Overexpression of the *Drosophila* ATR homologous checkpoint kinase Mei-41 induces a G2/M checkpoint in *Drosophila* imaginal tissue

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**Abstract**

**Background:** DNA damage generally results in the activation of ATM/ATR kinases and the downstream checkpoint kinases Chk1/Chk2. In *Drosophila melanogaster*, the ATR homologue *meiotic 41* (*mei-41*) is pivotal to DNA damage repair and cell cycle checkpoint signalling. Although various *mei-41* mutant alleles have been analyzed in the past, no gain-of-function allele is yet available. To fill this gap, we have generated transgenic flies allowing temporal and tissue-specific induction of *mei-41*.

**Results:** Overexpression of *mei-41* in wing and eye anlagen affects proliferation and a G2/M checkpoint even in the absence of genomic stress. Similar consequences were observed following the overexpression of the downstream kinase Grapes (Grp) but not of Loki (Lok), encoding the respective *Drosophila* Chk1 and Chk2 homologues, in agreement with their previously reported activities. Moreover, we show that irradiation induced cell cycle arrest was prolonged in the presence of ectopic *mei-41* expression. Similar to irradiation stress, *mei-41* triggered the occurrence of a slower migrating form of Grp, implying specific phosphorylation of Grp in response to either signal. Using a p53R-GFP biosensor, we further show that overexpression of *mei-41* was sufficient to elicit a robust p53 activation in vivo.

**Conclusion:** We conclude that overexpression of the *Drosophila* ATR homologue *mei-41* elicits an effectual DNA damage response irrespective of irradiation.

**Keywords:** ATR, DNA damage checkpoint, Mei-41, Overexpression, p53 activation

**Background**

Environmental and intrinsic stressors may impact the integrity of genome, i.e. the DNA, thereby provoking mutations eventually leading to cellular transformation or cell death. DNA damage is combated by a complex interplay of repair mechanisms ensuring the stability of the genome. Studies on DNA damage response (DDR) in a large variety of organisms, be it single cells like yeast or multicellular organisms like *Drosophila* or mammals, revealed that all organisms have evolved a core of components strikingly conserved across eukaryotes (reviewed in [1–3]). DDR can be envisaged as a cascade of signalling events, starting with the recognition of DNA lesions followed by the activation of the DNA damage checkpoint pathway to cause a temporarily cell cycle arrest thus enabling DNA repair processes to occur (reviewed in [1, 4]). Typical of signalling cascades, DDR is regulated by phosphorylation events mediated by different kinases all belonging to the conserved phosphatidylinositol 3-kinase related protein kinase (PIKK) superfamily (reviewed in [5, 6]). These kinases transmit the signals from the site of DNA damage to the cell cycle machinery by activating cell cycle checkpoints. The G2/M DNA damage checkpoint is critical for the maintenance of genome stability as unrepaired DNA double strand breaks (DSB) may directly cause mistakes in chromosomal segregation...
to the daughter cells if ending up in the M phase of the cell cycle. Hence, the final exit strategy in multicellular organisms before cellular transformation, and eventually cancer occurs as consequence of DDR is cellular suicide, i.e. apoptosis (reviewed in [7, 8]).

The Ataxia-Telangiectasia Mutated (ATM) and ATM and Rad3-related (ATR) kinases are the central mediators of DDR (reviewed in [5, 6]). ATM is known to orchestrate a global response to DSB in higher organisms including DNA repair, checkpoint activation and apoptosis. Well characterized targets of ATM are the Chk2 kinase and the tumour suppressor p53, the latter being stabilized upon DNA damage to further initiate specific target gene expression executing cell cycle arrest, DNA repair and apoptosis, respectively (reviewed in [1, 9]). Whereas ATM is involved primarily in the mammalian DSB response, ATR is activated by a much wider range of genotoxic stresses and appears to be a much more important player in DDR of yeast cells than ATM (reviewed in [2, 6]). Once activated, ATR phosphorlylates and activates the protein kinase Chk1, which effects a cell cycle arrest at the G2/M transition, allowing more time for DNA repair so that cells do not enter mitosis prematurely. Noteworthy, there is considerable crosstalk between the ATM/ATR signal transduction pathways (reviewed in [6, 8]).

