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Drosophila MOF regulates DIAP1 and induces apoptosis in a JNK dependent pathway

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Abstract Histone modulations have been implicated in various cellular and developmental processes where in *Drosophila Mof* is involved in acetylation of H4K16. Reduction in the size of larval imaginal discs is observed in the null mutants of *mof* with increased apoptosis. Deficiency involving *Hid, Reaper* and *Grim [H99]* alleviated mof^{RNAi} induced apoptosis in the eye discs. mof^{RNAi} induced apoptosis leads to activation of caspases which is suppressed by over expression of caspase inhibitors like *P35* and *Diap1* clearly depicting the role of caspases in programmed cell death. Also apoptosis induced by knockdown of *mof* is rescued by JNK mutants of *bsk* and *tak1* indicating the role of JNK in mof^{RNAi} induced apoptosis. The adult eye ablation phenotype produced by ectopic expression of *Hid, Rpr* and *Grim*, was restored by over expression of *Mof*. Accumulation of *Mof* at the *Diap1* promoter 800 bp upstream of the transcription start site in wild type larvae is significantly higher (up to twofolds) compared to mof^1 mutants. This enrichment coincides with modification of histone H4K16Ac indicating an induction of direct transcriptional up regulation of *Diap1* by Mof. Based on these results we propose that apoptosis triggered by mof^{RNAi} proceeds through a caspase-dependent and JNK mediated pathway.

Keywords Mof · H4K16Ac · Diap1 · Apoptosis · Diap1 promoter · Hid · Reaper · Grim

Introduction

Mof (males absent on the first) belongs to MYST family of proteins, characterized by highly conserved histone acetyl transferase domains, are involved in a wide range of physiological processes in mammals [1–3]. The MYST family of proteins are of particular interest as its members display diverse roles in various nuclear processes and some of them have been implicated in carcinogenesis [4]. *Mof* was originally described as one of the essential component of the X chromosome dosage compensation system in *Drosophila melanogaster.* Mutations in *mof* are lethal for male fruit flies [5]. Mof is one of the five mammalian MYST family histone acetyl transferases [6] and plays a vital role at multiple points in the cellular DNA damage response and double-strand break [DSB] repair pathways [7, 8]. In *Drosophila*, Mof is one of the members of the MSL (male sex lethal) complex which is involved in dosage compensation function [9–12]. Recent studies revealed that both *Drosophila* and mammalian Mof proteins reside in multi-protein complexes and there exists a high level of conservation among the interacting proteins. Both proteins exhibit histone acetyl transferase activity that is specific for histone H4 at K16 [5, 13–16]. Cells

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expressing the ΔhMof and cells with hMof knock down displayed about three fold higher frequencies of anaphase bridges compared to parental cells. In addition, decreased hMof activity was associated with loss of the cell cycle checkpoint response to DNA double-strand breaks [7]. Human Mof or TIP60 mediated acetylation of p53 at K120 determines whether the cell has to undergo apoptosis or cell cycle arrest [17]. Mutation of the *mof* gene in mice causes early embryonic lethality and embryos are unable to develop beyond expanded E4.5 blastocyst stage. Also cultures with $Mof^{-/-}$ homozygous mutant morphology die by apoptosis which is characterized by pyknotic nuclei indicating chromatin condensation. The appearance of aberrant chromatin morphology preceded activation of caspase 3 and DNA fragmentation [3]. Flies carrying *mof* mutation and as well as *mof* knockdown in SL-2 cells, undergo reduced post-irradiation survival. Depletion of Mof in SL-2 cells also resulted in an elevated frequency of metaphases with chromosomal aberrations, suggesting that the role of MOF in DNA damage response is conserved in *Drosophila* [18].

Programmed cell death or apoptosis in multi-cellular organisms is necessary both during normal development and tissue homeostasis and as well as to combat irreparable DNA damage [19]. 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 [20]. In mammals the caspase cascade can be initiated through two major branches, the intrinsic mitochondria pathway and external death receptor pathway [21, 22]. Apoptosis happens throughout fly development. For instance, during embryogenesis, most of midline glial cells are eliminated by apoptosis to achieve a well circuited central neural system [23, 24]. During metamorphosis, steroid hormone ecdysone triggers apoptosis and autophagy to remove the embryonic neural system, larval midgut, salivary glands and some other tissues that are no longer needed during adulthood [25, 26]. In addition, apoptosis occurs during the development of fly compound eye in larva and pupal stages. The fly retina provides a useful model to understand apoptotic events and to elucidate their physiological importance.

Different signals 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. Inhibitor of Apoptosis Proteins (IAPs) act as direct negative regulators of caspases by binding to them and inhibiting their activity [27]. IAPs were discovered in baculoviruses where they share redundant function with the caspase inhibitor protein p35 [28]. In *Drosophila*, RHG protein encoding genes *Hid*, *Rpr* and *Grim*, located contiguously on the third chromosome are indispensable for apoptosis in fly embryos. Deletion of these genes (RHG) abrogates embryonic apoptosis and cause developmental defects [29–31]. Conversely, over expression of any one of these proteins 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. 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 [32]. Every RHG protein binds 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 [27, 33]. In contrast to DIAP1, DIAP2 is reported to be dispensable for apoptosis and functions in innate immune response to Gram negative bacterial infection [34]. While evidence about the control of DIAP1 and apoptosis at the level of proteins is ample, not much light has been shed on the regulation of transcription at the *th* (*thread*) locus and on the factors involved therein. The 'guardian of the genome' p53, an important proapoptotic factor, is structurally conserved in both mammals and flies [35]. While it functions in stress induced apoptosis in both [36], in mammals it also induces cell cycle arrest and senescence [37]. In response to ionizing radiation induced DNA damage, dp53 triggers apoptosis via transcription of *rpr* [38]. Ectopic over expression of p53 in the *Drosophila* eye leads to apoptosis [36].

The apoptotic machinery in *Drosophila* is regulated through three evolutionarily 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 [39]. 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 is crucial to several biological processes like proliferation, differentiation and morphogenesis, regeneration, and positive regulation of apoptosis [40, 41]. Genetic and biochemical evidences indicate interaction between proapoptotic proteins Hid, Rpr, Grim and the JNK pathway. Apoptosis in eye discs of *Drosophila* induced by ectopic expression of Reaper can be partially rescued by increasing the dosage of Bsk (Basket), the *Drosophila* JNK protein. Also, Rpr mediated

degradation of DIAP1 inhibits degradation of DTRAF1, an upstream JNKKK (JNK kinase kinase) and triggers JNK mediated apoptosis [42].

Drosophila Mof plays a major role in maintaining genomic stability during early embryogenesis in addition to DNA damage response [43]. In the present study we have focussed on analysing the role of *dmof* in apoptotic process during development. Since homozygotes for *mof* mutation are late larval lethal, we have selected developing larval eye discs and adult eyes of *Drosophila* as the ideal in vivo tissue to study the interactions of Mof with cell death mediators in the apoptotic signal transduction pathways. Moreover, both larval eye discs and adult eyes do not affect cell viability or fertility and are hence optimal tissues to study functions of genes involved in crucial cellular processes. We found that homozygotes for *mof* mutation have abnormally small imaginal discs which we attribute to cell autonomous induction of apoptosis rather than cell proliferation. We also show that Mof knockdown in *Drosophila* eye discs causes phenotypes which resemble JNK gain of function including ectopic apoptosis.

