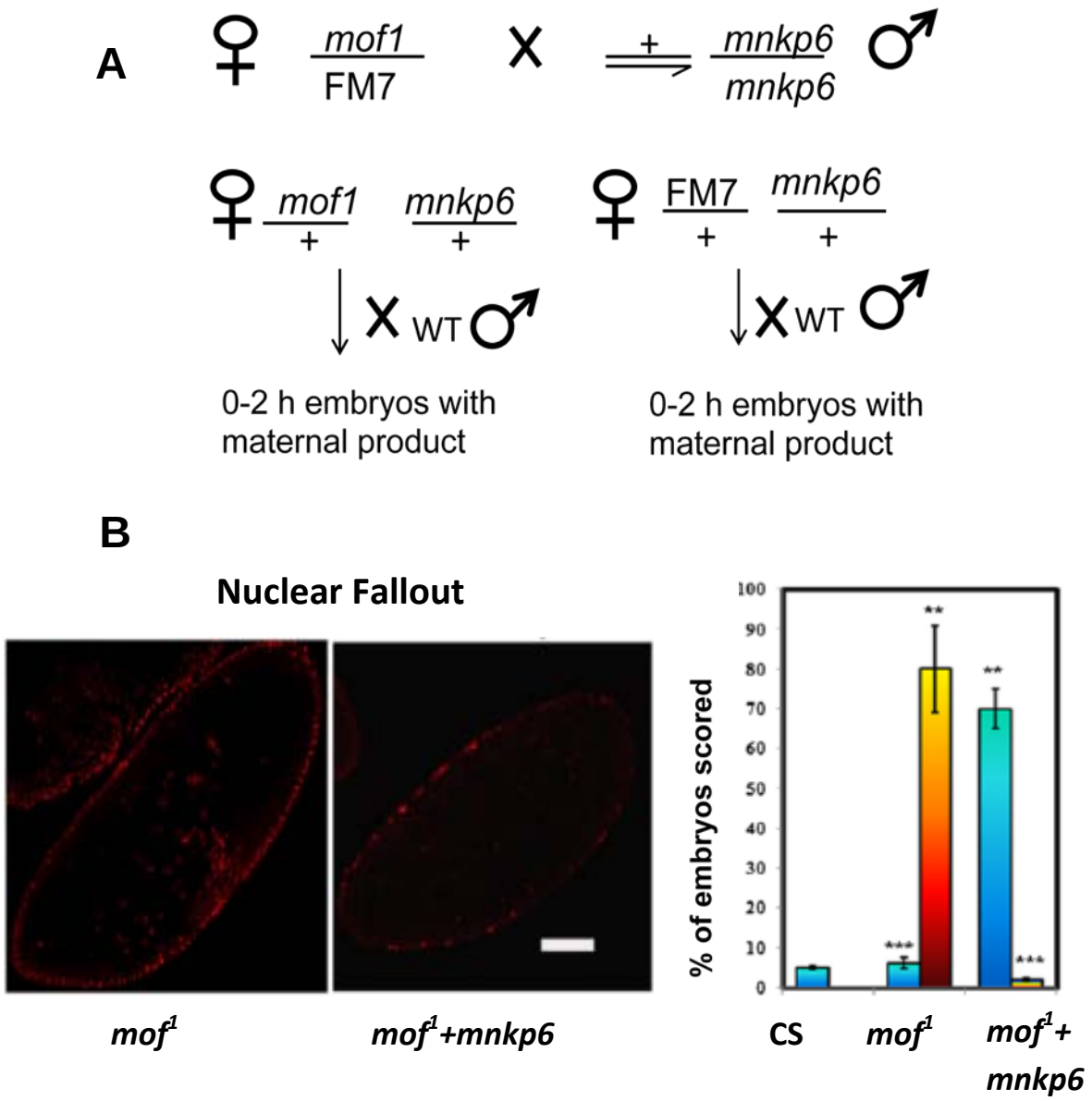


Fig. 4.94 Genetic study to confirm ATM/Chk2 mediated centrosomal inactivation. A. Genetic crosses to generate early embryos (0-2hrs) from *mof*^{1} and *mof*$_{1}$*/+; mnk*$_{p6}$*/+* females. **B.** Early embryos were collected and stained with DNA dye PI to study the severity of nuclear fallout. The high severity of nuclear fallout in *mof*$_{1}$ embryos was drastically reduced in the presence of *mnk*$_{p6}$ *(chk2)* mutation. Bar indicates 10 μm scale.

The data is graphically represented alongside. Statistical significance was assessed using student t-test. *** indicates P<0.001, ** indicates P<0.01, * indicates p<0.05. Blue and orange-red on histogram bars indicate low and high severity of fallout respectively.

4.91 Discussion

This study revealed for the first time the role of MOF during early embryogenesis in *Drosophila* apart from dosage compensation and response to ionizing radiation. We have already demonstrated the role of MOF in response to ionizing radiation is conserved in *Drosophila melanogaster*. In human cells, knockdown of hMOF results in loss of H4K16Ac and destabilization of nucleosomes that correlates with regions of chromatin decondensation. While acetylated H4 K16 appears to 'open up' the *Drosophila* male X chromosome to make it more accessible to transcription, which is an important part of the dosage compensation mechanism in the fly. Reduced levels of MOF in mammals correlate with decreased H4K16Ac, cell proliferation, cell survival and increased genomic instability. *Drosophila* haploinsufficency of maternal MOF causes several mitotic defects in the syncytial embryos and a large number of abnormal nuclei have been removed through the process of nuclear fallout. The increased number of abnormal or fall out nuclei correlated with reduced nuclear density in syncytial blastoderm embryos of *mof* heterozygotes.

Our study demonstrates that in response to spontaneous DNA damage (increased number of single and double stranded DNA breaks) in *mof* heterozygotes, Chk2 is activated leading to centrosomal inactivation and loss of damaged nuclei from the cortex of the syncytial embryos. Furthermore removal of one copy of Chk2 in the *mof* mutant background considerably reduced the number of fall out nuclei in the syncytial embryos indicating the restoration of genomic stability. Hence MOF seems to play a crucial role in ensuring genomic stability during early embryogenesis both in mammals and *Drosophila.*

CHAPTER-5

RESULTS-3

5. *Drosophila* MOF regulates transcription of DIAP1 and controls apoptosis through JNK -dependent pathway.

5.1 Introduction

Programmed cell death or apoptosis, in all multicellular organisms, is necessary both during normal development and tissue homeostasis as well as to combat irreparable DNA damage (Jacobson MD et al., 1997). The phenomenon is accompanied by a series of cellular, morphological, biochemical and molecular events. The direct executioners of apoptosis, are the caspases or cysteine proteases, which act in a cascade, get activated and can cleave a number of key cellular substrates leading to cell death (Hengartner, 2000). Likewise in *Drosophila*, apoptosis is both a natural developmental process (Lohmann I et al., 2002), as well as induced in response to genotoxic stress or malignancy (Brodsky et al., 2000).Majorly, apoptosis occurs during embryonic development in *Drosophila* (Stage 11), and then spreads to different tissues (Abrams JM et al., 1993).

Different signals trigger can trigger the regulators of *Drosophila* apoptosis, namely Hid, Rpr and Grim which serve as activators of caspases. However, Keeping caspases in check is vital for normal cell survival and the IAPs (Inhibitor of Apoptosis Proteins) are their direct negative regulators, binding to them and inhibiting their activity (Goyal L.et al., 2000). IAPs were discovered in baculoviruses where they share redundant function with the caspase inhibitor protein p35(Clem RJ et al., 1994).They are crucial in mediating cell's decision to proceed towards death and thus, need to be under strict regulation to ensure overall cellular stability in metazoans including *Drosophila*.

In *Drosophila*, RHG protein encoding genes Hid, Rpr, Grim, controlling embryonic apoptosis are located contiguously on the third chromosome. Indespensable for apoptosis in fly embryos, deletion of these genes abrogates embryonic apoptosis and causes developmental defects. (Grether M.E., 1995; White K., 1994; Chen P.,1996). Conversely, overexpression of any one of these leads to a reverse effect. The negative regulator of apoptosis, *Drosophila* Inhibitor of Apoptosis Protein1 (DIAP1), is encoded by the *thread* (*th*) locus. The major antagonists of DIAP1 are three pro-apoptotic proteins: Hid, Reaper and Grim. Further, various apoptosis inducing signals such as ionizing radiation and pathological stimuli can stimulate transcriptional activation of these proteins. Besides, an

additional level of post transcriptional regulation is imposed on Hid by the Ras-MAP kinase pathway where activated MAPK brings about an inhibitory phosphorylation on Hid (Bergmann et al., 1998). Each RHG protein can which bind to DIAP's BIR (baculoviral IAP repeat) domain via RHG motifs. This prevents DIAP's inhibition on the cellular caspases, Dronc (initiator caspase-9 ortholog or *Drosophila* NEDD-2 like caspase) and Drice (*Drosophila* interleukin converting enzyme, an effector caspase), leading to apoptosis (Wang S., 1999; Goyal L et al, 2000). In contrast to DIAP1, DIAP2 is reported to be dispensable for apoptosis and functions in innate immune response to Gram Negative bacterial infection (Huh et al., 2007).While evidence about the control of DIAP1 and apoptosis at the level of proteins is ample, not much light had been shed earlier on the regulation of transcription at the *th (thread)* locus and what factors might be involved therein.

Development of tissues and organs in any multicellular organism relies on production of large number of cells before it is destined for its fate. After cell fate determination, excess cells are eliminated by the process of cell death, mainly through the evolutionarily conserved process: apoptosis .In our earlier studies we have shown that *Drosophila* MOF, a member of MYST histone acetyltransferase and an essential component of male X hyperactivation, plays a distinct role in the DNA damage pathway. Our results had shown that reduced levels of MOF resulted in genomic instability leading to lethality in case of complete depletion. Therefore we extended our study to see the interaction of MOF with cell death mediators in the apoptotic signal transduction pathway.

We chose the developing larval eye discs and adult eyes of *Drosophila* as the ideal *in vivo* tissue system to genetically study the interactions of MOF with cell death mediators in the apoptotic signal transduction pathways. This is a region where cell death occurs naturally in the context of developing the adult eye structure. Coupling both extracellular signals and intercellular interactions, cell survival and death here is tightly regulated to generate the correct population of cells committed to eye organogenesis (Baker N.E et al., 2001). Besides, both the larval eye discs and adult eyes do not affect cell viability or fertility, and are hence optimal tissue systems to study functions of genes involved in crucial cellular processes.

