Differential DNA damage signalling and apoptotic threshold correlate with mouse epiblast-specific hypersensitivity to radiation

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ABSTRACT

Between implantation and gastrulation, the mouse pluripotent epiblast cells expand enormously and exhibit a remarkable hypersensitivity to DNA damage. Upon low dose irradiation, they undergo mitotic arrest followed by p53-dependent apoptosis, while the other cell types simply arrest. This protective mechanism, active exclusively after e5.5 and lost during gastrulation, ensures the elimination of every mutated cell before its clonal expansion, and is therefore expected to greatly increase individuals’ fitness.

We show that the insurgence of apoptosis relies on the epiblast-specific convergence of both increased DNA damage signalling and stronger pro-apoptotic balance. Although upstream Atm/Atr global activity and specific γH2AX phosphorylation are similar in all cell types of the embryo, 53BP1 recruitment at DNA breaks is immediately amplified only in epiblast cells after ionizing radiation. This correlates with a rapid epiblast-specific activation of p53 and its transcriptional properties. Moreover, between e5.5 and e6.5, epiblast cells lower their apoptotic threshold by overexpressing pro-apoptotic Bak and Bim and repressing the anti-apoptotic Bcl-xL. Thus even after low dose irradiation, the cytoplasmic priming of epiblast cells allows p53 to rapidly induce apoptosis via a partially transcription-independent mechanism.
INTRODUCTION

Maintenance of genomic integrity is important in stem cells for tissue differentiation and homeostasis (Blanpain et al., 2011). It is particularly crucial in the epiblast (Epi) which undergoes an explosive expansion and has the potential to contribute to all tissues including germline. Acquisition of post-zygotic mutations during embryonic development may alter organogenesis or favour tumour formation later in life (Greaves and Wiemels, 2003; Hafner et al., 2011; Marshall et al., 2014; Mori et al., 2002). In spite of their major importance, cell growth regulation and DNA damage response during embryogenesis remain poorly understood.

One stage of the early mouse development is particularly critical. As the embryo implants, it is composed of extra-embryonic annexes and a restricted pool of slow-dividing pluripotent cells, the Epi, from which the embryo proper will originate (Takaoka and Hamada, 2012). Soon after implantation at embryonic day 4 (e4.0), the Epi enters a phase of intense proliferation in order to produce the cells necessary for gastrulation, which initiates at e7.0 (Solter et al., 1971; Stuckey et al., 2011). From 120 cells composing the Epi of an e5.5 post-implantation embryo, it is estimated that about 14,500 cells are generated by the end of the gastrulation (Snow, 1977). Thus at e6.5-7.5, the Epi cells are dividing very actively and might therefore be particularly prone to accumulate mutations, which would be detrimental for further development. In fact, the post-implantation growth of the Epi corresponds to a very sensitive developmental window during which deletion of several genes instrumental for genome integrity is lethal (Brown and Baltimore, 2000; Dobles et al., 2000; Hakem et al., 1996; Jeon et al., 2011; Kalitsis et al., 2000). Remarkably, it has been reported that during this phase the mouse embryo is hypersensitive to DNA damage and undergoes apoptosis without cell cycle arrest when irradiated even with low doses (Heyer et al., 2000). This protective mechanism, eliminating every potentially dangerous mutation-carrier before its clonal expansion, is expected to greatly enhance individuals' fitness.

Upon induction of DNA double-stranded breaks, the DNA damage response is initiated on the local chromatin, spread into adjacent sites and amplified, ultimately leading to the activation of effector proteins, such as p53 (Panier and Boulton, 2014).
Depending on the cell context, the activation of p53 triggers arrest, repair, differentiation, senescence, or apoptosis (Carvajal and Manfredi, 2013). Several studies performed in human embryonic stem cells or peri-gastrulation embryos revealed that various mechanisms may prime stem cells for apoptosis, such as the presence of constitutively active Bax at the Golgi (Dumitru et al., 2012), an imbalance between pro- and anti-apoptotic factors lowering the apoptotic threshold (Liu et al., 2013), or the control of Bim expression by miRNAs which would allow a fast release upon stress induction (Pernaute et al., 2014).