Materials and methods

Drosophila culture and maintenance

mof¹ fly stock was a gift from Prof. John C. Lucchesi and *Canton S* was used as wild type strain. All crosses were maintained at 25 °C on standard culture media. 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 carried out using the UAS–GAL4 system [44]. The glass multiple reporter GMR-GAL4 line [45] was used to drive expression of UAS transgenes in the larval eye discs and adult eyes. 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).

Acridine orange staining

Eye discs from third instar larvae were dissected in PBS and stained with freshly diluted acridine orange solution (1 mg/ml) followed by wash in PBS. The stained discs were mounted in PBS, and viewed immediately under confocal microscope with the Rhodamine Red channel (Excitation wave length: 570 nm and emission wave length of 590 nm) using 10X objective.

Immunostaining of imaginal discs

Eye discs were dissected from well fed 3rd instar larvae, fixed for 20 min in 4 % paraformaldehyde in PBT and then washed in PBT for 10 min. The discs were treated with RNase A (10 mg/ml) at 37 °C for 15 min, washed in PBS, blocked in 2 % animal serum for 1 h and incubated with primary antibody overnight at 4 °C. They were further washed with PBT and PBS (5 min each) and stained with fluorescence conjugated secondary antibody for 4 h. The discs were mounted in Vectashield mounting media (Vector Laboratories, USA) containing DAPI or propidium iodide and viewed under confocal microscope (Olympus FV1000).

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 in eye disc was selected for analysis. 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.

Diap1-lacZ reporter assay

For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining, larval eye imaginal discs were fixed in 4 % formaldehyde in PBS for 10 min and then incubated in standard X-Gal staining solution for 4 h at 37 °C.

Total protein isolation and western blot analysis

Nearly 200 eye discs (or 20 larvae) were collected and thoroughly homogenized in lysis buffer (6 % SDS, 1 mM EDTA, 2 mM PMSF, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin). The lysed samples were boiled at 95 °C for 5 min and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was collected and western blot analysis was carried out following standard protocols using MOF (1:300), JNK (1:300), P-JNK (1:300), Diap1 (1:200) and ß-actin (1:500) antibodies (Abcam).

Chromatin immunoprecipitation assay (ChIP assay)

Wild type and *mof¹* mutant larvae were grown on standard *Drosophila* culture media. To detect the amount of association of Mof and H4K16Ac proteins with the *Diap1* promoter, chromatin immunoprecipitation was conducted as described previously [46]. The immunoprecipitated

sample was amplified by a standard PCR protocol and the ratio of amplified immunoprecipitated DNA to control DNA from three independent experiments was calculated by densitometry. Primers used for amplification are as follows:

Diap1[chip1]	Fwd: 5′-TTGAGGGAAGCCACAATTAGA-3′
	Rev: 5′-AATGCGTTCTTTTTGCATCC-3′
Diap1[chip2]	Fwd: 5′-ACCAGGCGAAAAGAGTGCTA-3′
	Rev: 5′-ATATTTTCGGTGGCGTTCAA-3′
Diap1[chip3]	Fwd: 5′-AAGCCCAGAGAGCACTGAAA-3′
	Rev: 5′-GCGGTATTGCACAAAATCCT-3′
Fwd: Forward primer; Rev: Reverse primer	

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-transcribed. Relative quantification was performed using SYBR Green Master Mix (Takara Bio) on Real time PCR (Applied Biosystems, 7900HT). Each experiment was repeated thrice independently. Using the $2^{-\Delta\Delta CT}$ method, fold change in product levels for each sample was determined, as described [47]. 18S rRNA was used for the normalization.

Name	Sequence
18S rRNA	Fwd: CCTTATGGGACGTGTGCTTT
	Rev: CCTGCTGCCTTCCTTAGATG
Mof	Fwd: CTGGGTAGGCTGAGCTATCG
	Rev: CCAGACGAGGTAATCGGTGT
Diap1	Fwd: CCGAGGAACCTGAAACAGAA
	Rev: GAATCGGCACTGACTTAGCC

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 $P < 0.05$.

Results

Mof mutant larvae have smaller imaginal discs than wild type due to increased apoptosis but not reduced cell proliferation

Male mof^1 larvae do not survive past the 3rd instar stage. The mof^1 mutant allele has a Glycine replaced by a Glutamic Acid at position 691 in the acetyl-COA binding motif (i.e. G691E), a change that eliminates its catalytic histone acetyl transferase activity. To understand the cause of lethality in mof^1 mutants, we used fly stocks that carry mof^1 mutation over the balancer *FM7GFP*. We observed that imaginal discs of mof^1 mutant larvae were significantly smaller in size when compared to wild type discs. The pouch of the wing disc reduced to around 40 % of normal size. We speculated that the small disc phenotype of mof^1 mutants might have resulted either due to reduced cell proliferation or increased apoptosis during the process of development. To assess the role of cell proliferation, we immunostained wing discs from mof^1 mutant larvae using antibody against anti-phospho histone H3 [mitosis marker] that detects mitotic cells. We did not observe any change in the number of mitotic cells in the mutant when compared to wild type [18]. To investigate whether apoptosis is the reason for reduced size of wing discs, mof^1 mutant discs were stained with acridine orange which selectively stains apoptotic cells. In contrast to wild type discs that show little or no apoptosis, mof^1 mutant wing discs displayed robust and wide spread apoptosis (Fig. 1a); clearly depicting apoptosis as the main cause of reduced imaginal disc size in the mof^1 mutants rather than reduced cell proliferation (data not shown). Since mof^1 mutants do not survive beyond the third instar larval stage, in the present study we used the UAS-GAL4 system to knockdown Mof in tissue specific manner using mof^{RNAi} and *GMR-GaL4* flies. Eye discs of GMR-GAL4/+; mof^{RNAi}/+ larvae were stained for acridine orange and as expected *GMR-GAL4* driven expression of mof^{RNAi} resulted in cell death in eye disc. This was further validated by a genetic rescue experiment by over expressing Mof using EP-Mof flies. The expression of *Mof* transgene in *GMR-mof^{RNAi}* background clearly abolished apoptosis caused by knockdown of *Mof* gene (Fig. 1b).