The ‘guardian of the genome’- p53, an important proapoptotic factor, is structurally conserved in both mammals and flies (Sogame et al., 2003). While it functions in stress induced apoptosis in both (Fan Y et al., 2000), in mammals it also induces cell cycle arrest and senescence (Zilfou JT et al., 2009).In response to ionizing radiation induced DNA damage, dp53 triggers apoptosis via transcription of *rpr* (Brodsky MH et al., 2000). Also ectopic overexpression of p53 in the eye leads to apoptosis (Fan Y et al., 2000). Present evidences point to dp53 as a player upstream in the apoptotic cascade.

The apoptotic machinery in *Drosophila* is regulated through three evolutionary conserved mitogen-activated protein kinase (MAPK) signalling pathways- the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 pathways (Schaeffer HJ et al., 1999). Presence of stress activates cell surface receptors, which transduce these signals to the nucleus via sequential phosphorylation of a series of kinases, headed terminally by MAPKs: ERK, JNK and p38.Activation of the JNK cascade, well established in mammalian systems, is crucial to several biological processes like proliferation, differentiation and morphogenesis, regeneration, and positively regulates apoptosis (Davis RJ 2000; Weston CR et al., 2002). Genetic and biochemical evidences indicate interaction between proapoptotic proteins Hid, Rpr, Grim and the JNK pathway. Apoptosis in eye discs induced by ectopic induction of Rpr can be partially rescued by dosage of *bsk* (*basket*), the *Drosophila* JNK protein. Also, Rpr mediated degradation of DIAP1 inhibits degradation of DTRAF1, an upstream JNKKK and triggers JNK mediated apoptosis (Kuranaga E et al., 2002).

There are reports about of a caspase-independent apoptosis pathway, possibly operating downstream of JNK pathway (Igaki T et al., 2002). Recent studies indicate a *dp53*/JNK dependent feedback amplification loop for stress-induced apoptosis (Shlevkov E et al., 2012). Hence, it was interesting to see where MOF is placed in this intricate apoptotic network and how it interacts with the cell death machinery to promote genomic integrity.

5.2 *Mof* mutant discs exhibit reduced size and elevated levels of apoptosis.

Dosage compensation, a regulatory process that ensures that equal amount of X chromosome gene products are present among both both sexes is maintained by 2 fold transcription of X linked gene in male *Drosophila*. MOF, a putative histone acetyl transferase plays an important role in specific histone acetylation during the process. The wild type gene product MOF is needed for male but not female viability (Hilfiker et al., 1997).The mof^1 mutant allele contains a Glycine replaced by a Glutamic Acid at position 691, a change that abolishes its catalytic HAT activity. Both male and female mof^1 larvae, (hemizygous males and homozygous females) do not survive past the 3rd instar stage. Our first clue pointing towards the involvement of MOF in cell survival came from observing imaginal discs from mof^1 mutant larvae. This suggested tolook for a role of MOF, beyond dosage compensation and a possible involvement in general transcriptional enhancement.

Larval eye imaginal discs from mutant and control 3rd instar larve were dissected out in *Drosophila* Ringers solution and observed under microscope at 10X and 20 X magnification (Olympus SZX16). Discs from both the sexes were compared to see whether there is any difference among males and females. We compared imaginal disc sizes of such dying larvae (mof^1/*FM7ActGFP*) and found them considerably reduced (about 4 folds) compared to wild type controls. Acridine orange staining, a commonly used marker for apoptotic cell death in *Drosophila* (Abrams et al., 1993, Bonini, 2000), was performed on live discs. Extremely high levels of spontaneous apoptosis were seen in cells of mof^1 males and females.

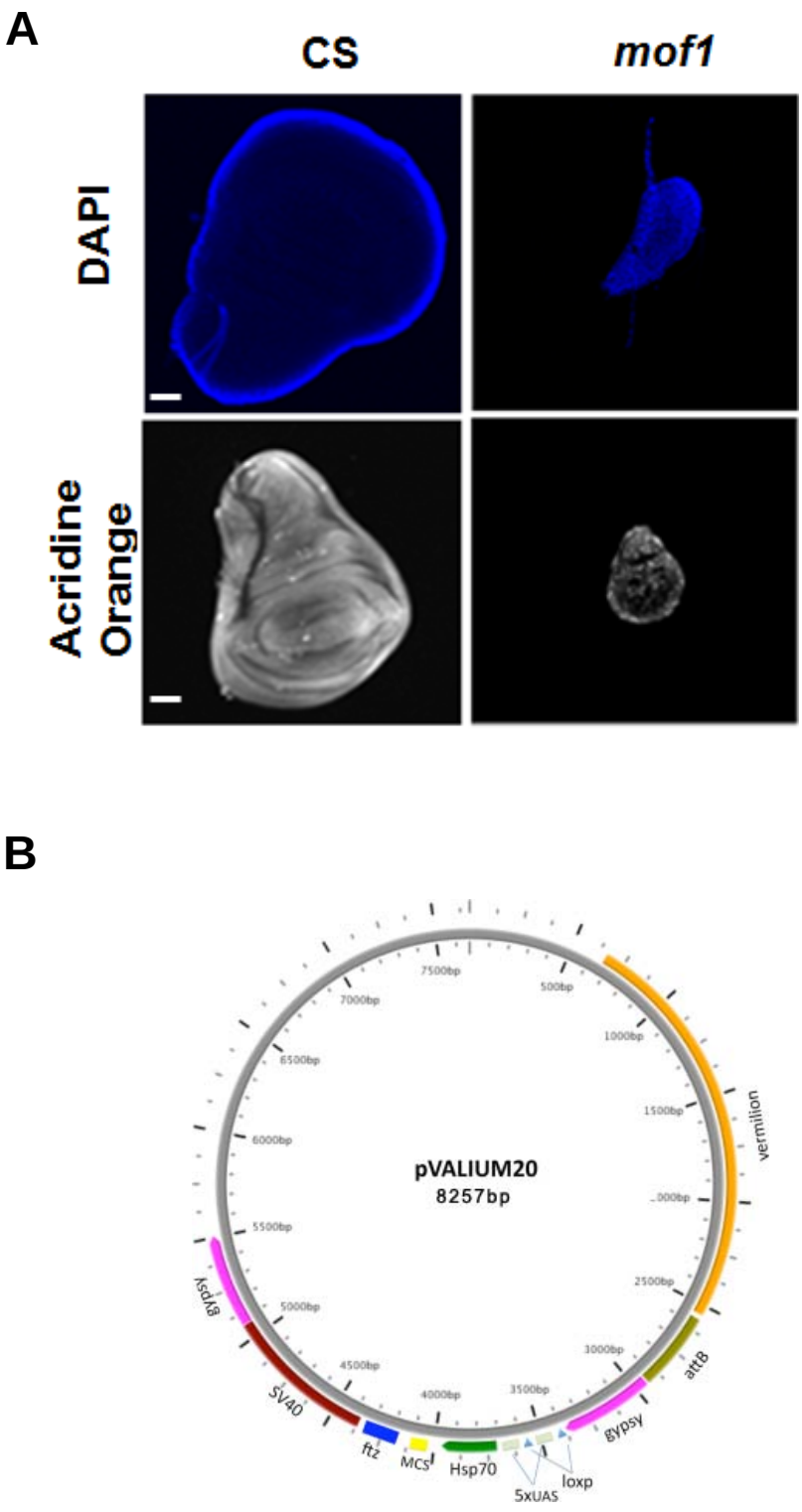
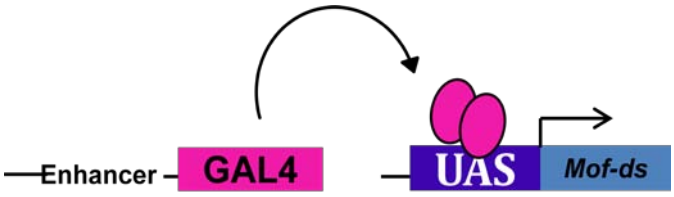


Fig. 5.1 A. Size differences between imaginal discs of wild type and *mof*^{1} larvae**.** Scale bar represents 50µm**. B.** Construct contained within transgenic fly stock UAS-RNAi-*mof.*

A



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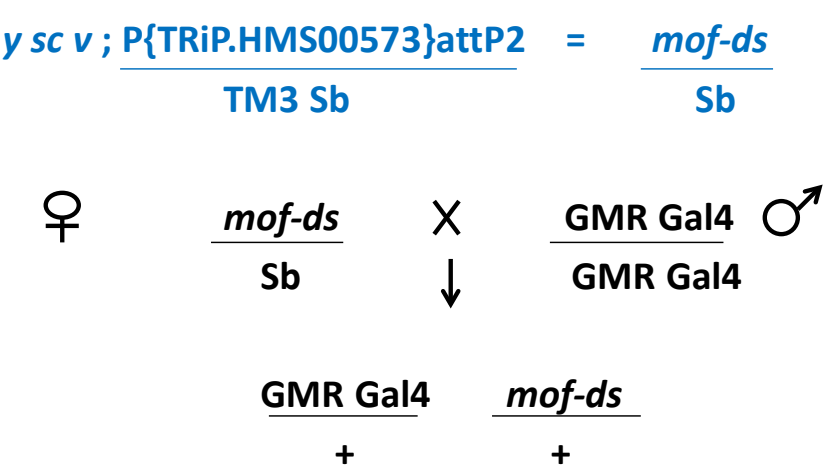


Fig. 5.2 A. Schematic representation of UAS-Gal4 tool in *Drosophila*, commonly used for tissue specific expression of genes. **B.** Scheme of genetic cross used to generate eye specific expression of RNAi- *mof*.

5.3 MOF depletion through RNAi induces apoptosis

Changes or modifications of genetic materials in the germ line of the parents in *Drosophila* leads to changes in the segregation and expression of the chromosomes. *Drosophila* MOF has shown to play regulatory role in transcription and chromatin modification. Total loss of function mutation mof^{1} resulted in lethality. In order to study its role in apoptosis further and due to the observed lethality, we continued our study with *Drosophila melanogaster* RNAi transgenes that enable the conditional inactivation of gene function in tissue specific manner expressed using the GAL4/UAS system .We carried out the further experiments with transgenic RNAi stock for *mof,* expressing a dsRNA of *mof* for undergoing RNAi under UAS control (mof^{ds}). Generated at the Transgenic RNAi Project (TRiP) at Harvard Medical School (Ni et al., 2010), it was obtained from Bloomington Stock Centre.(BDSC Stock no.33698; TRiP # HMS00573). By crossing the stock with the GMR GAL4 line (Glass multiple reporter, GMR), the expression of mof^{ds} was driven to the posterior region of the morphogenetic furrow in developing larval eye discs. Discs from wild type stock Canton S and GMR GAL4 served as control. Simultaneously, discs were also stained with acridine orange to see the level of apoptosis from transgenic lines with overexpression of mof^{ds}.