The Drosophila homologue of ATM is called telomere fusion (tefu), as it was originally identified by its essential role in telomere maintenance [10]. Although tefu is important for p53 activation and DNA damage-induced apoptosis, it has no evident role in cell cycle arrest in response to DNA damage [10–14]. Similar to vertebrates, the Drosophila Chk2 homologue loki (lok) regulates p53-mediated apoptosis in response to DNA damage as well as to telomere loss [15–21]. The ATR homologue in Drosophila is encoded by meiotic 41 (mei-41). Mutational analyses revealed that mei-41 is indispensable for meiotic recombination checkpoints as well as for DNA damage checkpoints in somatic cells [13, 22–27]. Like its target kinase grapes (grp) (the Drosophila Chk1 homologue), mei-41 is important to postpone the mitosis entry in larval cells after IR-stress [24, 25, 28]. Moreover, mei-41 and grp mutant flies are highly sensitive towards triggers that damage DNA or inhibit DNA replication, and are therefore essential to maintain genomic and chromosomal stability [29–33]. Overall in Drosophila, mei-41 appears to mostly fulfill the roles of both ATM and ATR with regard to DDR, whereas tefu’s primary role is the maintenance of telomeres and triggering apoptosis.

Although various mei-41 mutant alleles have been analyzed in the past in Drosophila, no gain-of-function allele is yet available. In order to fill this gap, we generated a mei-41 construct under UAS-control, which allows temporal and tissue-specific expression of mei-41 with the help of the versatile Gal4/UAS system [34]. We show that the overexpression of mei-41 in imaginal tissues is sufficient to induce a G2 arrest constraining the growth of affected tissues. Moreover, in the presence of ectopic Mei-41 cells are hampered to resume the cell cycle after irradiation (IR)-mediated arrest. Upon IR-stress, Grp protein shows retarded mobility, and likewise upon mei-41 overexpression, suggesting that ectopic Mei-41 protein is sufficient to phosphorylate Grp protein. Finally, using a p53-biosensor we show that overexpression of mei-41 effects p53 reporter gene expression in vivo, suggesting a link to the Chk2/lok pathway as well. Overall, our data provide evidence for a mei-41-induced cellular response independent of DDR-mediated mei-41 activation.

Results

Generation of a mei-41 overexpression construct

Aiming for a deeper understanding of the Drosophila DNA damage response, we concentrated on meiotic 41 (mei-41), the Drosophila checkpoint kinase ATR homologue and key player of this process [24, 25]. To study the consequences of mei-41 overexpression, we generated a pUAST-mei-41 construct allowing for tissue-specific induction during fly development with the help of the Gal4/UAS-system [34]. The mei-41 locus contains four small introns (59 bp, 57 bp, 74 bp, 64 bp) and covers more than 8 kb; there is no complete cDNA available (7899 bp) (see http://flybase.org for further details). We therefore decided to PCR-amplify genomic DNA in four smaller fragments to be fused to cover the entire open reading frame, and clone it into the pUAST vector (Fig. 1a). Transgenic lines were established, tested and a third chromosomal insertion (3.3) line was used for further experiments. Overexpression of mei-41 in the posterior compartment of wing imaginal discs using en-Gal4-GFP was demonstrated by in situ hybridization with a mei-41 specific probe (Fig. 1b). Moreover, qRT-PCR revealed about a 500-fold induction when UAS-mei-41 was ubiquitously induced with da-Gal4 during larval development relative to the endogenous mei-41 expression levels (Fig. 1c). We conclude that the UAS-mei-41 construct is well suited for overexpression studies during Drosophila development.