Fig. 1 Small imaginal discs in *mof* mutants due to increased apoptosis. **a** Size differences between wing imaginal discs of wild type (*Canton S*) and *mof¹* larvae depicted by acridine orange staining due to increased apoptosis. **b** Acridine orange stained eye imaginal discs from developing 3rd instar larvae. *GMR-GAL4* driven *mof^RNAi* eye discs show increased levels of apoptosis in region posterior to morphogenetic furrow compared to wild type control (CS) which is very much reduced in the presence of *Mof* transgene. *Scale bars* represent 50 μm
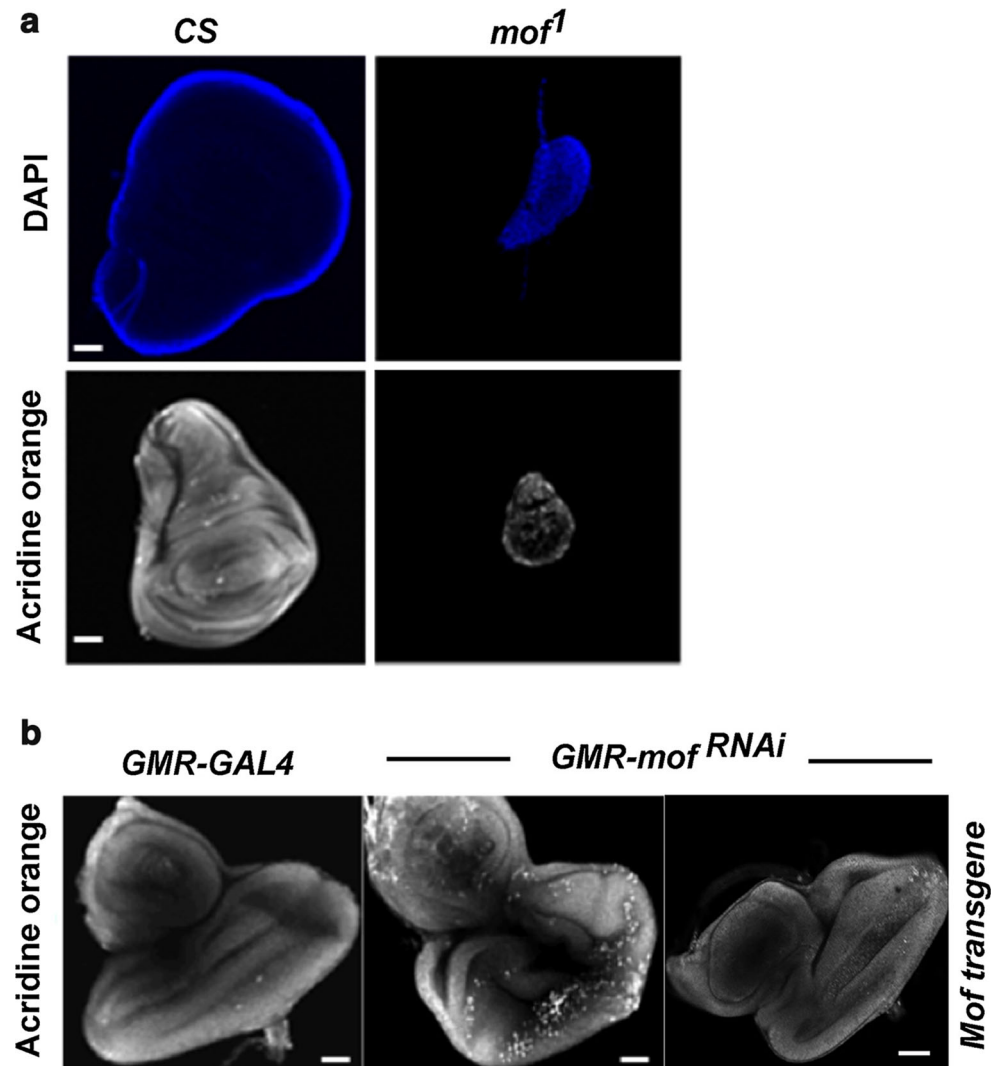
Drosophila pro-apoptotic genes: Hid, Reaper and Grim are required for *mof^RNAi* induced apoptosis

Three regulatory pro-apoptotic proteins Reaper, Hid and Grim (RHG), act as antagonists of Baculovirus, *Drosophila* and mammalian inhibitor of apoptosis (IAPs) and can predominantly induce apoptosis when ectopically expressed in transgenic animals and cultured insect and mammalian cells [48, 49]. These are a set of closely linked genes encoded by the 75C1region of the *Drosophila* third chromosome, also known as the H99 region, which when deleted, suppresses apoptosis post γ-irradiation and during embryogenesis [29–31]. To verify the involvement of RHG genes in apoptosis induced by *GMR-mof^RNAi*, we used the deficiency line *Df (3L) H99*. Interestingly we observed that dosage reduction of *reaper*, *hid* and *grim* genes alleviated *GMR-mof^RNAi* induced

apoptosis in the eye discs (Fig. 2a (a–c). Apoptosis in the eye discs induced by *GMR-mof^RNAi* was also considerably reduced in the *hid^109* mutant background but the expression of -Hid, Reaper and Grim did not change in the *mof* mutant at both transcript and protein levels [data not shown]. Ectopic expression of *Hid*, *Rpr* and *Grim* in the adult eye by *GMR-GAL4* resulted in ablated apoptotic eye phenotype [29, 50, 51]. To further confirm the genetic interaction of Mof with Hid, Reaper and Grim, we over expressed Mof (P [EP] mof) using GMR-GAL4 in the genetic background of GMR-hid, GMR-reaper and GMR-grim. We observed that over expression of Mof completely recovered the GMR-rpr induced apoptosis but partial recovery was observed in the case of GMR-hid and GMR-grim (Fig. 2b). This is explainable in support with the earlier findings that showed Reaper [and not Hid or Grim] promote the most significant DIAP1 degradation and apoptosis induction [52].
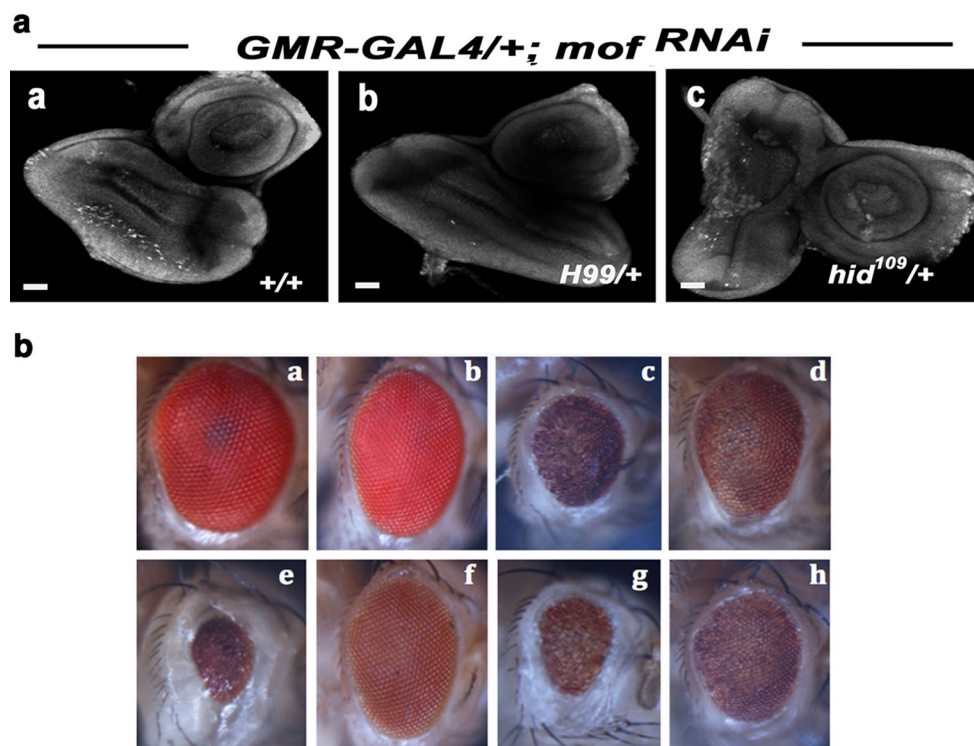
Fig. 2 Apoptosis in *mof* mutants is dependent on pro-apoptotic genes. **a** Confocal images showing acridine orange staining in eye discs of *a GMR-GAL4; mof^RNAi^/+, b GMR-GAL4; mof^RNAi^/H99, c GMR-GAL4; mof^RNAi^/hid^109^*. **b** Light microscopic pictures of adult fly eyes of the following genotypes. *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*/+

Apoptosis caused by down regulation of Mof is suppressed by caspase inhibitors and not p53 mutation

Over expression of baculovirus caspase inhibitor protein p35 and chemical caspase inhibitors inhibit RHG induced ectopic cell death, indicative of their role in caspase mediated cell death [31, 50, 51]. To investigate whether apoptosis induced by *GMR-mof^RNAi^* is mediated by caspases we over expressed baculovirus inhibitor of apoptosis P35 [*UAS-P35*] in the same genetic background. We observed significant reduction of apoptosis in the eye discs, while there was no change in *GMR-mof^RNAi^* eye discs in the presence of p53 mutation (data not shown) indicating that apoptosis induced by *GMR-mof^RNAi^* is independent of p53. *Ark (Apaf-1 related killer)* is the *Drosophila* homolog of mammalian *Apaf-1* an essential pro-apoptotic protein and most cell death is blocked in *ark* mutants [53]. We therefore expressed *mof^RNAi^* using *GMR-GAL4* in *ark^RNAi^* mutant background and observed considerable decrease in apoptosis suggesting the role of ark in *mof^RNAi^* induced apoptosis (Figs. 3a (a–c), 2b).