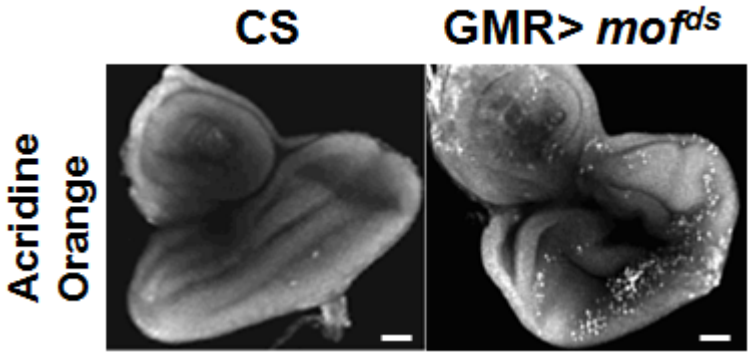


Fig .5.3 Acridine orange stained eye imaginal discs from developing 3rd instar larvae. GMR driven mof^{ds} line shows increased levels of apoptosis in region posterior to morphogenetic furrow compared to wild type control (CS). Scale bars represent 50µm.

Discs from overexpressed *mof*^{ds} exhibited high levels of apoptosis when stained with acridine orange as compared to discs from Canton S and GMR Gal 4 stocks. Introducing a [*mof* $^{t6.8}$]18H1 transgene in this background resulted in a total recovery of cell death.

Inexplicably, not much difference was noticed in the number of apoptotic cells between discs bearing a single and double copy of UAS–*mof*^{ds} transgene. When progeny of the cross between GMR-Gal4 and UAS-*mof*^{ds} were allowed to develop into adults, all of them showed normal eyes. We thus hypothesised that this apoptotic phenomenon might be developmental stage specific.

5.4 Modifiers of *mof*^{ds} induced apoptotic phenotype

To study whether caspases play a role in the *mof*^{ds} mediated apoptosis, we co-expressed the baculovirus caspase-3 inhibitor, p35 in larval eye discs. Baculovirus p35 protein functions to prevent apoptosis. It blocks the action of various kinds of caspases to prevent cell death. Here we have expressed p35 in the developing eye of *Drosophila* by crossing GMR Gal4 driven *mof*^{ds} flies to transgenic flies UAS-p35/UAS-p35 (homozygous for UAS-p35 transgene on chromosome 3) and collecting the F1 larvae (GMR/+; *mof*^{ds}/UAS-p35) for the cell death study.

p35 completely rescued the apoptotic phenotype by eliminating cell death, indicating that the process is caspase dependent. *Drosophila* Dronc is a key caspase mediating programmed cell death (Quinn, 2000). Ectopic expression of Dronc in the adult eye causes apoptosis and results in the ablated eye phenotype. It can be suppressed by coexpressing baculovirus caspase inhibitor p35. Dronc interacts genetically and biochemically with CED-4/Apaf-1 fly homolog Ark that plays an important role in Dronc processing.

drICE is a *Drosophila melanogaster* cysteine protease and functions as an effector caspase for programmed cell death in all stages of development. Acting downstream of Rpr, it cleaves baculovirus p35 and when overexpressed increases apoptosis. We therefore, co-overexpressed both *drICE*^{ds} and *dronc*^{ds} to see its involvement in MOF-depletion mediated cell death. For this, we separately crossed GMR driven *mof*^{ds} flies to transgenic flies to TRiP stocks for *drICE*^{ds} and *dronc*^{ds} (each TRiP stock is homozygous for transgene

UAS-RNAi-*dronc* or UAS-RNAi- *drICE*^{ds} contained on chromosome 3). All F1 larvae from these crosses were of our desired genotype (GMR/+; *mof*^{ds}/*dronc*^{d} or GMR/+; *mof*^{ds}/*drice*^{ds}). Co-overexpressed *drice*^{ds} and *dronc*^{ds} substantially reduced apoptosis, confirming further the involvement of apical (Dronc) and effector (DrICE) caspases in MOF-depletion mediated cell death.

Mof^{ds} induced cell killing can also be significantly rescued by overexpression of *Ark*^{ds}. Ark (Apaf-1 related killer) is the *Drosophila* homolog of mammalian Apaf-1 and physically interacts with the apical caspases Dronc (*Drosophila* caspase-9 homolog) and Dredd (death-related ced-3/Nedd2-like protein) (Rodriguez A., 1999; Quinn LM, 2000). Coexpression of UAS-dp53-RNAi did not affect the levels of apoptotic staining, indicating it might act upstream or independent of *mof*- mediated pathway of apoptosis. The *th4* allele of *diap1* is a loss-of-function allele with a point mutation in the second repeat of the BIR domain in DIAP1 protein (Lisi et al., 2000).

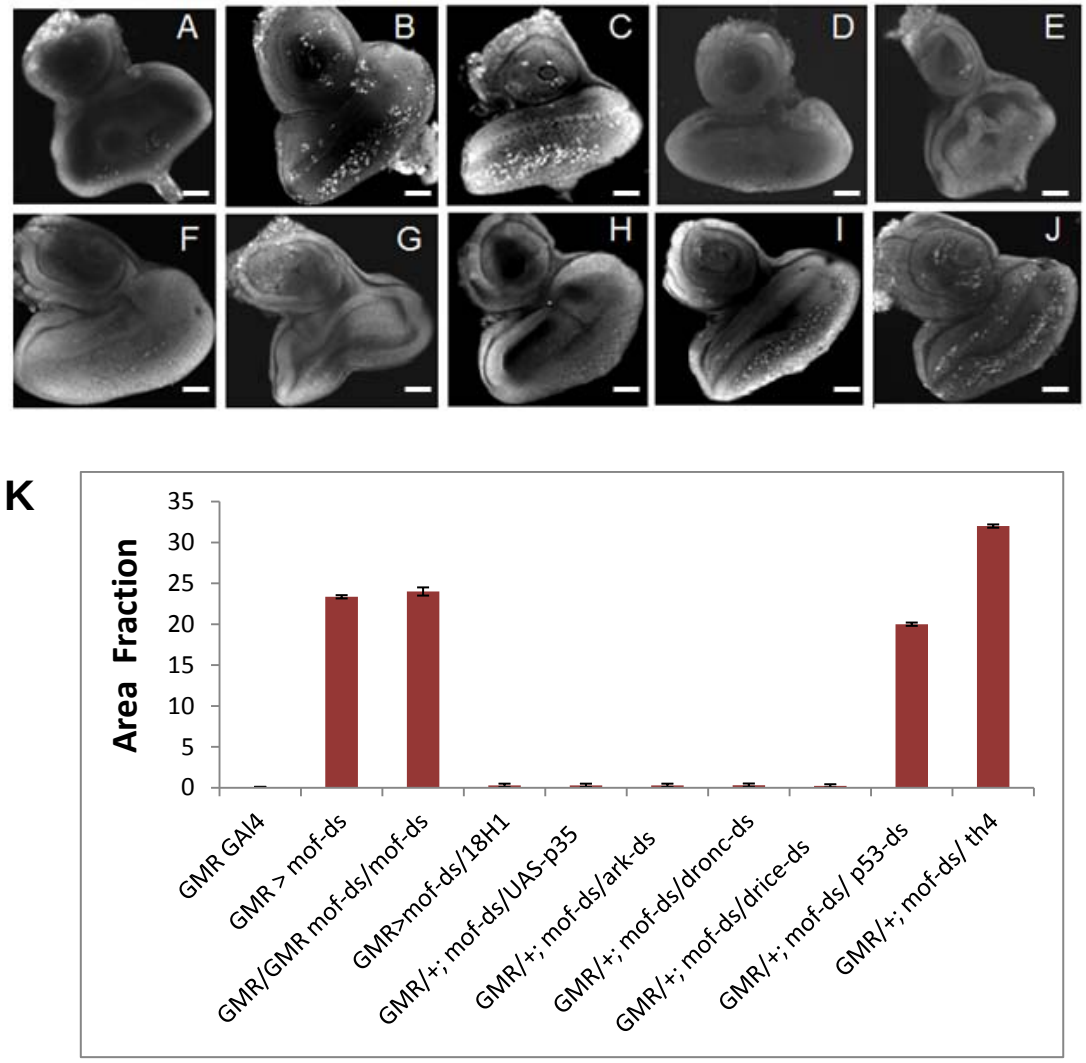


Fig. 5.4 Confocal images showing acridine orange staining in eye discs of **A.** GMR Gal4 **B.** GMR> *mof*^{ds} **C.** GMR/GMR; *mof*^{ds}/*mof*^{ds} **D**.GMR/+; *mof*^{ds}/ 18H1 **E.** GMR/+; *mof*^{ds}/UAS-p35 **F.**GMR/+; *mof*^{ds}/*ark*^{ds} **G.** GMR/+; *mof*^{ds}/*dronc*^{ds} **H.** GMR/+; *mof*^{ds}/*drice*^{ds} **I.** GMR/+; *mof*^{ds}/ *p53*^{ds} **J.** GMR/+; *mof*^{ds}/ *th4* .Scale bar represents 50µm. All images were taken with 10X objective (zoom= 1.8) on a Olympus confocal microscope (FV1000). **K.** Quantification of apoptotic cells. For each genotype 20 discs were analyzed from two independent experiments. Each bar for a genotype represents the mean reading of the fractional fluorescent area over a constant Region of interest (ROI) obtained with ImageJ (Mean ± SEM).

5.5. Caspase Activation in MOF-mediated apoptosis

We immunostained eye discs from GMR> *mof*^{ds} larvae with the cleaved caspase-3 antibody, that recognizes multiple epitopes- Dronc/Dronc-dependent caspases and DrICE, and is used for detecting apoptosis in *Drosophila*. Caspase overexpression was detected in the GMR region of the discs, confirming that *mof*^{ds} mediated apoptosis is caspase dependent. Discs from GMR-*hid* that display ectopic apoptosis in eye discs were taken as positive control.