Overexpression of mei-41 affects entry into mitosis

Primary to DDR is checkpoint activation, i.e. slowing down the entry into mitosis to allow time for repair [5]. We wondered whether the overexpression of mei-41 without further activation by DNA damaging compounds might suffice to affect cell cycle regulation. To this end, we induced mei-41 in the posterior compartment of the wing disc using en-Gal4-GFP, and monitored cells in M phase of the cell cycle (Fig. 2). Cells in mitotic phase were visualized with
anti-Phospho-Histone H3 (PH3) antibody staining and counted in the posterior compartment. Then their numbers were related to the whole wing disc size. As negative control, we overexpressed UAS-lacZ. Moreover, we included both downstream kinase Chk1/2 homologues grapes (grp) and loki (lok), since overexpression studies so far had not included cell cycle analyses in imaginal tissue [10, 16, 18]. It has been shown earlier that grp has a major role in the DNA replication checkpoint, whereas the primary role of lok is p53-mediated apoptosis in response to IR [16, 18, 19, 25, 28, 35].

Quantification of the results allowed us to uncover a significant reduction in the number of mitotic cells upon the overexpression of either mei-41 or grp, but not of lok (Fig. 2a-e). This is in agreement with earlier reports that mei-41 and grp are involved in cell cycle arrest in Drosophila cells and tissues, with a minor contribution of lok [16, 18, 25, 28]. We confirmed that the reduced number of mitotic cells after mei-41 or grp overexpression were not a consequence of apoptosis, as no increase of cleaved Caspase-3 activity was detected in the posterior compartment of the discs (see Additional file 1: Figure S1). Our results therefore demonstrate that both, mei-41 and grp, act on cell cycle regulation in a dose dependent manner, in contrast to lok. Our data conform to the requirements of mei-41 and grp in G2/M checkpoint function [24, 25]. Moreover, they demonstrate that our newly generated inducible mei-41 construct is a valuable tool for further analysis of mei-41 roles in a gain-of-function background, complementing the information gathered so far in a mei-41 loss-of-function background.
Ectopic induction of mei-41 during imaginal development reduces adult tissue size

To study the effects of mei-41 overexpression on adult tissue size, we ectopically induced mei-41 during proliferative phases of eye and wing development. Overexpression of mei-41 in the anterior part of the developing eye disc using ey-Gal4 resulted in a profound reduction of adult eye size (Fig. 3a). This growth defect is not restricted to the eye since overexpression of mei-41 in the posterior compartment of the developing wing disc using en-Gal4-GFP reduced its size within the adult wing significantly, whereas the size of the anterior compartment was unaffected (Fig. 3b). We did not observe any disturbance of wing and venation morphology, implying that growth but not differentiation or patterning of the tissue was affected (Fig. 3b).

So far, our data suggest that overexpression of mei-41 is sufficient for the induction of a cell cycle arrest without affecting development. The effect is very mild though, and only apparent in a quantitative approach, which may be explained by the lack of kinase activation by genotoxic stress. We cannot rule out the formal explanation, however, that the excessive amounts of Mei-41 kinase may generally interfere with other aspects of cell growth and proliferation. For example, ATM/ATR kinases must be tightly regulated in order to prevent aberrant activation of DDR. It is thought that the availability of specific protein cofactors, required for kinase recruitment to DNA damage sites, restricts kinase activity. ATR forms a heterodimer with its obligate partner ATRIP during the sensing of DNA damage in the course of DDR (reviewed in [6, 8]), and so does Drosophila Mei-41 with the ATRIP homologue Mus 304 [25]. We propose that Mus 304 may be limiting the effects of mei-41 overexpression, as the gene is expressed only about threefold of mei-41 (http://flybase.org).