Here, we characterize in mouse peri-gastrulation embryos the cell types heterogeneity of the radiation-induced DNA damage response. After e5.5, while all cell types undergo mitotic arrest upon exposure to ionizing radiations, only the Epi cells and their primitive streak (PrS) progeny exhibit an apoptotic response. We show the epiblast-specific convergence of an amplified DNA damage signalling at the level of 53BP1 recruitment, resulting in a fast and specific activation of p53, and a cytoplasmic priming due to increased Bak and Bim and decreased Bcl-xL expression, causing DNA damage-induced cell death.

RESULTS

The Epi of peri-gastrulation embryos is highly proliferative and shows replicative stress

First, we measured the mitotic indexes (MI) of the Epi, visceral endoderm (VE) and extra-embryonic ectoderm (ExE) at e6.5 and e7.5, using Ser10-phosphorylated Histone H3 (P-H3) as a mitotic marker (Hendzel et al., 1997), and Oct4 (Palmieri et al., 1994) and T-Brachyury (Herrmann et al., 1990) as specific markers of the Epi at e6.5 and of the PrS at e7.5, respectively. For each cell type, the MI was calculated as the percentage of the P-H3+ among dapi+ cells. Both at e6.5 and e7.5, the MI of the Epi was significantly higher than that of extra-embryonic tissues. At e6.5, we measured an MI of 13.2% in the Epi versus 5% and 2% in the ExE and VE, respectively (Fig. S1A) and at e7.5, an MI of 10.7%, versus 4% and 5.5% in the ExE and the VE (Fig. S1B). At e7.5, among the T-Brachyury+ cells, those localized in the embryonic region had an MI of 6.5% while those in the extra-embryonic mesoderm territories had an MI of 3.5% (Fig. S1B). This confirms that at peri-gastrulation stage the whole embryo is expanding consistently in both embryonic and
extra-embryonic compartments, but the Epi and the embryonic PrS are the fastest dividing tissues.

Rapid cell cycles may induce replicative stress, stalling and collapse of replication forks or interference between transcription and replication machineries (Zeman and Cimprich, 2014). The phosphorylated form of the histone H2AX (γH2AX), which has mostly been used to detect DNA damage foci at double-stranded breaks, i.e. after irradiation, is however also accumulated on the chromatin under replicative stress. Therefore γH2AX may be used as a marker of replicative stress in fast dividing cells, such as cancer cells (Seo et al., 2012; Ward and Chen, 2001). In untreated e6.5 embryos, we detected endogenous γH2AX both in the Epi and in the ExE, suggesting that these tissues are dealing with replicative stress (Fig. S1C). In contrast, VE cells, with a lower MI, appeared negative for this marker. The basal phosphorylation of H2AX observed in the Epi and ExE, however, does not correlate with the formation of 53BP1 foci (Fig. S1D), which is a documented marker of DNA damage (Anderson et al., 2001). This result supports the hypothesis that in the early embryos, detection of γH2AX is linked to replicative stress, and not to the activation of the DNA damage response pathway.

**At e6.5 ionizing radiation induces general mitotic arrest and Epi-specific apoptosis**

To explore the effect of exogenous DNA damage on cell cycle and survival, e6.5 embryos were X-rays irradiated with 5 Gy and cultured for 10 minutes to 2 hours before fixation and immunostaining. Unlike a previous report (Heyer et al., 2000), the MI of treated embryos was decreased to 2.7% 30 minutes after irradiation and 0.3% after 1 hour, while non-irradiated samples had an MI of 9.5% (Fig. 1A). The mitotic arrest was observed in both Epi and extra-embryonic tissues, suggesting that at e6.5, all embryo cells have active cell cycle checkpoints, including the fast-cycling Epi cells.

In addition, irradiation quickly induced apoptosis in the embryos, evidenced by a cleaved Caspase-3 (Cl Casp-3) staining (Fig. 1B). Indeed, the apoptotic index of the embryos drastically increased from 1-2.5% in non-irradiated controls to 15.5% 1.5 hour after 5 Gy irradiation and 29% after 2 hours. Using a 10-fold lower radiation dose of 0.5 Gy, we measured apoptotic indexes of 10.2% and 16.7% at respectively
1.5 and 2 hours after exposure to radiation (**Fig. 1B**). Finally, Caspase-3 cleavage after irradiation was associated with Bax activation (**Fig. 2A**), indicating that the DNA damage-induced apoptosis observed in the e6.5 embryos is driven by the mitochondrial pathway, in agreement with reports in other cell types (Hsu et al., 1997).