It was earlier reported that mechanism of activation of caspases in *Drosophila* is mediated by an exclusively important antagonist molecule: the IAPs [33, 54]. This conserved family of inhibitors was first identified in baculovirus [55] by means of their ability to suppress viral-infection. Subsequently homologs were identified in *Drosophila melanogaster, C. elegans*, mammals, and yeast [56]. To study the role of IAPs, we over expressed *Drosophila inhibitor of apoptosis 1 (DIAP1)* using *GMR-GAL4* in *mof^RNAi^* flies. We observed complete rescue of apoptosis confirming that *mof* mutants induce apoptosis through caspase mediated pathway. We also analysed the effect of loss of function mutants of *diap1* [*th^4^/+* and *diap1^11−3e^/+*] in the *mof^RNAi^* induced apoptosis. As anticipated we observed increased apoptosis in the eye discs of *GMR-mof^RNAi^* further validating the antagonistic function of IAPs (Figs. 3a (d–f), 2b).

GMR-*mof^RNAi^* induced apoptosis is caused by activation of caspases in the eye discs

Dronc and drICE are *Drosophila melanogaster* cysteine proteases that function as initiator and effector caspases for programmed cell death at all stages of development. We therefore, co-overexpressed *drICE^RNAi^* and *dronc^RNAi^* independently with *mof^RNAi^* to study their role in cell death. Co-overexpression of *mof^RNAi^* along with *drice^RNAi^* and

Fig. 3 Apoptosis in *mof* mutants is caspase dependent. **a** Confocal images showing acridine orange staining in eye discs of *a GMR-GAL4; mof^RNAi/ +*, *b GMR-GAL4; mof^RNAi/UAS-P35*, *c GMR-GAL4; mof^RNAi/Ark^RNAi*, *d GMR-GAL4; mof^RNAi/GMR-Diap1*, *e GMR-GAL4; mof^RNAi/diap1^{11−3e}*, *f GMR-GAL4; mof^RNAi/th^4*, *g GMR-GAL4; mof^RNAi/dronc^RNAi*, *h GMR-GAL4; mof^RNAi/drice^RNAi*. *Scale bar* represents 50 μm. All images were taken with $10\times$ objective (zoom = 1.8) using Olympus confocal microscope [FV1000]. **b** 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) in the eye discs* [Mean ± SEM]
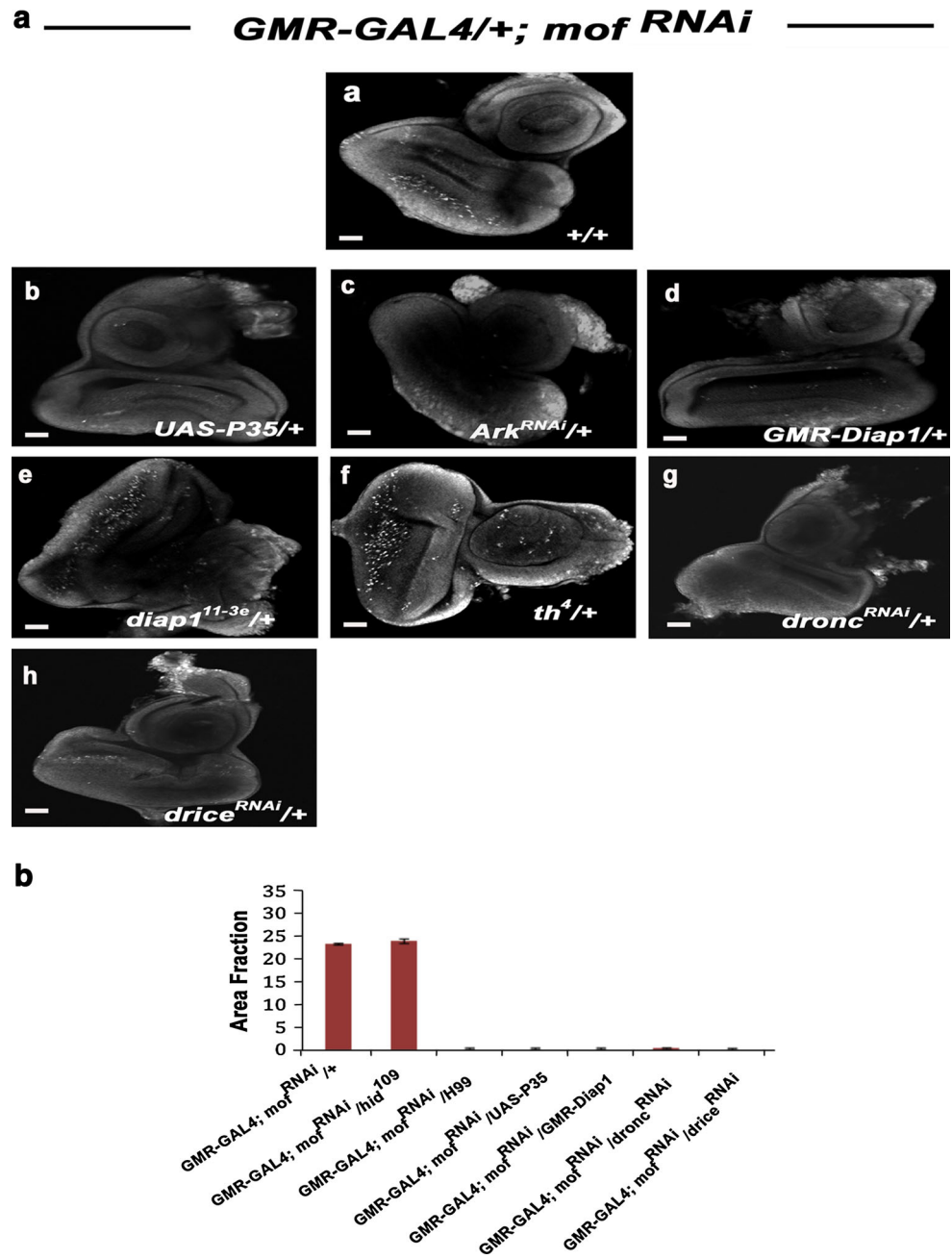
dronc^RNAi using *GMR-GAL4* in two separate experiments substantially reduced apoptosis (Figs. 3a (g–h), 2b). To visualize and detect apoptosis we immunostained eye discs from *GMR-mof^RNAi* larvae using cleaved caspase-3 antibody that recognizes multiple epitopes of Dronc/Dronc-dependent caspases and DrICE. Overexpression of caspases in the GMR region of the eye discs expressing *mof^RNAi* using *GMR-GAL4* driver confirmed that caspases mediate *mof^RNAi* induced apoptosis. Discs from GMR-*hid* that display ectopic apoptosis in eye discs served as positive control (Fig. 4a).