Irradiation induced G2/M checkpoint is extended in mei-41 overexpressing cells

We know from the literature that mei-41 is indispensable for the irradiation induced G2/M checkpoint [13, 22, 25].
Likewise, the combined activity of *grp* and *lok* is required, with a minor contribution of the latter [16, 18, 25, 28]. Accordingly, we wondered how overexpression of *mei-41*, *grp* or *lok* may affect the cellular response to irradiation. Larvae overexpressing the respective constructs in the posterior compartment of imaginal discs were exposed to 40 Gy IR. PH3 staining uncovered cells in early mitosis in wing imaginal discs: as expected, IR-induced a G2/M checkpoint as very few cells entered mitosis 1 h after irradiation (Fig. 4a, b). Cell cycle arrest is induced and maintained to allow sufficient time for DNA repair, and eventually the cell to recover from DNA damage and resume cell cycle. Accordingly, cells start to re-enter mitosis, seen by increasing numbers of PH3 positive cells over time. In the control, we observed rising numbers of PH3 positive cells already at 4 h post-IR, becoming considerably numerous 6 h later (Fig. 4a, b). A likewise increase was observed in the anterior compartment of the irradiated discs from larvae overexpressing either *mei-41*, *grp* or *lok*. In the posterior compartment, however, i.e. in cells overexpressing *mei-41*, re-entry into the cell cycle was considerably hampered even after 6 h recovery time (Fig. 4a, b), presumably by keeping the DSB damage checkpoint active. We expect both, the endogenous as well as the overexpressed Mei-41 kinase to be activated in response to IR-stress. Consequently, cells are expected to be flooded by activated Mei-41, keeping them from resuming mitosis entry. Even several hours post-IR, activated Mei-41 levels apparently surpass the threshold for a G2/M checkpoint.

Overexpression of *grp* had a much milder effect: 4 h after irradiation, cell cycle had not yet resumed, whereas after 6 h, the number of mitotic cells was approaching that of the control (Fig. 4a, b). These data indicate that *grp* overexpression slowed down cell cycle re-entry, but not as efficiently as *mei-41* overexpression. This result may be expected, since *grp* encodes the effector kinase downstream of *mei-41*: IR-stress activates endogenous Mei-41 kinase which phosphorylates and activates the effector kinase Grp. In the presence of abundant Grp protein, this response will be intensified and longer lasting. Therefore, we expect cell recovery to lag behind, i.e. *grp* to mirror but not to match *mei-41* overexpression.
Fig. 4 (See legend on next page.)
Overexpression of lok, however, had a similar effect as mei-41 overexpression (Fig. 4a, b), which, at first glance, came as a surprise: cell cycle re-entry appeared delayed even at 6 h after irradiation, i.e. the number of PH3 positive cells was still reduced. However, we had not observed any effect of lok overexpression on cell cycle regulation in the absence of IR-stress (Fig. 2a), in agreement with the many reports on the supplemental role of lok for the G2/M checkpoint [25, 28]. Instead, the specific role for lok in the activation of p53-mediated cell death is well established [15, 16, 25]. Thus, we wondered whether the lack of PH3 positive lok overexpressing cells several hours post-IR indeed reflected failure of cell cycle re-entry, or alternatively might be explained by IR-induced lok-mediated apoptosis. To this end, although no signs of cell death were observed in un-stressed discs where lok was overexpressed (Fig. 2), we repeated the experiment, now addressing apoptosis induction by IR-stress with 6 h recovery time. Indeed, in response to IR-stress accumulation of cleaved Caspase-3 was detected most prominently in the central part of the wing disc in all genotypes (see Additional file 1: Figure S2). In contrast to the discs overexpressing either lacZ, mei-41 or grp, however, lok overexpressing cells showed a much stronger apoptotic response than any of the others (see Additional file 1: Figure S2). We conclude that the lower numbers of PH3 positive cells present in lok overexpressing tissue may result primarily from an increase in apoptosis rather than from a delay of cell cycle re-entry.