**Radiation hypersensitivity of peri-gastrulation embryos is cell-type and stage-specific**

DNA damage-induced apoptosis was cell type-specific. Indeed, 2 hours after exposure to 0.5 Gy ionizing radiations (**Fig. 2A**), or 1.5 hours after 5 Gy (**Fig. S2**), only the Epi cells stained positive for Cl Casp-3 and active Bax. Therefore Epi cells are more sensitive than the adjacent ExE and VE cells to the same physical stress.

We then wondered whether the specific sensitivity of the Epi to irradiation can be observed at an earlier developmental stage or if it is acquired during its maturation. In e5.5 embryos, irradiation with 0.5 Gy did not trigger apoptosis (**Fig. 2B, upper panel**), but it induced a general mitotic arrest (**Fig. 2B, lower panel**). Indeed, P-H3 staining was completely negative in treated embryos, while controls showed an MI of 7.6%. We conclude that DNA damage signalling occurs in e5.5 embryos which respond with a mitotic arrest. However at this stage Epi cells are resistant to apoptosis.

Conversely, in the gastrulating e7.5 embryo, the PrS cells, which originate from the Epi but are committed toward differentiation, showed two types of behaviour upon exposure to 0.5 Gy radiation. Among the **T-Brachyury** population located in the embryonic area (the mesendoderm progenitors), posterior cells underwent massive apoptosis (red double arrow) while those which had reached the anterior-most region of the embryo, were clearly Cl Casp-3 (green double arrow) (**Fig. 2C**). The cells contributing to the extra-embryonic mesoderm were also Cl Casp-3. This result suggests that cells lose their hypersensitivity as they migrate away from the streak and differentiate. As in e6.5 embryos, ExE and VE cells did not show hypersensitivity, whereas Epi cells underwent massive apoptosis.

Altogether, our data argue that at e6.5-7.5, the Epi and PrS are hyperproliferative and hypersensitive to DNA damage. To elucidate the molecular basis of this differential response to irradiation, we studied the DNA damage response
cascade in e6.5 embryos, which leads to Epi-specific Bax activation and Caspase-3 cleavage.

**The Atm/Atr pathway is equally active in the Epi, VE and ExE upon irradiation**

Upon DNA damage, the major upstream kinases Atm and Atr phosphorylate a large number of substrates at an S/TQ consensus sequence (Kim et al., 1999). In e6.5 embryos, immunofluorescence using an anti-phosphorylated S/TQ motif (P-S/TQ) antibody showed a similar staining in the Epi, VE and ExE after exposure to 0.5 Gy radiation (Fig. 3A). The same result was observed upon a 10-fold higher irradiation dose (5 Gy) (Fig. S3A). These results suggest that the Atm/Atr pathway is equally active in all three tissues of the embryo. Of note, the signal observed in the non-irradiated control embryos corresponds to a staining of the mitotic chromosomes in the Epi cells, independent of exogenous DNA-damage (see the control staining in Fig. S3B).

In addition, among Atm/Atr targets, H2AX is rapidly phosphorylated at the double-stranded breaks and typical γH2AX foci can be detected. Similarly to P-S/TQ, 10 minutes after 0.5 Gy irradiation, γH2AX foci formation was comparable in Epi and extra-embryonic cells (Fig. 3B). Quantifications of the amount of fluorescence per cell showed no significant difference between cell types. Therefore no difference in the activity of Atm/Atr between Epi and extra-embryonic cells was detectable, neither when testing P-S/TQ motifs, nor when investigating the specific phosphorylation of H2AX.

Finally, although Atm is considered as the kinase of choice in the response to double stranded breaks, it was not essential to radiation-induced apoptosis. Indeed, we observed a strong Cl Casp-3 signal in Atm-null embryos after 0.5 Gy irradiation (Fig. 3C). Unlike previous findings (Heyer et al., 2000), our result agrees with most of the literature, where Atm deficiency was shown to sensitize cells to DNA damage (Williamson et al., 2010). Since very few cells remained in the Epi of Atm-null embryos 2 hours after irradiation, the TUNEL analysis presented by Heyer et al. may have not detected them. We conclude that radiation-induced apoptosis in the Epi does not strictly depend on Atm, which may be compensated by Atr and/or DNA-PK kinases (Callen et al., 2009; Gurley and Kemp, 2001; Siliciano et al., 1997).
53BP1 focal recruitment at double-stranded breaks is amplified in the Epi