Apoptosis induced by *mof^RNAi* is mediated by JNK pathway

Several recent studies revealed the role of *Drosophila* c-Jun N-terminal kinase (JNK) signalling in regulation of apoptosis [57–60]. JNK pathway is activated either in apoptotic or in proliferating cells depending on the apoptotic stimuli received [61–63]. Genetic and biochemical analyses have also indicated that c-Jun amino-terminal kinase (JNK) signaling pathway is specifically activated by Transforming growth factor beta (TGF-beta) activated
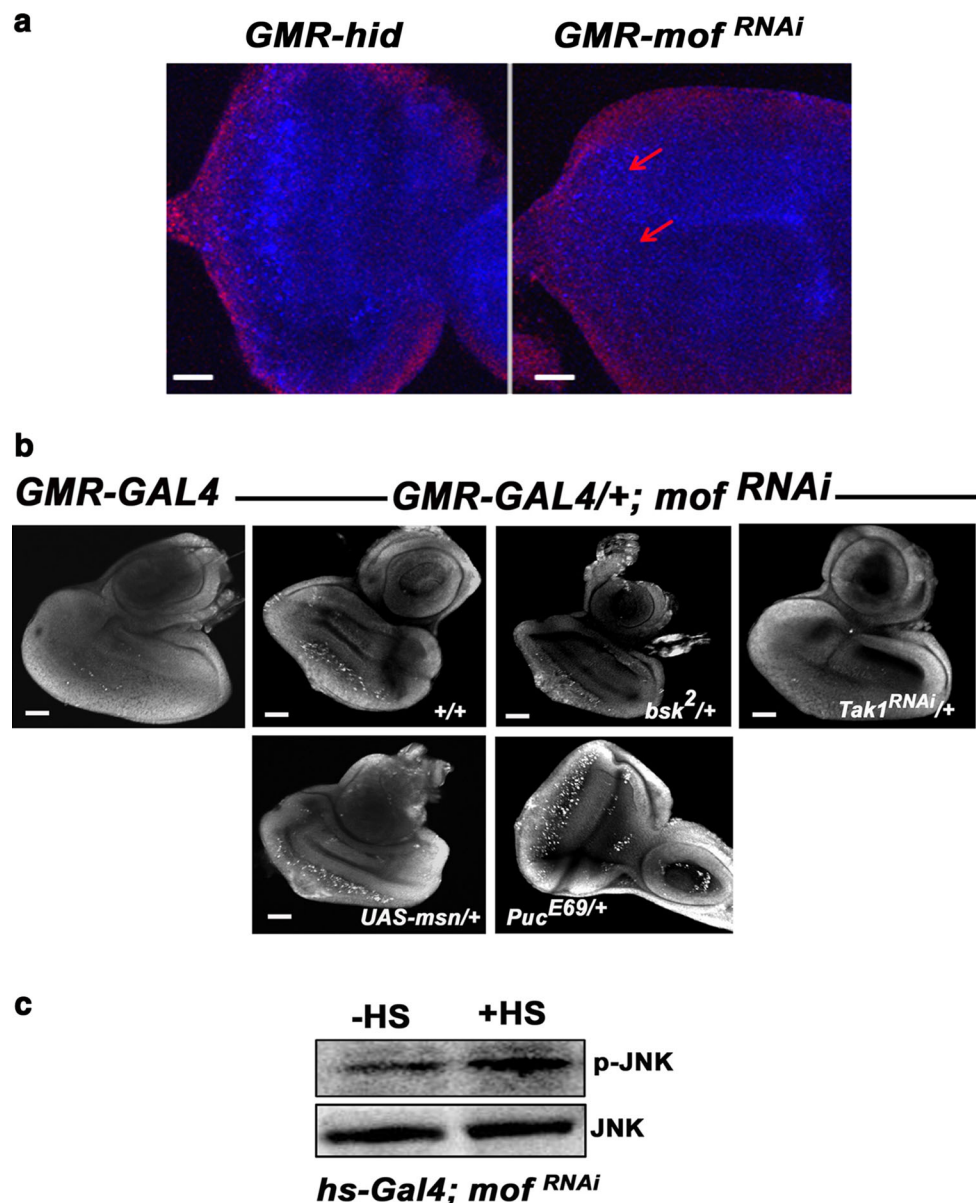
Fig. 4 Apoptosis in *mof* mutants is dependent on JNK pathway. **a** Immunostained eye discs from GMR-*hid* [positive control] and GMR > *mof*^{RNAi} eye discs with anti-caspase3 antibody. Considerable caspase overexpression was observed in the experimental eye disc. *Arrows* depict fluorescent apoptotic cells. Images taken with a 60× objective (zoom = 1) of Olympus confocal microscope [FV1000]. *Scale bar* 100 μm. **b** Acridine orange staining of larval eye discs of GMR-GAL4, *GMR-mof*^{RNAi} [control], GMsR-*mof*^{RNAi} in the presence of JNK mutant's *bsk*^{2}, *Tak1*^{RNAi}, *msn* and *puc*^{E69}. **c** Western blot analysis of total protein isolated from *hs-GAL4; mof*^{RNAi} larvae in the presence and absence of heat shock using antibodies against phosphor-JNK and JNK (loading control)

kinase 1 (TAK1 or JNKKK) signaling [64] that regulate cell shape and apoptosis. Ectopic activation of *TAK1* also leads to small eye phenotype which is the result of ectopically induced apoptosis. Further *Tak1* acts upstream of *Bsk* in the *Drosophila* JNK signalling pathway [65]. To determine whether JNK pathway is involved in Mof depletion-induced apoptosis, we blocked JNK activity in the Mof knockdown tissues [*GMR-mof*^{RNAi}] using JNK mutants. Three lines of evidence have shown that apoptosis

is dependent on JNK activity. Firstly apoptosis induced by *mof*^{RNAi} using *GMR-GAL4* in the eye disc was significantly reduced in the presence of *bsk*^{2} (JNK) and *TAK1*^{RNAi} (JNKKK) mutants. Second apoptosis in Mof knock down eye discs was elevated when Msn (MAPKKKK) was over expressed using *UAS-Msn* flies and in the genetic background of *Puc*^{E69} [inhibitor of JNK] (Fig. 4b). Taken together, results demonstrated that Mof knockdown requires JNK signalling pathway to induce cell death in

Drosophila. Thirdly, we also addressed JNK activity directly by analysing the expression of phosphorylated JNK, which is an activated form of the, protein in wild type versus the *hs-mof*[RNAi]. A drastic increase in the expression of P-JNK in *hs-mof*[RNAi] larvae upon heat shock was observed when compared to wild type further confirming the involvement of JNK pathway in Mof induced apoptosis (Fig. 4c).

Drosophila Mof activates *Diap1*

The JNK signaling has been reported to be controlled by either *Drosophila inhibitor of apoptosis* (*DIAP1*) or the initiator caspase *Dronc* [66–68]. Drastic reduction of DIAP1 transcript was observed in the *mof*[1] mutation, but surprisingly, *dronc* (*Drosophila* caspase-9 homolog), which is regulated directly by DIAP1, did not show any change in transcript levels. Similar results were obtained with *drice* transcripts posing a probability that caspase activation immediately precedes cell death (Fig. 5a). Similar to the mRNA levels, DIAP1 protein was also decreased in *mof*[1] mutant larvae (Fig. 5b). Over expression of Mof in the larval eye discs using *GMR-Gal4* resulted in increased expression of DIAP1 which was further confirmed by immunostaining of the eye discs with anti-DIAP1antibody. This pattern of DIAP1 staining is similar to earlier published reports [55]. Also *Lac Z* staining of eye discs of *GMR-Gal4* driven *EP-Mof/diap1-LacZ* flies has shown a similar expression pattern of *diap1* (Fig. 5c). All the above studies clearly confirmed that Mof activates *Diap1*.