Mobility shift of Grp after mei-41 overexpression

Using Drosophila Schneider S2 cells, it has been demonstrated that Grp is phosphorylated in response to DNA damage or incomplete DNA replication, and that this phosphorylation was dependent on the presence of Mei-41 [28]. Presumably, the Drosophila ATR homologue Mei-41 phosphorylates Grp in the process of DDR, comparable to what is known from other organisms like yeast, Xenopus and also mammals [2, 3]. As we had seen a dose dependency for mei-41 on checkpoint induction (Fig. 2), we wondered, whether the sole overexpression of mei-41 may suffice to induce Grp phosphorylation, i.e. induce a bona fide DNA damage response. To this end, mei-41 was ubiquitously overexpressed together with a HA-tagged form of Grp using da-Gal4. Larval protein extracts from imaginal discs were separated by Phos-Tag™ PAGE to increase the separation of phosphorylated from unphosphorylated proteins, followed by Western blotting. For negative control, larvae just overexpressing HA-tagged Grp were used; for positive control such larvae had been subjected to 40 Gy IR-stress. Indeed, overexpression of mei-41 resulted HA-tagged Grp to migrate more slowly compared to the negative control, but similar to the one detected 1 h post-IR (Fig. 5). These results suggest, that overexpression of mei-41 is sufficient to induce DDR at low levels, i.e. to activate, respectively phosphorylate its downstream target Grp in vivo.
Activation of the p53R-GFP biosensor can be achieved by overexpression of mei-41

As outlined above, mei-41 mutants display checkpoint defects that are only matched by grp; lok double mutants, implying that Mei-41 may not only act on Grp but also, to a lesser degree, on Lok [16, 25, 28]. Genetic and molecular data imply that Lok, but not Grp, is an activator of p53 in response to DNA damage in Drosophila [16, 25]. To investigate a potential cross-regulatory effect of the Drosophila ATR homologue Mei-41 on p53 activity we made use of a p53R-GFP biosensor, where p53 activation is reflected by nuclear GFP accumulation [36]. This p53R-GFP biosensor is activated during meiosis in the female germline, as well as in response to genotoxic stress in somatic tissues [36–38]. Thus, it is well suited to determine whether mei-41 overexpression alone may suffice to effect p53 activation. UAS-mei-41 was ubiquitously overexpressed with da-Gal4 in the background of the p53R-GFP reporter, and the giant salivary gland nuclei were examined for accumulation of GFP. GFP signal intensity was measured, using nuclear Putzig (Pzg) protein [39] as internal standard. For control, p53R-GFP nuclear localization was evaluated after ectopic induction of either Lok or Grp as well. As expected from its ability to activate p53 [16, 18, 19], overexpression of lok caused a strong nuclear GFP signal, which was well above lacZ control, demonstrating the reliability of our test system (Fig. 6a, b). Interestingly, overexpression of mei-41 was sufficient to induce nuclear p53R-GFP accumulation, albeit much weaker than that of lok (Fig. 6a, b). In contrast, Grp was unable to trigger measurable GFP signals (Fig. 6a, b). In addition to the visual assessment, qRT-PCR measurements of GFP expression levels were conducted on larval imaginal discs. They uncovered a 27-fold and 5-fold increase of p53-GFP reporter expression, respectively, in response to ubiquitous lok and mei-41 overexpression in comparison to the lacZ control (Fig. 6c). These data provide unambiguous evidence that ectopic Mei-41 is able to induce p53 activity. Whether this activation is direct, as shown for ATM as well as ATR (reviewed in [6, 8]), or indirect via Lok, requires further investigations. Our newly established UAS-mei-41 construct is well suited to facilitate future analyses.