In the absence of any differential activation of the upstream DNA damage signalling, we then analysed 53BP1, which acts downstream of Atm/Atr and H2AX phosphorylation. 53BP1 is essential for the activation of the effectors Chk2 and p53 (Wang et al., 2002; Ward et al., 2003). Widely expressed in the nucleus under normal conditions, it is recruited at the DNA damage foci upon irradiation. Remarkably, 10 minutes after 0.5 Gy irradiation, clear typical 53BP1 foci were detectable in the Epi (Fig. 3D), but not in the VE and ExE cells, where the staining was more diffuse and foci weaker and smaller. In fact, quantification of the amount of fluorescence per cell revealed a 3.5-fold stronger response in Epi cells versus extra-embryonic tissues. This difference persisted over time since the same observation was made 30 minutes and 1 hour after irradiation (Fig. S4A). A similar result was obtained 30 minutes after a 10-fold higher radiation dose (Fig. S4B). Since 53BP1 basal level is the same in the Epi, VE and ExE, its higher focal recruitment in Epi cells cannot be due to overexpression (Fig. S1D). Therefore, the Epi, ExE and VE cells present different signalling activities on the site of DNA lesions, since in Epi cells γH2AX-positive foci are more efficient in recruiting 53BP1.

After the appearance of γH2AX-positive foci, several enzymes are required to set up the epigenetic environment responsible for 53BP1 recruitment: Rnf8, Rnf168, Tip60, Hdac1, Hdac2, Nsd1, Nsd2 and UbcM4. To get insight into the molecular basis of Epi-specific 53BP1 recruitment, we compared the endogenous expression of these genes in the Epi versus extra-embryonic tissues. To this end, we dissected non-irradiated embryos at the embryonic/extra-embryonic border, extracted RNA and performed qRT-PCR in the two independent Oct4⁺- and Oct4⁻-enriched cell populations, below designated distal and proximal portions, respectively (Fig. S5A-C). For feasibility and ethical needs to restrict the number of embryos, we used e7.5 embryos. Rnf8, Tip60, Nsd1, Nsd2 and UbcM4 mRNAs were not differentially expressed (Fig. S5D). However, Rnf168, Hdac1 and Hdac2 were overexpressed in the distal cell population at respectively 1.35-, 1.3- and 1.4-fold, closer to our personal experience of biological relevance (1.5). Therefore the high responsiveness of the Epi chromatin to radiation-induced DNA damage may also depend on the over-expression of these enzymes.
Irradiation induces Epi-specific activation of p53 and its transcriptional properties

P53 is the ultimate coordinator of DNA damage sensing and cell fate decision (Kracikova et al., 2013). The stronger focal recruitment of 53BP1 at the DNA breaks suggests that Epi cells can achieve a greater amplification of the DNA damage signalling, resulting in differential activation of its main effector p53. Among the various post-translational modifications of p53, we chose to study the phosphorylation of Ser18 (which corresponds to Ser15 in Human p53) because it is central in its activation and triggers a sequential series of additional phosphorylation events in the protein (Carvajal and Manfredi, 2013; Dumaz et al., 1999; Saito et al., 2002; Siliciano et al., 1997).

Remarkably, Ser18-phosphorylated p53 (P-p53) was detected in 100% of the Epi cells as early as 10 minutes after 0.5 Gy irradiation (Fig. 4A), while in ExE cells a consistent signal for P-p53 was observed only after 1 hour. At this time, still only a fraction of the VE cells were P-p53+. Irradiation of the embryos with a 10-fold higher dose (5 Gy) led to a similar observation: although slightly faster, the activation of p53 in the ExE and VE was reproducibly delayed compared to the Epi (Fig. S6 A). This result is relevant since the irradiation-induced apoptosis of the Epi is p53-dependent. Indeed, we confirmed previously reported data (Heyer et al., 2000) showing that apoptosis is drastically reduced in p53-null embryos 1 hour after 5 Gy-irradiation (Fig. 4B). Therefore we conclude that Epi cells' hypersensitivity toward apoptosis may rely on their unique capacity to rapidly activate p53 upon DNA injury. In addition, immunoreactivity for p53 total protein was as intense in the ExE, VE and Epi (Fig. S6B), suggesting that the abundant P-p53 found in the Epi is the consequence of the increased DNA damage response (in particular, amplified 53BP1 focal recruitment) and not of endogenous p53 overexpression.