Mof regulates the transcription of *diap1* by binding to its promoter sequence

Since *Mof* interacts genetically with *Diap1*, the next question was to study the mechanism of regulation of *Diap1*. Till date, transcriptional regulation of *Diap1* has not been explored except reports of positive regulator STAT92E binding the *Diap1*promoter at a distance of 3.3 kb upstream from the transcription start site (TSS) [69]. Berkeley Genome Project Promoter Prediction Tool has been used to predict around 8 possible promoter stretches, lying between the +1TSS and the STAT site-bounded 1.2 kb region. PCR primers of 100–250 bp amplicons spanning the promoter stretch were designed for carrying out ChIP assay (Fig. 6a, b). ChIP assay with antibody against Mof in wild type *Canton S* third instar larvae revealed enrichment of Mof at all the amplicons. Similar levels of enrichment at all the stretches were obtained using anti-H4K16Ac antibody in wild type larvae confirming that histone modifications are the result of binding of Mof. In parallel, *mof*[1] mutant larvae as negative control did not

show any binding at the *Diap1* promoter (Fig. 7a–c). These results clearly established the role of Mof as a transcriptional activator of *Diap1* (Fig. 8).

Discussion

In this study to address the novel function of *Drosophila* Mof in the regulation of apoptosis, we carried out in vivo experiments involving both loss and gain of function experiments. We attributed the reasons for the reduced size of larval imaginal discs upon Mof knockdown to increased apoptosis rather than reduced cell proliferation. The fact that apoptosis induced by depletion of Mof occurs through an activated JNK pathway raises questions about the possibility of Mof interaction with downstream regulators of JNK. Mof, a MYST histone acetyltransferase is an essential component of the *Drosophila* Dosage Compensation Complex (DCC) and shares orthologs from yeast to humans [70]. Until recent times, only few studies have been carried out to explore the possible functional roles of *Drosophila* homolog of human *Mof*. In mammals Mof is involved in embryonic development and maintenance of normal epigenetic signature during early stages of development [71, 72]. 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 [8, 73].

We have demonstrated for the first time that depletion of Mof in *Drosophila* induces apoptosis leading to reduced size of the larval imaginal discs. Since *mof* mutants do not survive beyond the larval stage, *mof*[RNAi] flies have been used to study the depletion of Mof in larval imaginal discs. Depletion of Mof in *GMR-mof*[RNAi] flies in the genetic background of *Df (3L) H99* (deletion of *Reaper*, *Hid* and *Grim*) rescued apoptosis in the GMR region of the eye disc indicating the role of RHG genes in *Mof* induced apoptosis. The evolutionary conserved pathway of cell death is intrinsic and performed by an army of caspases, *Apaf-1* and *Bcl-2* members in most of the organisms [74, 75]. 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 [76]. The role of caspases in apoptosis induced by *GMR-mof*[RNAi] was elucidated by co-expression of caspase inhibitors *P35* and *Diap1*. Similar to mammalian p53, over expression of *Drosophila* p53 leads to apoptosis but does not cause G1 arrest. Moreover, p53 mutants do not affect X ray-induced cell cycle arrest [77]. Apoptosis induced by *GMR-mof*[RNAi] remained unchanged in the presence of p53 mutation clearly demonstrating that apoptosis induced by *GMR-mof*[RNAi] is independent of p53 in *Drosophila*. Apoptosis in *GMR-mof*[RNAi] discs was significantly reduced in the

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Fig. 5 Mof interacts genetically with *Diap1*. **a** RT-PCR analysis of total RNA extracted from *Canton S* and *mof¹* larvae using primers against genes *Diap1*, *Dronc* and *Drice*. *18S rRNA* is used as loading control. **b** Western blot analysis of total protein isolated from *Canton S* and *mof¹* larvae using antibodies against Mof and Diap1. β-actin is used as loading control. **c** Immunostaining of eye discs from larvae over expressing of Mof (GMR > EP-*mof¹* with anti-DIAP1 antibody). *Red colour* 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. Lac Z staining of larval eye discs of genotype GMR > EP-*mof*; *diap1-lacz*. *Scale bar* 50 μm (Color figure online)
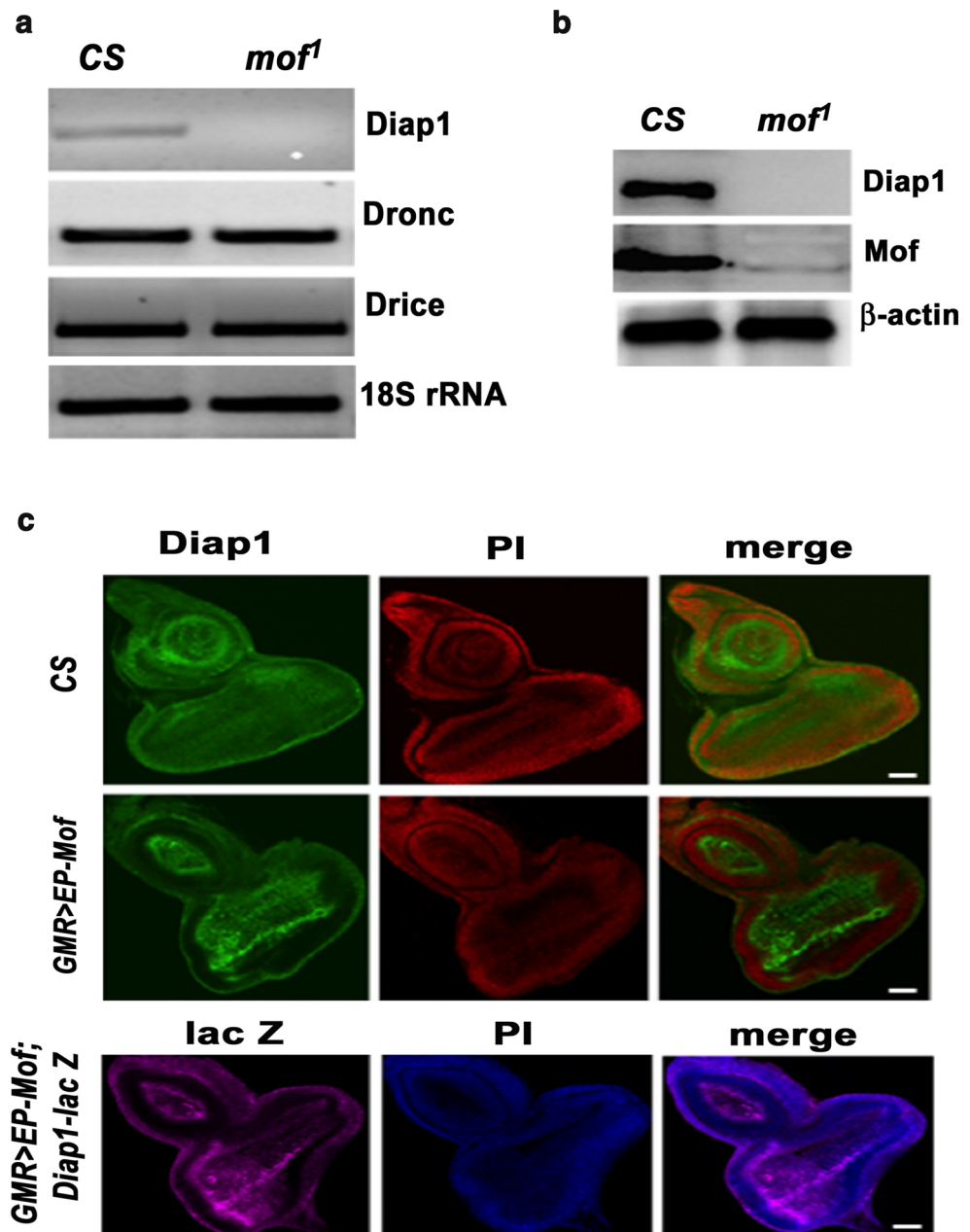
presence of *drICE^RNAi* and *dronc^RNAi* elucidating caspase mediated cell death in the eye discs of *GMR-mof^RNAi* larvae. Loss of function mutations of *diap1* are embryonic lethal while ectopic expression can inhibit apoptosis [51]. The primary mode of regulation occurs through its targeted ubiquitination and proteosomal degradation of initiator caspase DrONC [78]. Ectopic expression of pro DRONC in the *Drosophila* eye generates a severe eye ablation phenotype, which was completely rescued by co-expression of *Diap1*. However such reversion fails when the ablation phenotype was induced by prodomain lacking DRONC, thus confirming the specific interaction between *Diap1* and prodomain of *Dronc*. We observed drastic increase in the