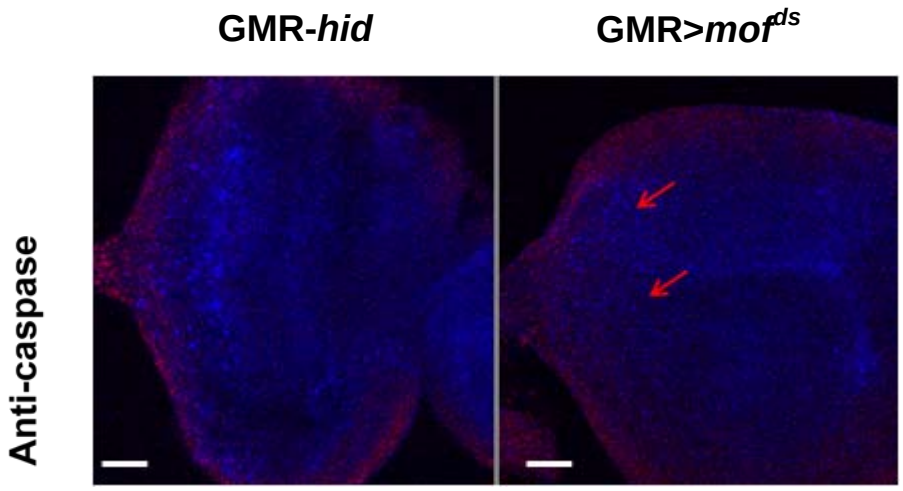


Fig. 5.5 Immunostained eye discs from GMR-*hid* (positive control) and GMR>*mof*^{ds} eye discs with anti-caspase3 antibody. Considerable caspase overexpression was observed in the experimental sample. Arrows depict fluorescent apoptotic cells. Images taken with a 60X objective (zoom=1) on a Olympus confocal microscope (FV1000). Scale bar represents 100 µm.

5.6 Genetic Interaction between MOF and DIAP1

To investigate whether MOF interacts at the transcriptional and protein level with any of the crucial components of the apoptotic pathway, notably the proapoptotic RHG proteins –Hid, Reaper and Grim, or DIAP1 (*Drosophila* Inhibitor of Apoptotis Protein1), we performed RT PCR and Western blot studies for both control and *mof* mutant stocks at the larval level. While the transcript levels of the RHG proteins from the mutant larvae did not change, those of DIAP1 were drastically reduced. Surprisingly, *dronc*, (*Drosophila* caspase-9 homolog), which is regulated directly by DIAP1, did not show any rise in mRNA levels in mutants. Similar observation was obtained with *drice* transcripts posing a probability that caspase activation immediately precedes cell death. Similar to the mRNA levels, DIAP1 protein also showed a drastic decrease in mof^{1} mutant larvae.

We further overexpressed MOF in the larval eye discs through GMR Gal4 driven enhancer trap line, EP-*mof* (Bloomington #26610).This transgenic line contains UAS binding sites for GAL4 inserted in a P-element construct immediately upstream of endogenous *mof* transcription unit. Immunostaining of these discs with anti-DIAP1 antibody showed a strip of labelled cells in the region posterior to the morphogenetic furrow (characteristic of GMR). The pattern of DIAP1 staining correlates with earlier reports (Ryoo et al., 2002).This was confirmed by LacZ staining in discs of a GMR Gal4 driven UAS-*diap1-LacZ* stock that showed a similar expression pattern of *diap1*. The results confirmed that *mof* and *diap1*interact genetically.

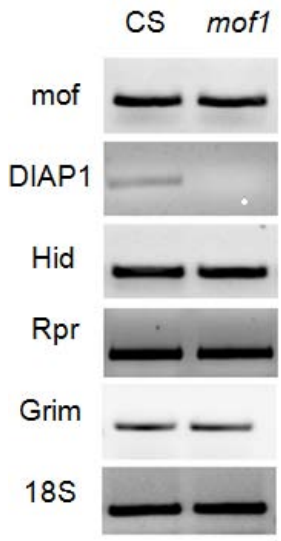


Fig. 5.6 RT-PCR experiments in wild type and *mof*^{1} larval extracts. mRNA levels of *diap1* drastically fall in *mof*^{1} mutants.

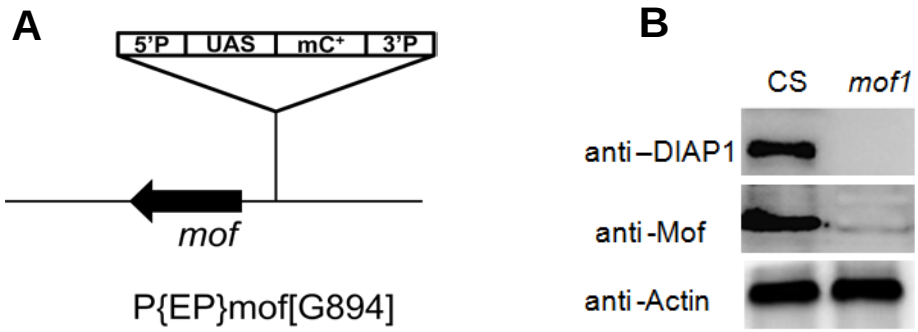


Fig. 5.7 A. Schematic view of the transgene construct in EP-*mof* fly stock. A P-element with mini-white marker and UAS sequences for Gal4 binding, is inserted proximate to the transcription unit of endogenous *mof*. **B.** Western analysis on total protein from wild type and *mof*^{1} larvae.

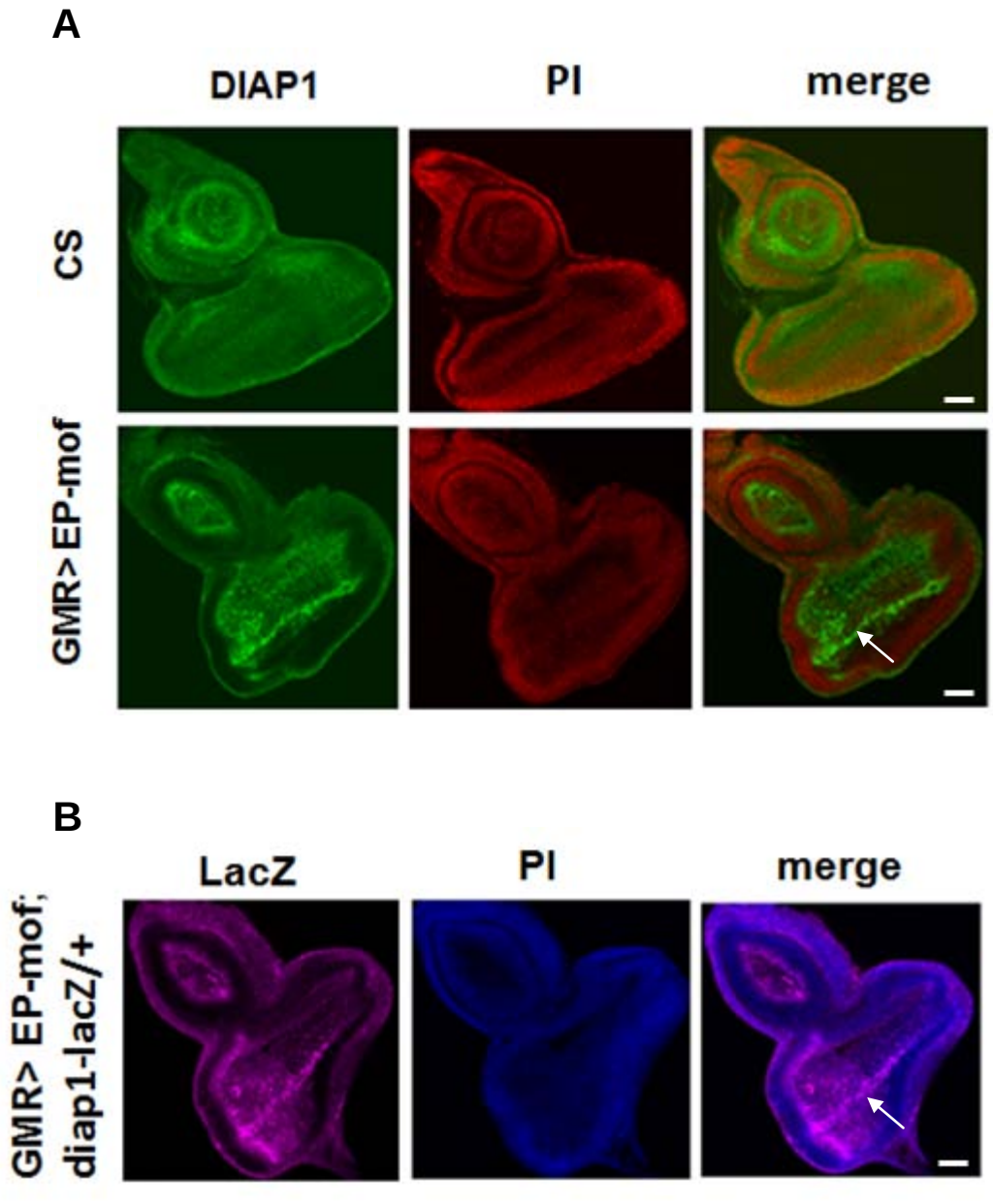


Fig. 5.8 Immunostaining of eye discs of larvae having overexpression of MOF. **A.** Immunostained GMR>EP-*mof* discs with anti- DIAP1 antibody. Red color indicates propidium iodide staining, green indicates anti DIAP1 stain and merge is the superimposition of PI stained eye discs with the green to show the exact location of the expression of DIAP1. **B**. LacZ staining of GMR>EP-*mof*; *diap1-lacz*. Arrows show cells with DIAP1 overexpression. Error bars represent 50µm.

5.7 MOF regulates *diap1* transcription by binding to its promoter

We next questioned the mode of regulation of DIAP1 by MOF. Till date, transcriptional control of DIAP1 gene expression has not been explored except reports of positive regulator STAT92E binding to sites in the *diap1*promoter, at a distance of 3.3kb upstream from the transcription start site (TSS) (Betz et al., 2008).We downloaded around 4.5 kb of sequence data, directly upstream of the +1TSS, from the publicly accessible database at NCBI (National Center for Biotechnology Information). Using the Berkeley Genome Project Promoter Prediction Tool, we predicted around 8 possible promoter stretches, lying between the +1TSS and the STAT site-bounded 1.2 kb region. PCR primers of 100-250bp amplicons spanning the stretch were designed for ChIP (Chromatin Immunoprecipitation) assay.

ChIP assay with antibody against MOF on cross-linked and sonicated chromatin isolated from wild type CS third instar larvae, revealed strong enrichment of MOF at all the amplicons. Similar levels of enrichment at all the stretches were obtained using anti-H4K16Ac antibodies in wild type larvae, confirming that such histone modifications are the result of MOF binding. ChIP-DNA pulled down was analyzed by both standard reverse transcriptase PCR as well as quantitative real-time PCR. In parallel, mof^{1} mutant larvae used as negative control did not show any binding at the DIAP1 promoter. These results clearly establish the role of MOF as a transcriptional activator of *diap1* gene.