We then tested whether the rapid phosphorylation of p53 correlated with a transcriptional induction of its target genes. We therefore extracted RNA from 0.5 Gy irradiated e7.5 embryos and performed RT-PCR to assay the expression of some p53 targets. Embryos were dissected at the embryonic/extra-embryonic border prior to lysis, as described before (Fig. S5A-B). In the distal cell population (containing the Epi cells), p21 and Noxa genes were induced respectively 2.35 and 1.5-fold 1 hour after 0.5 Gy irradiation (Fig. 4C), while Bax and Mdm2 were not changed to a significant level (1.20 and 1.17-fold respectively). Bim, which is not known as a p53
target, was unaffected (1.12-fold). On the other hand, no increase in the expression of p21 and Noxa was observed in the proximal cell population.

**p53-induced apoptosis is transcription-independent**

Since a fraction of p53 also non-transcriptionally controls the mitochondrial apoptotic pathway (Vaseva and Moll, 2009), we tested the role of transcription for DNA damage-induced apoptosis. E6.5 embryos were incubated in α-Amanitin-containing serum for 30 minutes prior to 5 Gy irradiation and further cultured for 90 min in α-Amanitin. The apoptotic phenotype was then analysed using a Cl Casp-3 immunostaining. Treated embryos exhibited a lower average apoptotic index than the controls incubated in the presence of DMSO (Fig. 4D), but the reduction was modest and not fully penetrant since only one out of four embryos presented an apoptotic index significantly reduced to 7%. Hence, control of apoptosis by p53 in the Epi is only partly dependent on transcription.

Thus e6.5 Epi cells exhibit a stronger DNA damage signalling. Their amplified local response on chromatin lesions at the level of 53BP1 recruitment correlates with a unique capacity to rapidly activate p53 upon DNA damage induction. Although p53 transcriptional program is quickly activated in the Epi, transcription-independent properties of p53 are also involved in the apoptotic process.

**p53 is activated in e5.5 Epi cells upon irradiation**

Since the Epi cells are prone to undergo apoptosis upon low dose irradiation and maintain this hypersensitivity as they enter the PrS, but lose it when they migrate away and differentiate, we thought that the peculiar epigenetic environment of pluripotent cells might be responsible for the amplification of the DNA damage signalling. This prompted us to analyse p53’s behaviour in e5.5 embryos, which possess pluripotent Epi cells, but are resistant to irradiation. Exposure of e5.5 embryos to 0.5 Gy induced the Epi-specific phosphorylation of p53 (Ser18) as early as 10 minutes after irradiation, as observed in e6.5 embryos (Fig. 4E). Hence this particularly rapid activation of p53 seems to be a common feature of pluripotent Epi cells. Importantly, this result shows that p53 activation is essential but not sufficient to explain Epi-specific radiation-induced apoptosis at e6.5.
Epi cells are primed for apoptosis

We reasoned that if the DNA damage-induced apoptosis observed in the Epi is not dependent on transcription, it must depend on a reservoir of constitutive pro-apoptotic effector proteins. In fact, the comparative analysis of transcripts coding for pro- and anti-apoptotic factors in e7.5 distal and proximal cell populations revealed that pro-apoptotic Bim and Noxa were over-expressed in the Epi-containing cell population 2- and 9-fold, respectively, while Bak, Bax and Bid were not affected (Fig. 5A and S5A-B). On the other hand, the anti-apoptotic factors Bcl-xL and Mcl-1 did not exhibit any differential expression. In addition, immunoblots of e7.5 distal and proximal cell populations showed equal expression of Bax but showed a 2-fold higher level of Bak and Bim in the distal cells (Fig. 5B and S5A-C). On the contrary, Bcl-xL was down-regulated 1.9-fold in this tissue. Unfortunately, no antibody was available to test for Noxa expression. Therefore, at e7.5, Epi cells express higher levels of pro-apoptotic factors Noxa (at the RNA level), Bak (at the protein level) and Bim (at both RNA and protein levels), while the anti-apoptotic Bcl-xL is down-regulated (at the protein level).

Since these analyses were performed in e7.5 embryos, we used immunofluorescence to test the expression of Bak, Bim and Bcl-xL at e6.5. Interestingly, Bak staining was stronger in the Epi cells and largely colocalized with the Mito-ID mitochondrial marker (Fig. 5C). We also observed a striking difference of intensity for Bim immunostaining, which was much weaker in the ExE, and completely absent from the VE (Fig. 5D). Unlike Bak, Bim signal was more diffuse and colocalized more rarely with Mito-ID dye, suggesting that Bim is involved both in mitochondrial and cytosolic complexes in Epi cells. Conversely, Bcl-xL showed a stronger expression in the extra-embryonic tissues and was found both at the mitochondria and in the cytosol (Fig. 5E). In conclusion, these results suggest that the apoptotic threshold is lower in the Epi than in the extra-embryonic cell types, which primes Epi cells to rapidly undergo apoptosis upon p53 activation.