Fig. 6 Response of the p53R-GFP reporter on mei-41 overexpression. (a) In response to p53 activation, nuclear GFP is expressed from the p53R-GFP reporter [36]. This system was used to assay p53 activation in consequence of the overexpression of either lok, mei-41 or grp; lacZ served as control. Salivary glands were analyzed; their nuclei visualized with the nuclear marker Pzg [39]. In contrast to lacZ and grp, overexpression of lok and to a lesser degree mei-41 resulted in a robust induction of the p53R-GFP reporter. Size bar represents 100 μm in all panels. (b) Quantification of nuclear p53R-GFP intensity was determined relative to the mean intensity of the nuclear marker protein Pzg (n = 84). Induction of lok strongly induced p53-GFP nuclear accumulation. Also mei-41 caused a significant nuclear accumulation of p53R-GFP, whereas grp did not. *** p < 0.001; ** p < 0.01; ns, not significant according to ANOVA two tailed Dunnet’s approach. (c) Expression levels of GFP were quantified by qRT-PCR in third instar larvae. Overexpression of lok and mei-41 with da-Gal4 considerably increased p53R-GFP reporter gene activity in relation to the lacZ-control: lok 27.5-fold, mei-41 4.9-fold and grp 1.6-fold. Data were assembled from four biological and two technical replicates. Mini-max depicts 95% confidence, median corresponds to expression ratio. As reference genes, cyp33 and Tbp were used. Efficiencies for GFP (0.91), for cyp33 (0.96) and Tbp (0.95) were accounted for in determining relative quantities [44].
Discussion
In this work, we show that mei-41 and grp act on cell cycle regulation in a dose dependent manner, conforming to the requirements of mei-41 and grp in G2/M checkpoint function [24, 25]. Whereas developmental patterning is unaffected, a cell cycle arrest and proliferation defects result from the overexpression of mei-41. Moreover, we unambiguously show that p53 activity is induced by ectopic Mei-41. We do not know, however, whether this activation is direct, as shown for ATM as well as ATR (reviewed in [6, 8]), or indirect via Lok. The defects resulting from Mei-41 overexpression are very mild, however, both regarding cell cycle delay, cell proliferation or apoptosis, compared to a normal irradiation response. There are several explanations. For example, during genotoxic stress Mei-41 kinase is activated by phosphorylation, which does not follow the overexpression. Moreover, Mei-41 heterodimerizes with its obligate partner Mus 304 [25] during damage response [6, 8]. In our overexpression experiments, Mus 304 may be limiting, allowing only for the mild effects observed. Finally, excessive amounts of Mei-41 kinase resulting from the overexpression may generally interfere with other aspects of cell growth and proliferation.

Conclusions
Overexpression of the Drosophila ATR homologue mei-41 during imaginal development is sufficient to initiate a cellular response resembling the DNA damage response, which is reflected by the induction of a G2/M checkpoint, growth retardation, apparent phosphorylation of the effector kinase Grp (Drosophila Chk1), as well as the activation of p53. The rather subtle effects presumably result from a lack of Mei-41 kinase activation by genotoxic stress, as IR resulted in long-lasting checkpoint activation. Moreover, the observation of p53 activation in response to mei-41 overexpression indicates cross-talk of ATR/ATM pathways also in Drosophila that may involve the effector kinase Lok (Drosophila Chk2).

Methods
Cloning of pUAST-mei-41 and generation of transgenic flies
Four fragments covering mei-41 were PCR-amplified from genomic DNA, and subcloned individually in pBT vector (Stratagene, La Jolla, USA) (fragments I-III) or pGX-attP [40] (fragment IV) to obtain the following subclones: pBT 2.3 kb NgoMIV/Xmal - EcoRI fragment I (translation start to EcoRI); pBT 2.2 kb EcoRI-Xhol fragment II; pBT 2.7 kb Xhol-Acc65I fragment III; pGX-attP 0.8 kb Acc65I - BglII fragment IV (translation stop). Subsequently fragments I-III were fused by successive cloning in pBT vector. The insert was excised as SacII/Acc65I fragment and cloned into likewise opened pGX-attP harbouring fragment IV. The full length genomic mei-41 construct was excised with BamHI/AvrII and subsequently shuttled into BglII/XbaI of pUAST vector [34] and sequence verified. Three independent transgenic fly lines were established by P-element mediated germline transformation [41]. All three lines were functionally tested for ectopic expression of mei-41 RNA by in situ hybridization of imaginal discs. The subsequent functional assays were performed with line UAS-mei-41 (3.3) inserted on the third chromosome.