A

Start	End	Score	Promoter Sequence
794	844	0.9	ATTAACATCGTTAAAAATATCGGTCCATAACAGCACTGCT**A**AGTGTTAAC
896	946	0.98	AAAAGAGTGCTAAATATACCGCGCATTGCCCTCCGATTCA**C**ATCGTCAAA
1251	1301	0.81	GGAGTGGAATGTAAAATGTGGGGCGATGTAAAAATGCCTT**T**ACATAAGTG
1268	1318	0.94	GTGGGGCGATGTAAAAATGCCTTTACATAAGTGCACTCGC**A**TTTGCAAGG
1367	1417	0.98	TCAGTGCGTGAAAAAATACGCGGTTTCCATTTTTGTTCTA**T**TTACTCGCG
1568	1618	0.98	ACAAAGTGTAAAAAAAACAAGCGGCTGCTAAAATGCCCGC**A**AGTGTTTTT
1584	1634	0.94	ACAAGCGGCTGCTAAAATGCCCGCAAGTGTTTTTAATTGA**T**ATGAAAATG
2049	2099	0.95	AGCATTTTCTATAAATAGTGCAAAATCAAAACCCAATTC**C**CATTGCAAA

B

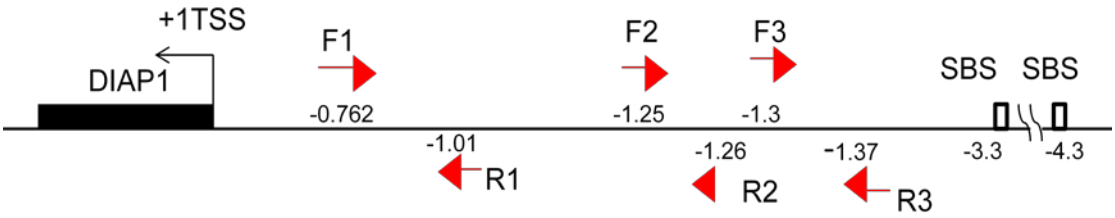
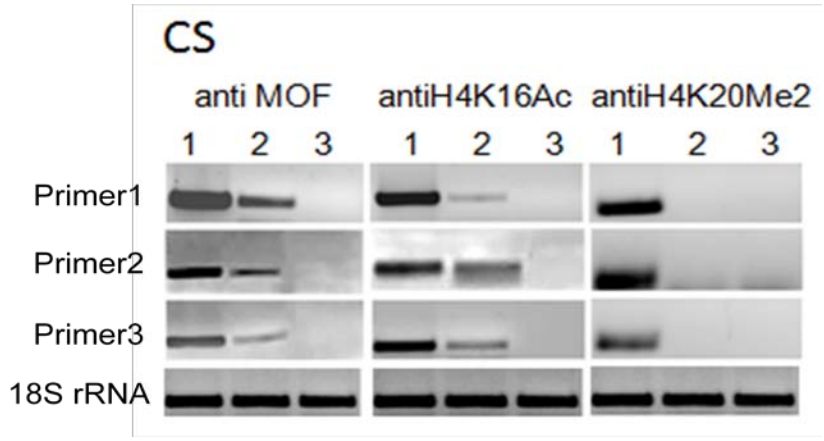


Fig. 5.9 Chromatin Immunoprecipitation (ChIP) assay designed to detect MOF binding at the *diap1* promoter. **A.** *diap1* promoter as predicted by BDGP promoter prediction tool (http://tools.genome.duke.edu/generegulation/McPromoter/). **B.** Primer positions on the DIAP1 promoter, upstream of the +1 transcription start site (TSS). F=forward primer, R=reverse primer. The previously reported STAT binding sites (SBS) lie further upstream.

A



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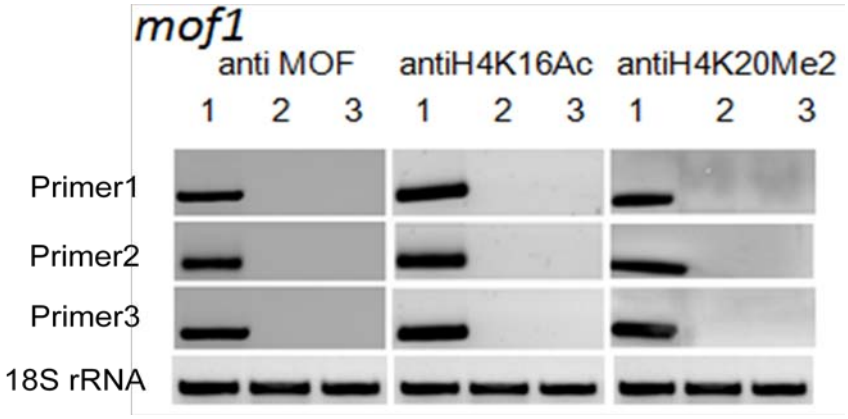

Fig. 5.10 Reverse transcriptase PCR on ChIP DNA from CS and *mof*^{1} mutant larvae. Lane1- Input (5% of cross-linked chromatin), Lane2- ChIP DNA, Lane3- No Antibody control.

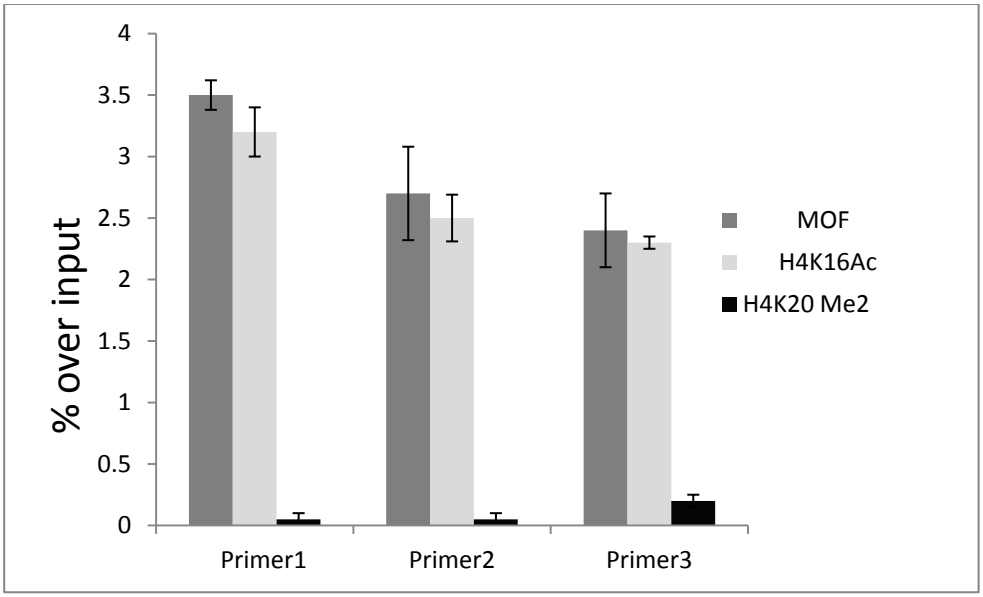

Fig. 5.11 Quantitative PCR of DNA immunoprecipitated from wild-type CS larvae. Antibodies used: MOF, antiH4K16Ac and antiH4K20Me2. Each bar represents the mean fold enrichment from two independent experiments (with error bars denoting + SEM). Enrichment was calculated as the fold difference (2^{-DDCt}) between DC_t of target sample (C_t value of ChIP sample normalized to input sample) and DCt of negative control (calibrator sample).No antibody control was chosen as the negative control since no deletion mutant of *mof* was available. P-value <0.05 (two-tailed Student's t-test).

5.8 Balance between anti-apoptotic MOF and proapoptotic Hid, Reaper and Grim decides apoptosis.

In *Drosophila*, the proapoptotic proteins-Reaper, Hid, Grim, and Sickle [also called IAP-binding motif (IBM) proteins] physically interact with the BIR domain of DIAP1; disrupting its binding to Dronc, releasing Dronc from DIAP1 and thereby triggering apoptosis (Goyal L et al., 2005; Kornbluth S et al., 2005; Chai J et ., 2003). In addition, Reaper also binds to DIAP1 and promotes its significant auto-ubiquitination and degradation (Ryoo et al., 2002). In comparision, Hid and Grim do not promote auto-ubiquitination of DIAP1 *in vitro*. Ectopic expression of Hid, Rpr and Grim in the adult eye through the eye specific Glass Multiple Reporter (GMR) driver results in ablated apoptotic eye phenotype (Grether et al., 1995; Hay et al., 1995). In larval eye imaginal discs, such apoptosis could be detected by live disc staining with acridine orange, a common dye that stains apoptotic cells. Several modifier alleles of this popular apoptotic phenotype have already been reported (Goyal L et al., 2005, Ryoo et al., 2002).

To further confirm the interaction of MOF with Hid, Rpr and Grim, we overexpressed MOF in a transgenic enhancer trap line P{EP}*mof* (Bloomington stock #26610) that contains UAS binding sites for GAL4 transcriptional regulator inserted proximal to the endogenous *mof* transcription unit. We were interested in analysing whether the apparent negative regulator of apoptosis-MOF was capable of reverting ablated eye phenotype produced by ectopic expression of Hid, Rpr or Grim in the adult eyes. Performing genetic crosses with this MOF transgenic line to an eye specific GAL4 driver stock (GMR-GAL4) allowed overexpression of MOF in the adult eye. The result was confirmed by performing Western blot analysis in comparison to control level.

We found that such MOF overexpression completely recovered the GMR-*rpr* induced apoptosis but only partially with GMR-*hid* and GMR-*grim*. This is explainable in support with the earlier findings that showed Rpr (and not Hid or Grim) promote the most significant DIAP1 degradation and apoptosis induction (Ryoo et al., 2002).

We used two modifier alleles that significantly suppressed mof^{ds} induced apoptosis in the eye discs. *Df(3L)H99* is a deletion mutant allele for the 75C1, C2 region of the 3rd chromosome (that contiguously encodes *hid, rpr, grim*) (White et al., 1994) and completely rescued cell death. *Hid109* is a loss of function allele. However partial

apoptosis still persists due to functional *rpr* and *grim*. These results confirm that *mof* acts antagonistically to the proapoptotic proteins in mediating cell death.