expression of diap1 upon immunostaining of GMR > *Mof* eye discs with Diap1 antibody and *diap1-LacZ* staining. A drastic decrease in *Diap1* transcripts and protein was observed in the *mof¹* mutants indicating that Mof regulates the expression of *Diap1*.

In wild type, Mof as Histone acetyl transferase enhanced *Diap1* promoter acetylation leading to its activation but was highly decreased in the case of *mof* mutation. Hence increased expression of *Diap1* leads to decreased apoptosis. In General RHG proteins such as Hid, Reaper and Grim degrade the Diap1 by interacting with BIR domain of Diap1 protein in the background of *mof* mutation. But in case of wild-type flies as a genomic stability maintainer,
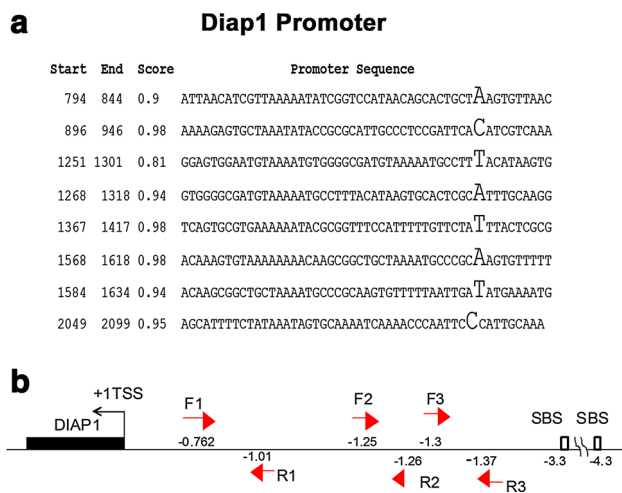
a

Diap1 Promoter

Start	End	Score	Promoter Sequence
794	844	0.9	ATTAACATCGTTAAAAATATCGGTCCATAACAGCACTGCTAAGTGTTAAC
896	946	0.98	AAAAGAGTGCTAAATATACCGCGCATTGCCCTCCGATTCACATCGTCAAA
1251	1301	0.81	GGAGTGGAATGTAAAATGTGGGGCGATGTAAAAATGCCTTTACATAAGTG
1268	1318	0.94	GTGGGGCGATGTAAAAATGCCTTTACATAAGTGCACTCGCATTTGCAAGG
1367	1417	0.98	TCAGTGCGTGAAAAAATACGCGGTTTCCATTTTTGTTCTATTTACTCGCG
1568	1618	0.98	ACAAAGTGTAAAAAAACAAGCGGCTGCTAAAATGCCCGCAAGTGTTTTT
1584	1634	0.94	ACAAGCGGCTGCTAAAATGCCCGCAAGTGTTTTTAATTGATATGAAAATG
2049	2099	0.95	AGCATTTTCTATAAATAGTGCAAAATCAAAACCCAATTCCCATTGCAAA

b

Fig. 6 Promoter sequence of *Diap1* gene. **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

Mof increases the Diap1 expression levels and decreases the pro-apoptotic proteins such as Hid, Reaper, Grim and caspases Drice and Dronc. Interestingly JNK signalling has been reported to be controlled by either *Drosophi*la DIAP1 or the initiator caspase Dronc [66–68].

It has been reported earlier that extracellular death-inducing signals might be transduced via JNK pathway in *Drosophila* [79]. Cell death induced by GMR > *mof*[RNAi] is accompanied by activation of JNK pathway in the eye discs. Cell death induced by GMR > *mof*[RNAi] is rescued in the presence of mutants of the JNK pathway or its negative regulator. Our genetic assays point towards the involvement of *Tak1*, *Hep* and *Bsk* as crucial JNK components in Mof-induced apoptosis. Increased phosphorylation of JNK molecules in GMR > *mof*[RNAi] tissue definitively points towards the involvement of JNK signalling in apoptosis. Thus our results clearly indicate that Mof regulates Diap1 and Diap1 in turn is regulated by JNK signalling (i.e. particularly the key components of JNK pathway such as Tak1 and Bsk). This provides a different dimension to that of existing one that Diap1 regulates JNK signalling [66–68]. We suspect that a feed-back or interlinking mechanism exists between Diap1 and JNK signalling during apoptosis.

We show that rough eye phenotype induced by *GMR-Rpr* in the adult eye was completely rescued by over expression of Mof and partially in the case of *GMR-hid* and *GMR-grim*. These results clearly elucidated the genetic interaction between pro-apoptotic genes and *Mof*. It has also been well documented that certain interdependent regulatory loop exist between Diap1 proteins with RHG components ubiquitinating and destroying Diap1 and also
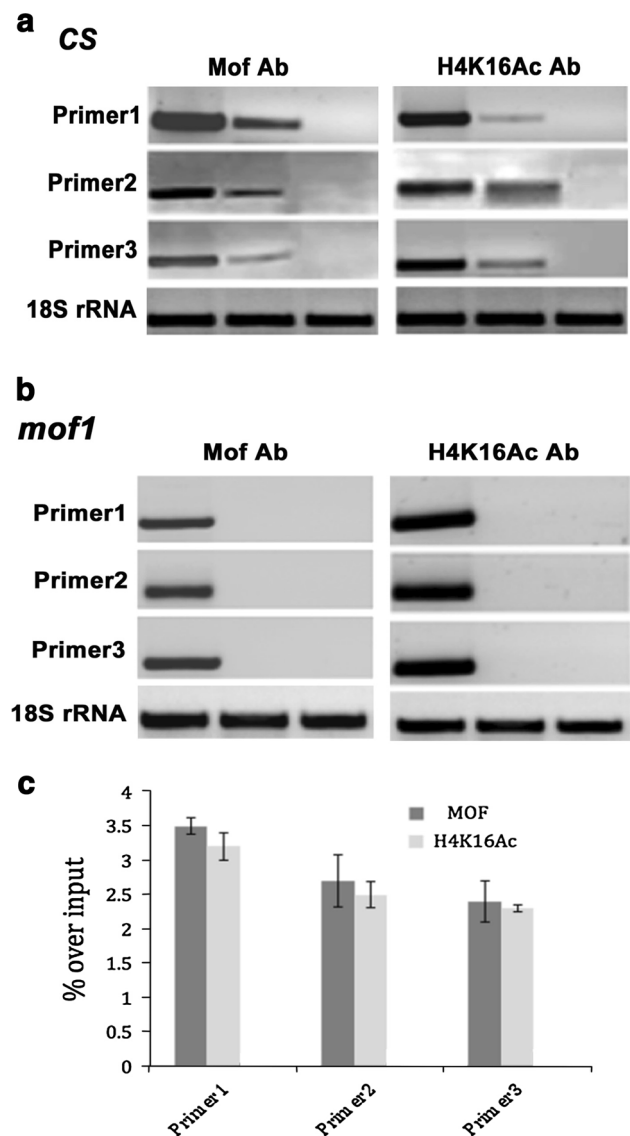
a