RNA expression analyses: In situ hybridization and qRT-PCR
In situ hybridization on larval wing discs was performed with digoxigenin-labelled DNA probes of mei-41 according to standard protocols [42]. Quantitative RT-PCR was performed as outlined earlier [38, 43]. With the PolyA-tract System 1000 kit (Promega Mannheim, Germany) poly(A)+ RNA was isolated from 20 total third instar larvae for the quantification of UAS-mei-41 expression, and from imaginal discs only attached to the mouth hook from 20 third instar larvae for pS3R-GFP biosensor quantification. Real time qPCR was conducted with Blue S'Green qPCR kit (Biyozym, Hessisch-Oldendorf, Germany) on 6 ng of cDNA in 10 μl end volume using MIC magnetic induction cycler (bms, Pots Point, Australia) including target and no-template controls. Absence of genomic DNA was tested in a non-RT control. As internal references for mei-41 expression, βTub56D and gapdh2 were used, whereas cyp33 and Tbp served as internal references in the case of pS3R-GFP biosensor quantification. The references were selected based on variance and Cq values. Relative quantification of the data was performed with micPCR software Version 2.6.0 based on REST taking target efficiency into account [44]. At least three biological and two technical replicates were performed. The following primer pairs were used (5′ - > 3′):

| Primer | Sequence |
|--------|----------|
| mei-41 | upper, CTC CTG CAA GAC TTT AAT TCG CTC AC lower, GCG TTG GCT GCA TGT ACT TCT CA |
| βTub56D | PP17563 DRSC FlyPrimer bank [45] |
| gapdh2 | PP2976 DRSC FlyPrimer bank [45] |
| GFP | upper, TCAAGGACAGCGCAACTA CAAGAC lower, TCACCTTGATGCCGTTCTT CTGC |
| cyp33 | PP14577 DRSC FlyPrimer bank [45] |
| Tbp | PP1556 DRSC FlyPrimer bank [45] |

Fly work and immunochromene
Flies were raised on standard corn-molasses food at 25 °C. The following strains were used: da-Gal4 (BL55849); en-Gal4-GFP [46], UAS-lacZ (BL8530), UAS-mei-41 (this
work), UAS-grapes.ORF.3xHA (F000934; obtained from FlyORF; Zürich, Switzerland) [47], UAS-chk2 (lok) (gift of U. Abdu) [48], UASp-lacZ [49], p53R-GFP [36]. Staining of third instar wing imaginal discs or salivary glands was done according to standard protocols as described earlier [38, 50] using the following antibodies: rabbit anti-GFP (1:100; Santa Cruz Biotech, Dallas, USA), guinea pig anti-Pzg (1:1000) [39], rabbit anti-cleaved Caspase-3 (1:250; Cell Signaling, Germany) and rabbit anti-Phospho-Histone H3 (PH3) (1:50; Cell Signaling, Germany). Secondary antibodies from goat or donkey, coupled to FITC or Cy3, were obtained from Jackson Immuno-Research Laboratories (Dianova, Hamburg, Germany). Larval tissue was documented by confocal microscopy using a MRC1024 confocal scan head coupled to a Zeiss Axiohot (Carl Zeiss AG, Oberkochen, Germany) and LaserSharp 2000 imaging software. Pictures were compiled with Corel Photo Paint and Corel Draw software.