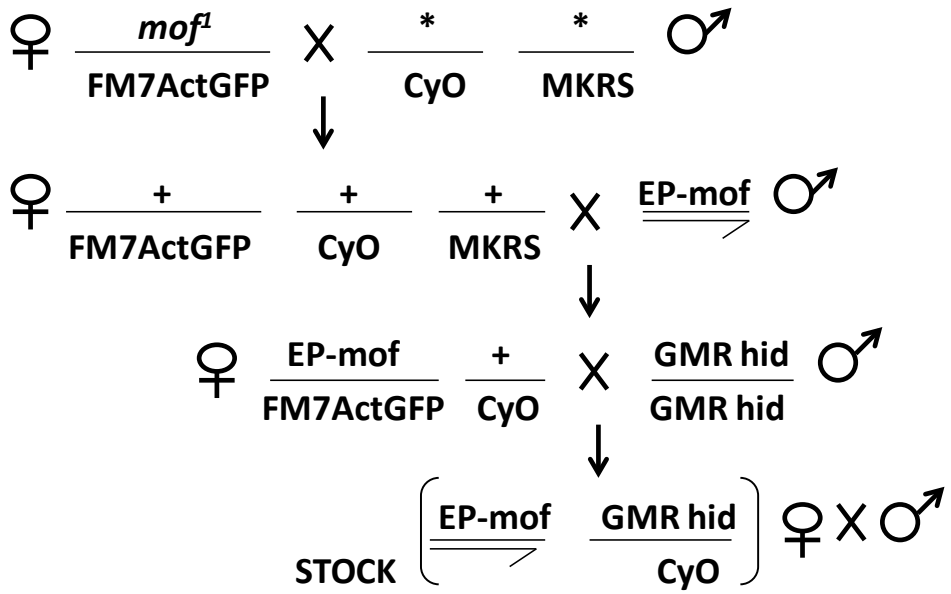

Fig. 5.12 Scheme of genetic cross to generate combination of EP-*mof* with GMR-*hid*. Identical schemes were used with GMR-*rpr* and GMR-*grim*. The derived stock was crossed to Gal4 driver and adult eye photographed under bright field microscope (zoom= 8) to look for cell death.

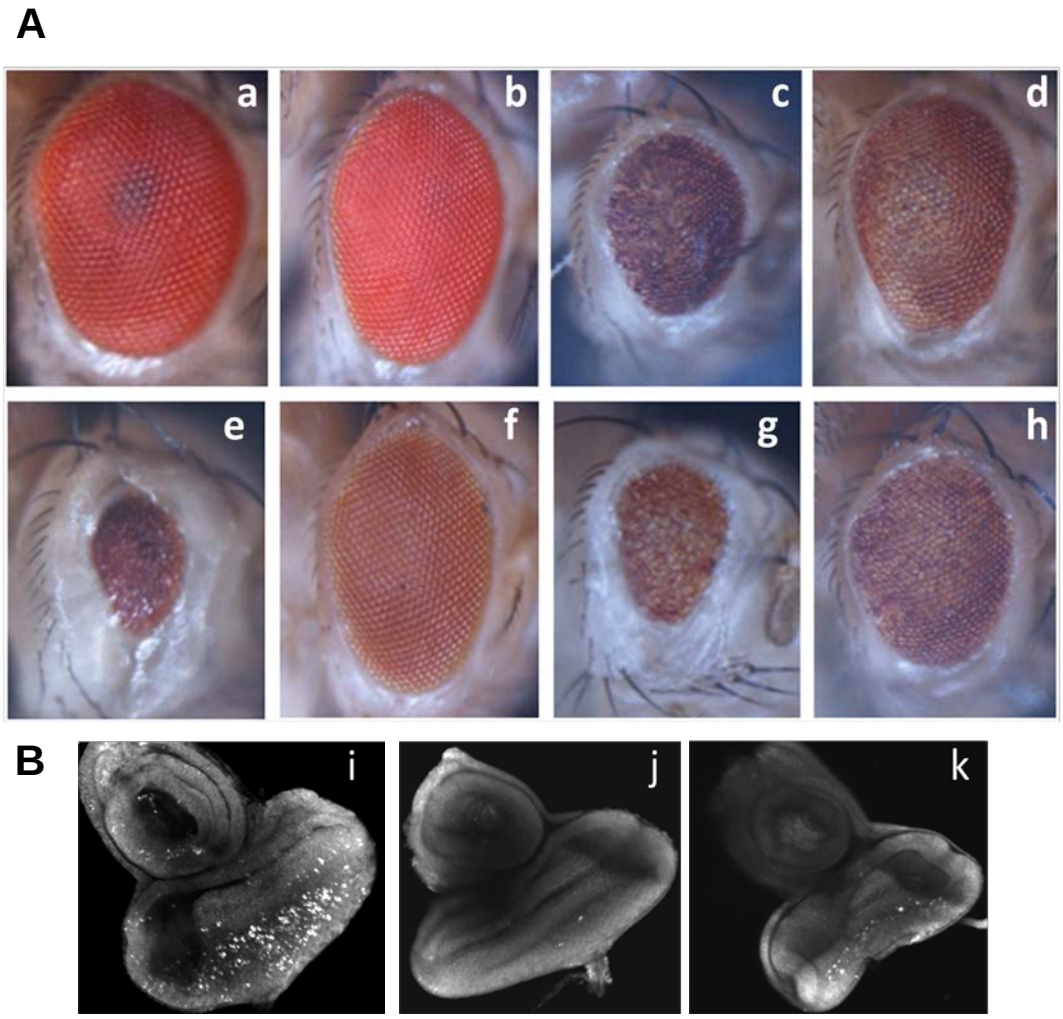

Fig. 5.13 Studies of genetic interactions. **A.** Light microscopic pictures of adult fly eyes of the following genotypes**. a.** CS control **b.** EP-*mof* **c.** GMR-*hid* **d.** EP-*mof*/+; GMR Gal4/+; GMR-hid/+ **e.** GMR-*rpr* **f.** EP-*mof*/+; GMR Gal4/GMR-*rpr* **g.** GMR-*grim* **h**. EP-*mof*/**+;** GMR Gal4/+; GMR-*grim*/+.**B.** Acridine orange staining of live eye discs from **i.** GMR Gal4/+; mof^{ds} **/+ j .**GMR Gal4/+; mof^{ds} /*H99* **k.** GMR Gal4/+; mof^{ds} /hid^{109}

5.9 Activation of JNK pathway is involved in MOF regulated apoptosis

To test whether the *Drosophila* JNK (DJNK) pathway is involved in *mof* induced cell death, we examined the alleles of JNK pathway to see their effect in modifying the apoptotic phenotype induced by GMR> mof^{ds}. We chose two mutant alleles for DJNK, *bsk* (basket). bsk^{2}*is* a point mutant allele at codon 316 *where* AAG (Lys) is replaced by TAG (stop). *UAS–DJNKDN* expresses a dominant-negative form of DJNK) and is catalytically inactive (Kuranaga E et al., 2003). Crossing both these alleles with GMR>mof^{ds} showed a great deal of suppression of apoptosis. *hemipterous*^{1} (hep^{1}) is a viable hypomorphic allele of *hep* (encoding DJNK kinase), having a P-element inserted about 179 bp upstream of its start codon. $Tak1^{ds}$ is an RNAi stock (TRiP stock no.33404) for dTAK1 (TGF-beta activated kinase1), an upstream JNKKK. This transgenic stock contains siRNA hairpin against Tak mRNA, under a UAS element. While hep^{1} rescued apoptosis partially, complete recovery was obtained with $Tak1^{ds}$.

Immunoblotting analyses on total protein extracts from both wild type and GMR> mof^{ds} eye discs using antibodies against showed an increase in the phosphorylated (active) JNK in the latter. These results imply that mof^{ds} mediated apoptosis occur through an activated JNK pathway.

In *Drosophila*, *puckered (puc)*, a MAPK phosphatase, is the only known antagonist of the JNK pathway. *puc* expression is a consequence of activated JNK cascade and serves to mediate a negative feedback JNK-regulatory loop (Martin-Blanco et al.,1998).We found that transcript levels of *puc* drop drastically in mof^{1} mutants. This confirms a heightened and uncontrolled JNK activation. Though our results doesn't comply with the negative feedback model, a possible explanation might be a negative regulation of *puc* expression by MOF.

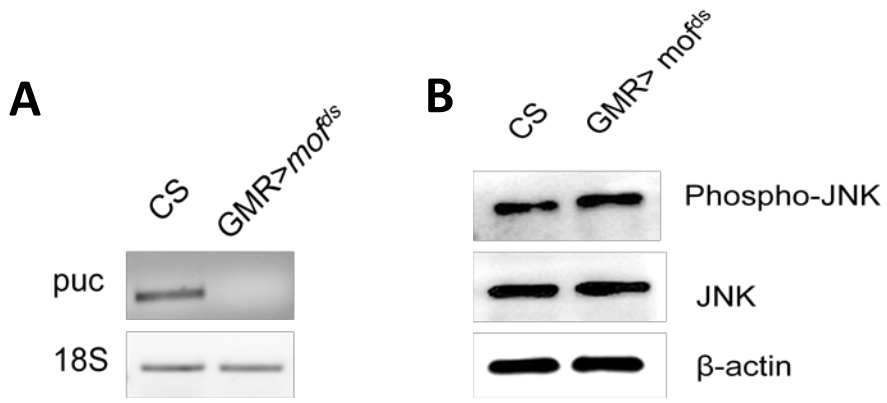

Fig. 5.14 A. *puc* transcript levels decrease sharply in GMR>*mof*^{ds}. **B.** DJNK undergoes phosphorylation during *mof*^{ds} mediated apoptosis in eye discs. This is indicative of JNK signalling active in MOF mediated apoptosis.