CS

b

mof1

c

Fig. 7 Chromatin immunoprecipitation assay to detect Mof binding at the *diap1* promoter. RT-PCR on ChIP DNA from *CS* and *mof*[1] mutant larvae has been carried out. *Lane 1* Input (5 % of cross-linked chromatin), *Lane 2* ChIP DNA, *Lane 3* No antibody control. **a**, **b** Quantitative PCR of DNA immunoprecipitated from wild-type *CS* and *mof*[1] mutant larval discs Antibodies used: Mof and antiH4K16Ac. **c** *Each bar* represents the mean fold enrichment from two independent experiments (*with error bars* denoting + SEM). Enrichment was calculated as the fold difference ($2^{-\Delta\Delta C_t}$) between DC_t oftarget sample (C_t value of ChIP sample normalized to input sample) and DC_t of negative control (calibrator sample). No antibody control was chosen as the negative control since no deletion mutant of *mof* is available. *P* value <0.05 (two-tailed student's *t* test)

Diap1 in maintaining RHG stability to a certain extent [79]. *Drosophila melanogaster* tumour-necrosis factor receptor-associated factor 1 (DTRAF1) has been identified as a dominant suppressor of Reaper-induced cell death. It was also shown that Reaper modulates the JNK pathway through *Drosophila* DIAP1, which negatively regulates
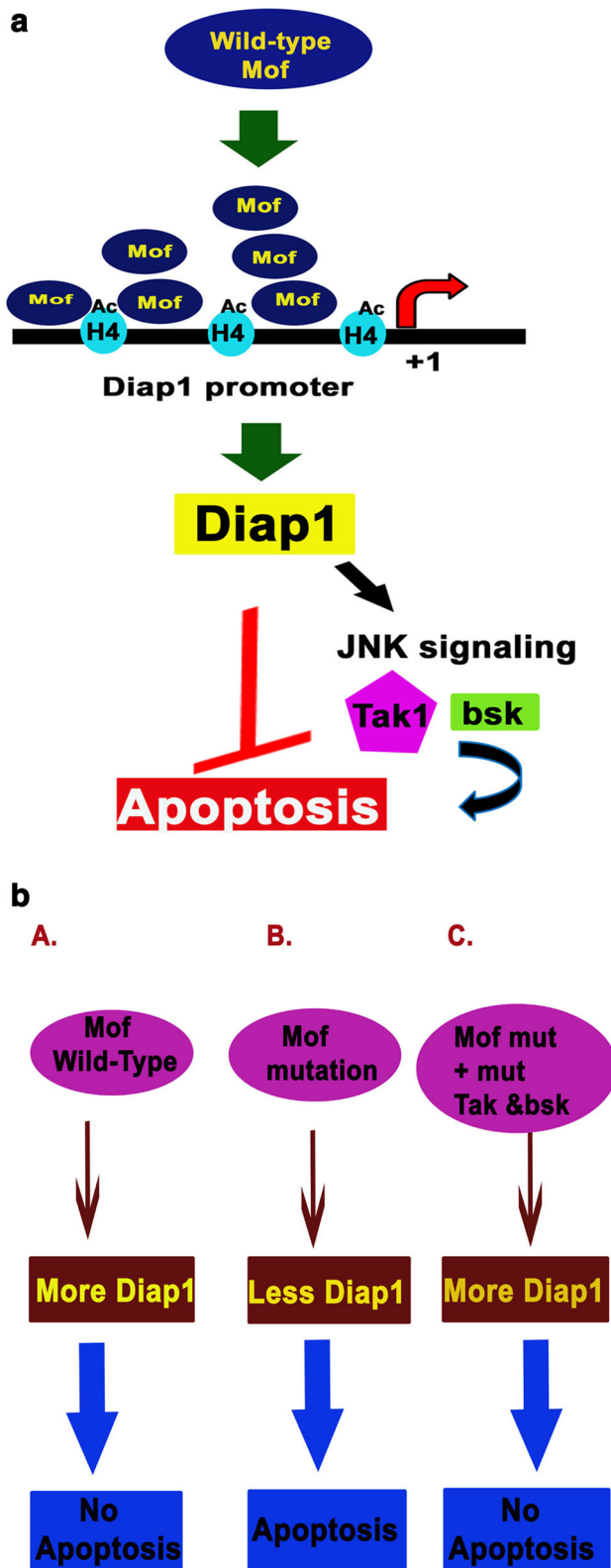
◀**Fig. 8** Hypothesis depicting the role of *Drosophila* Mof in Apoptosis. **a** In wild type Mof binds to Diap1 promoter thereby acetylating at H4K16 leading to increased expression of Diap1 there by inducing apoptosis in JNK mediated pathway. **b** (*A*). In the wild type Mof, expression of Diap1 is more and it inhibits apoptosis by regulating hid, reaper, Grim via BIR domain of Diap1. Hence apoptosis does not occur. *B* In *mof^RNAi* flies the production Diap1 is less since H4K16Ac is less at Diap1 promoter. Higher expression of Hid, Reaper and Grim (RHG) degrades diap1 protein. This leads to more production of Dronc and Drice and ultimately apoptosis via Tak1-Bsk signalling molecules that play key role in JNK signalling pathway. Thus there is direct regulation between Diap1 mediated apoptotic signalling through JNK pathway. In simple Mof controls Diap1 and Diap1 modulates apoptotic signalling via Tak1 and Bsk protein that play pivotal role in JNK signalling. *C* Mof mutation or *mof^RNAi* (having reduced Diap1 protein expression) in the background of JNK mutants *tak1* and *bsk* mitigates apoptosis

DTRAF1 by proteasome-mediated degradation [42]. Moreover, Diap1 also possesses the ability to bring about in vitro ubiquitination of the caspase Dronc [80, 81]. We have demonstrated using chromatin immunoprecipitation assays that Mof binds to the promoter regions of *Diap1* and thereby regulate its transcriptional activity. *Hid*, *Grim* and *Reaper* are the essential death–inducing genes (required for the destruction of larval tissues), functioning through destruction of Diap1 during development. However, no true antagonist proteins have been identified till date. As a chromatin modifier, Mof promotes transcriptional activation of DIAP1 and act to antagonize unnatural cell death. Functioning against apoptosis promoting proteins-Hid, Reaper and Grim, Mof helps in maintaining optimal cellular levels of DIAP1 especially in the cell's decision to undergo apoptosis. Our studies led us to hypothesise through genetic and biochemical evidences that Mof is one of those candidate proteins which actually induce *Diap1* transcription. It probably helps to maintain a threshold level of Diap1 in the cell and overproduce it upon receiving appropriate signals. We believe, however that this may not be the sole method of regulation of Diap1 by Mof. Further studies need to be carried to determine if Mof can inhibit Diap1 by other mechanisms, such as inhibiting its ubiquitination.

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