Ionizing radiation (IR) treatment and Phos-Tag™ based mobility shift detection

Third instar larvae were irradiated with 40 Gy using Elektra Versa HD linear accelerator (Elektra Instrument AB; Stockholm; Sweden) at the Marienhospital Stuttgart. To investigate phosphorylation of Grp protein, 25 third instar larvae of the genotype da-Gal4/+; da-Gal4/ UAS-grp-HA were irradiated with 40 Gy. After 1 h recovery time, imaginal discs connected to the mouth hook were isolated and homogenized in 50 μl binding buffer (20 mM HEPES pH 7.6, 150 mM KCl, 2.5 mM MgCl2, 10% glycerol, 0.05% NP-40, 1 mM DTT, ROCHE complete ULTRA protease inhibitor mini tablet). The unirradiated controls as well as the da-Gal4/+; da-Gal4/ UAS-grp-HA UAS-me1-41 larval tissues were treated likewise. The homogenates were separated in 10% SDS-PAGE including 50 μM Phos-tag™ Acrylamide solution (#AAL-7, Wako Chemicals GmbH, Neuss, Germany) and 100 μM MnCl2 at 70 V for 22-24 h at 8 °C. After blotting on PVDF membrane (BioRad, Munic, Germany), HA-tagged Grapes was detected with rat anti-HA (1:2500, Roche Diagnostic, Basel, Switzerland), and secondary anti-rat antibody coupled to alkaline phosphatase (1:1000; Jackson Immuno-Research Laboratories via Dianova, Hamburg, Germany).

Documentation and statistical evaluation of larval and adult tissue

Cells in M phase within the posterior compartment were counted based on PH3 signals. The posterior compartment was determined by GFP labelling from en-Gal4-GFP [46]. Cell number was related to total size of the respective wing discs using ImageJ. p53R-GFP expression in salivary glands was examined by measuring signal intensity of 12 nuclei from seven different glands each (n = 84 nuclei), using the mean intensity of Pzg signals as internal standard. Wings from female flies were dehydrated in ethanol, mounted in Euparal (Roth, Karlsruhe, Germany) and documented with an ES120 camera (Optronics, Goleta CA, USA) connected to a Zeiss Axiohot (Carl Zeiss AG, Jena, Germany) using Pixera Viewfinder software, version 2.0. Female flies were etherized before taking pictures from the heads with an ES120 camera coupled to a Leica Wild M3C Stereomicroscope (Leica, Wetzlar, Germany). Size of female eyes (UASp-lacZ/+; ey-Gal4/+ and ey-Gal4/+) or wings (UASp-lacZ/+; en-Gal4-GFP/+ and en-Gal4-GFP/+; UAS-me1-41/) was measured using Image J. Statistical analysis was conducted by ANOVA using a two-tailed Tukey-Kramer or Dunnett’s test for multiple comparisons. ***p < 0.001 highly significant; **p < 0.01 very significant; *p < 0.05 significant; not significant (ns) p > 0.05. Box plots were compiled using the online plotting tool BoxPlotR (http://shiny.chemgrid.org/boxplotr/).

Additional file

**Additional file 1:** Analysis of apoptosis. (PDF 9148 kb)

**Abbreviations**

ATM: Ataxia-Teleangiectasia Mutated; ATR: ATM and Rad3-related; Chk1, Chk2: Checkpoint kinase 1, 2; da: Daughterless; DDR: DNA damage response; DSB: DNA double strand breaks; en: Engrailed; GFP: Green fluorescent protein; grp: Grapes; IR: Ionizing radiation; lok: LokI; mei-41: Meiotic 41; PH3: Phospho-Histone H3

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**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article and its additional information file. Materials are available from the corresponding author on reasonable request.

**Authors’ contributions**

ACN and AP conceived and designed the experiments; FEB, MZ, AP and ACN collected and analyzed the data and performed the statistical analysis; ACN and AP wrote the manuscript; and all authors have approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.
Competing interests
The authors declare that there are no competing financial, personal, or professional interests.

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