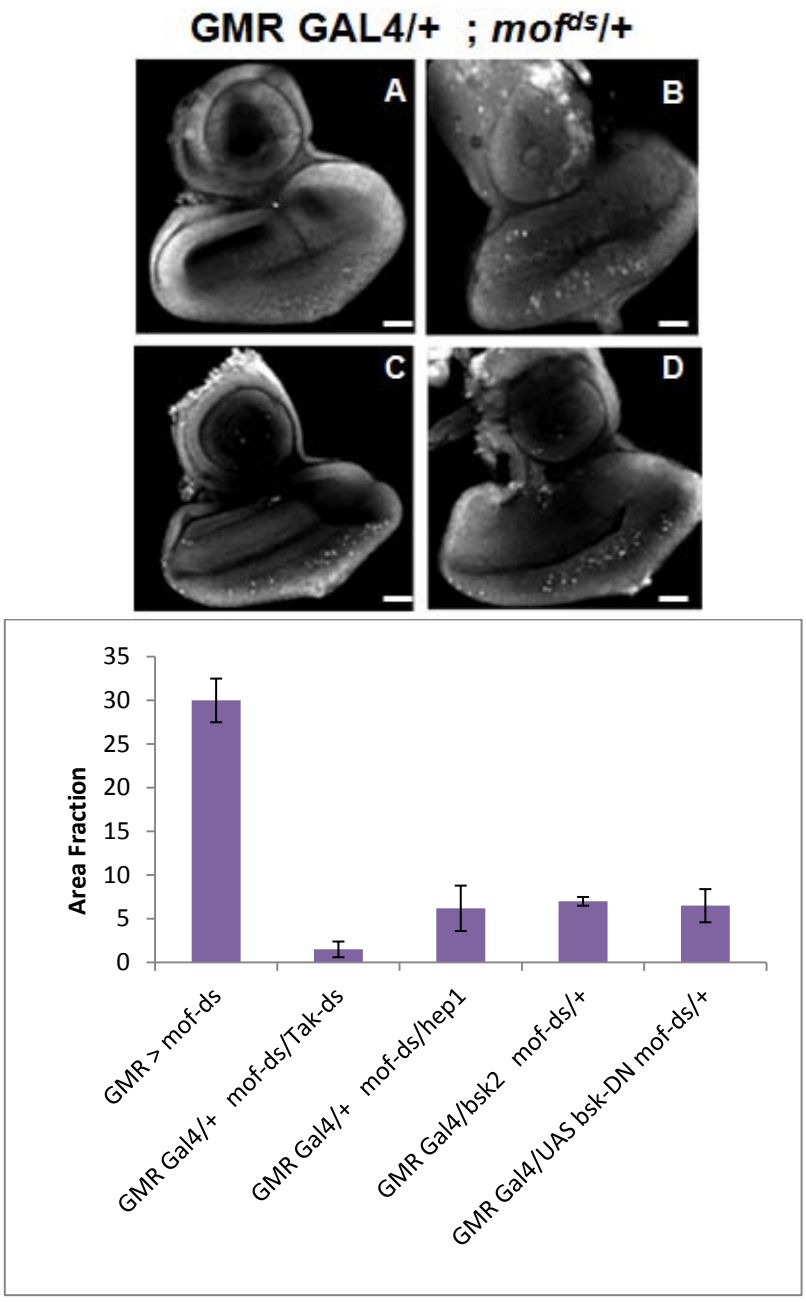

Fig. 5.15 Acridine orange phenotypes of GMR>*mof*^{ds} suppressed completely by **A.** *Tak1*^{ds} and partially by **B.** *hep1* **C.** *bsk*^{2} **D.** *UAS–DJNKDN*. The histogram below shows fold change in apoptotic cells. For each genotype 20 discs were analyzed from 2 independent experiments. Each bar for a genotype represents the mean reading of the fractional fluorescent area over a constant Region of interest (ROI) obtained with ImageJ (Mean ± SEM).

5.10 Proposed model for MOF regulated apoptosis in *Drosophila*

All our above results confirm the importance of MOF in the *Drosophila* cell death pathway. As a chromatin modifier, it promotes transcriptional activation of DIAP1 and acts to antagonize unnatural cell death. Functioning against apoptosis promoting proteins-Hid, Rpr, Grim, it thus helps maintain optimal cellular levels of DIAP1 and participates crucially in the cell's decision to undergo apoptosis. That apoptosis induced by MOF depletion occurs through an activated JNK pathway raises questions about the possibility MOF interacting with upstream regulators of JNK.

There are reports hinting the involvement of HDACs like Sir2 in mediating JNK dependent apoptosis in transgenic flies (Griswold et al., 2008). Ectopic overexpression of Sir2 in the adult eye triggers p53 independent, JNK mediated apoptosis (similar to mof^{ds} induced cell death). However, how Sir2 activates the JNK pathway is unknown. We hypothesize that probably MOF and Sir2 interact (genetically or protein-protein) such that such interaction sequesters MOF molecules from the cellular pool in a Sir2 overexpressed condition. This leaves little MOF molecules available to bind to DIAP1 promoter and activate transcription of this inhibitor. All of this might lead to uncontrolled apoptosis.

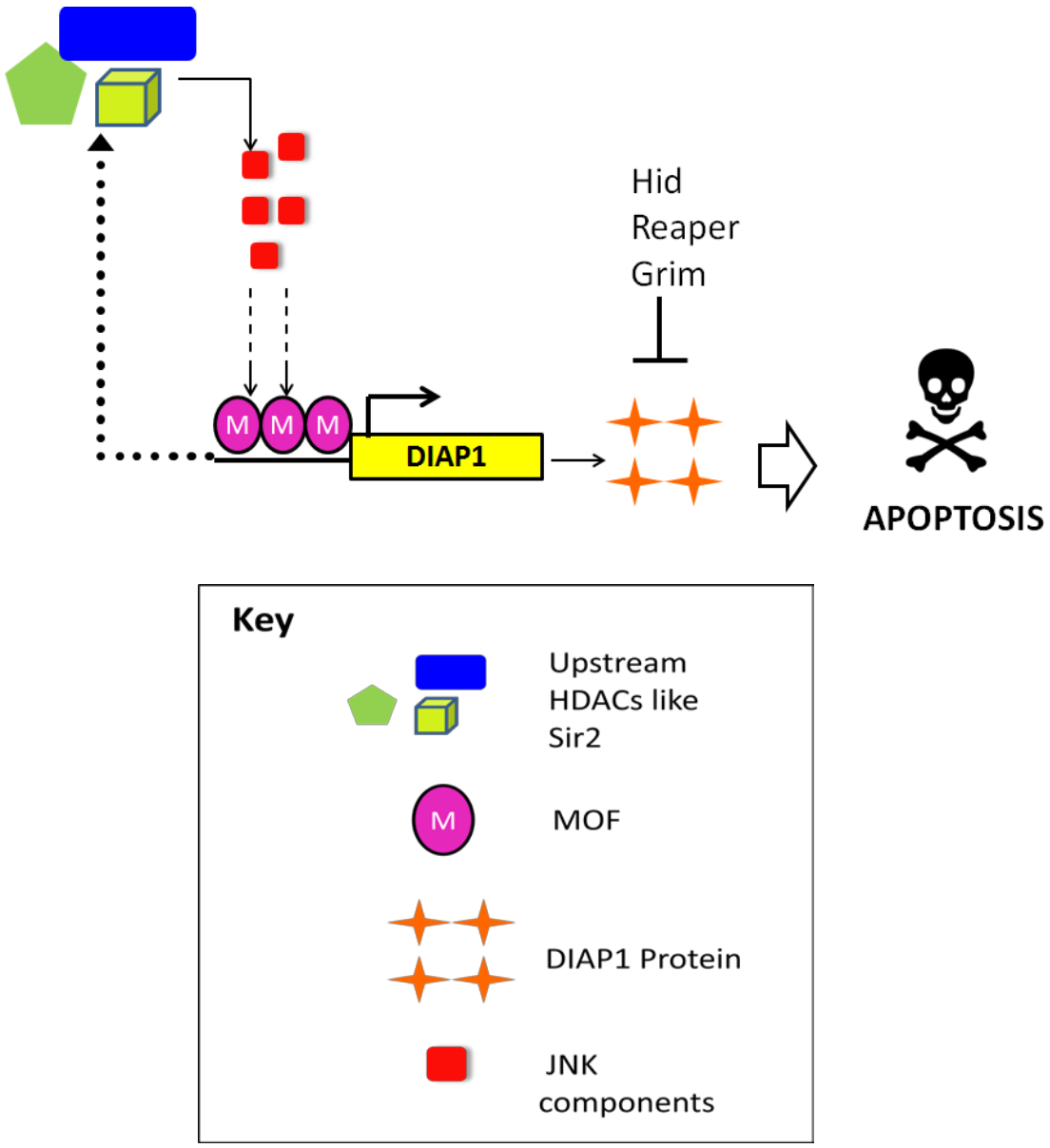

Fig. 5.16 Our proposed model for apoptosis in *Drosophila* with MOF mediating key roles in the pathway. Upstream HDACs might serve to regulate MOF and also trigger death inducing signals via JNK pathway. Regulated MOF binding to DIAP1 promoter serves to maintain cell specific threshold levels of DIAP1 against the antagonistic action of apoptosis promoting RHG proteins. The finally determined DIAP1 levels promote or block cell death.

5.11 Discussion

Cell-destroying caspases have to tightly controlled to prevent any unwanted calamity. Also, upon receiving death inducing signals a cell can only meet its destined death through caspase activity rising to a proper threshold. The onus for enabling this rests on the pro-apoptotic proteins and DIAP1. In fact, a certain interdependent regulatory loop exists between both these proteins, with RHG components ubiquitinating and destroying DIAP1 and also DIAP1 maintaining RHG stability to a certain extent (Bergmann A et al., 2003). Also, DIAP1 possesses the ability to bring about *in vitro* ubiquitination of the caspase Dronc (Wilson R et al., 2002). Almost ubiquitously, and throughout development, Hid, Grim and Reaper are the essential death–inducing genes (required for example, for the destruction of larval tissues), functioning through destruction of DIAP1. However no true antagonist against such role have yet been identified. We propose, through genetic and biochemical evidence, that MOF is one such likely candidate, which actually induces DIAP1 transcription and helps maintain a threshold level of DIAP1 in the cell, overproducing it upon obtaining appropriate signals. We believe, however that this may not the sole method of regulation of DIAP1 by MOF. It would be interesting, to further determine if MOF can inhibit DIAP1 by multiple mechanisms, such as inhibiting its ubiquitination.

The evolutionary conserved pathway of cell death is intrinsic and performed by an army of caspases, Apaf-1 and Bcl-2 members in most organisms (Vernooy et al., 2000, Shi et al., 2001). In *Drosophila* and mammals, unlike *C. elegans* the regulating signals for apoptosis are extrinsic called ‘social control of cell death’ (Raff, 1992). It has been reported that extracellular death-inducing signals might be transduced via JNK pathway in *Drosophila* (Bergmann et al., 2003).Our genetic assays point towards the involvement of Tak1, Hep and Bsk, crucial JNK components in MOF-induced apoptosis. Also, phosphorylation of JNK molecules in GMR> mof^{ds} tissue definitively points towards the involvement of JNK signalling in this apoptotic phenomenon.

Till date, few reports exist regarding the detailed involvement of chromatin modifiers in *Drosophila* apoptosis. Maintaining proper balance between acetylation and deacetylation of histones on chromatin through regulated activity of HATs and HDACs is crucial to vital cellular processes such as DNA damage repair, cell cycle progression and

prevention of cancer (Carrozza MJ et al., 2003; Gupta A et al., 2008, Sharma GG et al., 2010). Several evidences confirm that these antagonistic proteins function together, and often in complexes to decide higher-order chromatin organization. dMOF appears a promising candidate for regulating cell death at more than one implicated points. Recently, it was proposed that HDACs like SIRT1 could regulate autoacetylation of hMOF at K274 and thus directs the binding of hMOF to DNA binding sites (Lu L et al., 2011). Direct interactions have been confirmed between SIRT1 and human Tip60 and MOF (Yamagata K et al., 2009; Peng et al., 2012).We hypothesise that upon genotoxic stress or activating signals promoting upregulation of Sir2 like HDACs, dMOF molecules are rapidly deacetylated. This inhibits MOF from activating apoptotic inhibitor proteins and causes excessive apoptosis.

CONCLUSION

MOF, a MYST histone acetyltransferase, and an essential component of the *Drosophila* Dosage Compensation Complex (DCC), shares orthologs from yeasts to humans (Conrad T et al., 2012).Until recent times, scarce reports existed with regard to other possible functional roles of *Drosophila* MOF(dMOF) . Emerging studies in mammalian MOF now indicate the necessity of MOF in embryonic development and maintenance of normal epigenetic signature during early stages of development (Gupta A et al., 2008; Dixon TT et al., 2008). Its role in transcriptional regulation has also been implicated where MOF-mediated H4K16Ac promotes PolII progression from mammalian gene promoters (Zippo A et al., 2009). Interestingly, when present with protein partners in multi-subunit complexes, mammalian MOF can also acetylate non-histone substrates like Tip5 for promoter associated RNA-dependent gene silencing (Zhou, Y. *et al.* 2009).

Evidences regarding the involvement of MOF in DNA damage pathway to ensure genome stability has been steadily accumulating in recent years (Gupta, A. *et al,* 2005; Li, X. *et al,* 2010; Sharma, G.G. *et al,* 2010). A similar possibility in *Drosophila* had been totally unexplored, in contrast to its extensively studied role as a gene dosage compensating chromatin modifier. All of our observations obtained in studying the role of MOF in the *Drosophila* response to IR are consistent with those obtained in mammalian systems and therefore support the conclusion that the role of MOF in the DDR is conserved between mammals and *Drosophila*. Furthermore, we have shown for the first time that MOF is required for IR response in a whole animal.

Previous experiments have shown that depletion of MOF results in decreased levels of H4K16ac in both mammals and *Drosophila* with associated alterations in gene transcription but with minimum affect on transcription of DNA repair genes (Kind et al. 2008; Sharma et al. 2010). Thus, MOF appears to be involved in the DDR primarily through the effect of H4K16 acetylation on chromatin structure. Based on this conclusion, *Drosophila* is an appropriate screening model for the identification of MOF inhibitors as well as inhibitors of H4K16ac deacetylases relevant to the mechanism of DDR. Furthermore, while the dosage compensation role of MOF in *Drosophila* is not observed in mammals, where transcriptional balance is regulated by X chromosome inactivation, the role of MOF function in the DDR is conserved in *Drosophila* and mammals.

All MOF deficient mouse embryos fail to develop the expanded blastocyst stage and die at implantation *in vivo* (Kumar R et al., 2011). We have observed loss of maternal MOF in *Drosophila* embryos caused mitotic defects during early syncytial cycles and chromosomal aberrations, visualized by presence of chromatid bridges and lagging chromosomes. These defects occur spontaneously in *mof* heterozygyotes without stress and resemble the defects induced by X-ray irradiation or chemical treatment of wild type embryos (Callaini G et al., 1992). Our data has shown that endogenous DNA damage occurs during the process of development as shown by the presence of single and double stranded DNA breaks. Similar phenotypes like spontaneous mitotic defects and chromosomal aberrations in *Drosophila* were also observed in RecQ5 DNA helicase mutants that are involved in DNA replication and maintainance of genomic integrity (Nakayama M et al., 2009). MOF is an essential component of dosage compensation complex and homozygotes for the mutation do not survive beyond late larval stages. The nuclei in mof^{1} early embryos are large and fragmented resembling mitotic catastrophe. The typical mitotic wave in wild type embryos is disrupted due to the presence of abnormal nuclei in the *mof* heterozygotes. Such abnormal nuclei are PH3 negative and appear in clusters (Sakurai H et al., 2011). Cellularisation occurs during the interphase of 14th nuclear division. During this stage the actin filaments in mof^{1} embryos loose the typical honeycomb like structures often leading to empty cages without the chromosomes. The empty cages are indicative of the presence of abnormal nuclei that are PH3 negative and have been eliminated by nuclear fallout while the centrosomes are still retained in the cortex. Similar mitotic defects were also observed in the case of another *mof* allele (mof^{3}). The late larval lethality of *mof* homozygotes were rescued to 100% with the addition of *mof* transgene in the mutant genetic background while the mitotic defects in the *mof* heterozygote embryos were partially rescued.

Usually every organism tries to protect itself by preventing these abnormal nuclei from being incorporated into forming adult structures (Sullivan W et al., 1993). Mutations in PcG genes which are components of chromatin remodeling aiding in maintenance of transcriptional state during embryogenesis, also resulted in the formation of abnormal nuclei (Dor E et al., 2006). Also the severity of the fall out nuclei in the mof^{1} heterozygotes was high containing more than 5 fall-out nuclei per embryo and this was reverted back to

normal with a wild type *mof* transgene. The response to DNA damage and mitotic defects maintain genomic stability by blocking chromosome segregation and removing the abnormal nuclei by nuclear fallout mechanism. Staining with PH3 antibody and DNA dye in the syncytial embryos is a good system to detect irregular or damaged DNA in *Drosophi*la and also for studying maternal genes required for mitosis and genomic stability. The abnormal nuclei that stain negatively for PH3 are asynchronous and are seen during nuclear cycles 11–13. Following mitotic failure the defective nuclei drop into the interior of embryos and free centrosomes are seen in the cortex. In the wild type embryos all the nuclei remain in actively dividing phase compared to *mof* heterozygotes wherein nondividing nuclei are also present.

In a variety of systems, cell cycle checkpoint defects lead to progression into mitosis with damaged DNA or incompletely replicated DNA leading to "mitotic catastrophe", a process that is distinct from apoptosis (Roninson IB et al., 2001). Failure to achieve this inevitably commits the cell to programmed cell death or apoptosis. In syncytial *Drosophila* embryos damaged or incompletely replicated DNA triggers centrosome inactivation during mitosis leading to defects in spindle fibre assembly and chromosome segregation (Takada S et al., 2003). The hallmark of DNA damage response (DDR) involves the phosphorylation of histone variant H2AX that play an essential role in the recruitment and retention of downstream proteins involved in DNA repair. In addition to this γ–H2AX is also involved in the transduction and amplification of DDR from megabase domains surrounding the damage site (Polo SE et al., 2011). In our studies using *mof*^{1} heterozygotes we observed increase in single and double stranded DNA breaks and as well as H2Av phosphorylation revealing the occurrence of DNA damage event.

DNA damage leads to increased localization of Chk2 to centrosomes and spindle fibres and also Chk2 is the signal for mitotic catastrophe that disrupts centrosome function leading to elimination of the abnormal nuclei (Takada S et al., 2003). *Drosophila* Chk2 plays a vital role in response to stress. It was also reported that mutation of Mnk gene (*Drosophila* homolog of Chk2) prevents centrosome inactivation and suppresses defects associated with chromosome segregation in response to damaged or incompletely replicated DNA. In our study we observed increased level of Chk2 in the *mof* heterozygotes in response to the damaged nuclei causing centrosome inactivation resulting

in elimination of the damaged nuclei. The number of abnormal or damaged nuclei was reduced in the embryos of *mof*^{1}*/+; mnkp6/+* mothers indicating that the mutant allele *mnkp6* prevents inactivation of centrosomes and hence loss of nuclei from the cortex. Unlike the cell cycle delays that occur to repair the damaged DNA or incompletely replicated DNA, *Drosophila* embryonic system utilizes the delay to identify and discard those abnormal nuclei. When the DNA lesions enter mitosis, Chk2 is activated as a response and leads to centrosomal inactivation and delinks the chromosomes from their centrosomes which ultimately results in loss of the nuclei (Sakurai H et al, 2011). It has been proposed that Chk2 functions at two points during early embryogenesis in response to genotoxic stress. At the onset of mitosis DNA lesions leads to activation of Chk2 that target proteins involved in centrosomal spindle activity and in maintaining γTURC localisation. This causes failure in anaphase chromosome segregation. Once failure of mitotic division occurs, Chk2 causes centrosomal inactivation and disrupts the link between centrosomes and nuclei. Since centrosomes anchor nuclei to the cortex, Chk2 response to DNA damage results in loss of nuclei from the cortex (Raff JW et al., 1989).

Sensing of DNA lesions by DDR machinery occurs in a complex and heterogeneous chromatin environment (Misteli T et al., 2009; Shi L et al., 2012). Earlier reports also emphasized on the alteration in the chromatin structure that helps in the sensing and as well as spreading of the DNA damage response apart from double strand breaks (Bakkenist CJ et al., 2003; Bencokova Z et al 2009; Hunt CR et al., 2007). DNA damage induces the activation of chromatin bound Chk2 by a chromatin derived signal resulting in the dissociation of the activated Chk2 from the chromatin. Chk2 is phosphorylated at T68 by ataxia telangiectasiamutated (ATM) and transmits the DNA damage signals from the upstream phosphatidylinositol 3’-kinase like kinases to the effector substrates including p53, Braca1, Cdc25A and Cdc25C (Li J et al., 2005). In addition Chk2 has been reported to phosphorylate p53, thereby enhancing the transcriptional activity of p53 responsive genes (Takai H et al., 2002). Further the functional link between p53 and Chk2 during DNA damage occurs through the phosphorylation and acceleration of degradation of Hdmx, a negative regulator of p53 (Chen LD et al., 2005).

Not much light has yet been shed upon the direct role of chromatin modifiers in regulating *Drosophila* apoptosis. The chromatin remodelling complex BRM (comprising BRM, OSA and MOR- members of the *trithorax* family), the *Drosophila* analog of yeast SWI2/SNF2 complex, has been implicated to play a role, possibly through positively regulating DREF, over expression of which causes cell death in the adult eye (Hirose F et al., 2001). Molecular mechanism of such regulation has not been worked out yet. The prevailing model of *Drosophila* apoptosis emphasizes mostly on amplifying cell death signals through the RHG proteins,that in turn degrade the sole major ubiquitous inhibitor, DIAP1 in order to release caspases (Kornbluth S et al., 2005). Our study points towards an essential regulatory step in maintaining optimum DIAP1 concentrations within the cell. The presence of MOF as a transcriptional activator of DIAP1 is critical to maintaining the right levels of DIAP1, hence also regulating cell fate. Not surprisingly, upstream factors and stimuli affecting MOF levels would also contribute immensely to ultimately determine life or death of a cell. Future studies in these lines look promising to unravel a chromatin based mechanism in regulating DNA damage repair and apoptosis.

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