Epi cytoplasmic priming is set between e5.5 and e6.5

At e5.5, on the other hand, the expression patterns of Bak, Bim and Bcl-xL were different. In fact, Bak was detected at extremely low levels at e5.5, therefore not demonstrating any overexpression in the Epi (Fig. 5F), while Bim was expressed evenly in the Epi and ExE (Fig. 5G) and Bcl-xL was detected with an equal intensity
in all cell types (Fig. 5H). This observation contrasts with the results obtained in e6.5 and e7.5 embryos and suggests that the resistance of e5.5 embryos to irradiation, in spite of p53 activation in the Epi, is due to the lack of a permissive apoptotic balance. Thus the expression of pro- and anti-apoptotic factors is tightly controlled in the peri-gastrulation mouse Epi, allowing a remodelling of the apoptotic threshold between e5.5 and e6.5, via an increase of Bak and Bim expression and decrease of Bcl-xL.

DISCUSSION

As they enter a phase of high proliferation, mouse Epi cells show a remarkable sensitivity to exogenous DNA damage, undergoing apoptosis at very low radiation dose. This protective mechanism, operative in a rather sharp window of development, is expected to eliminate potentially dangerous mutation-carriers before their clonal expansion. This hypersensitivity toward apoptosis relies on the convergence of several regulatory mechanisms. Indeed, we have shown that Epi cells exhibit an amplification of 53BP1 DNA damage signalling on the chromatin, leading to a strikingly quick and specific activation of p53 in this tissue. In addition, after e5.5, Epi cells lower their apoptotic threshold by overexpressing pro-apoptotic Bak and Bim and down-regulating Bcl-xL, which primes and sensitizes cells to undergo apoptosis upon p53 activation (Fig. 6).

Hypersensitivity to radiations correlates with hyperproliferation in the Epi

Epiblast cells become hypersensitive as they enter a phase of intense expansion (Fig. S1A-B). Therefore, they may suffer from replicative stress, topological constrains of replication or replication/transcription conflicts (Helmrich et al., 2011; Postow et al., 2001). Part of the complexity of the DNA damage response derives from the important cross talk with the replication machinery. Components of the DNA damage response pathway also play roles in replication or replication/transcription coupling, independently of DNA damage induction (Gamper et al., 2012; Olcina et al., 2013; Shechter et al., 2004; Turinetto et al., 2012). Hence one could argue that the DNA damage response may be altered in Epi cells during this developmental window, because of their high proliferation rate. Indeed mitotic chromosomes are P-S/TQ positive in the Epi, independently of exogenous stress
induction (Fig. S3B) and Epi cells show noticeable levels of replicative stress (Fig. S1C-D). However we showed that the ExE exhibited similar levels of stress without exhibiting hypersensitivity. Therefore, the replicative stress alone cannot justify the differential apoptotic behaviour of the Epi upon low dose irradiation.

Epi cells possess a highly responsive chromatin

We found that Epi cells differ in their early DNA damage response, showing increased 53BP1 focal recruitment on the site of DNA breaks (Fig. 3D). Importantly, the measurement of similar amounts of γH2AX immunofluorescence in the Epi and extra-embryonic tissues rules out the possibility that radiations cause more damage in the Epi than in the other cell types (Fig. 3B). Instead, pluripotent cells are often reported to possess loose, plastic and highly dynamic chromatin, which may facilitate fast reorganization and response to environmental cues. In fact, Murga and colleagues have shown that the relaxation of chromatin by depletion or inhibition of Histone H1 in mouse ES cells triggers an amplified response to DNA damage (Murga et al., 2007). Differences in epigenetic backgrounds between Epi, ExE and VE may interfere with 53BP1 recruitment and/or stability upon DNA damage.

The increased 53BP1 recruitment is of particular interest when considering the position of 53BP1 in the cascade of the DNA damage response. In addition to its function as mediator of the DNA damage signalling, 53BP1 is also an amplifier of the response, as it can interact directly with the upstream sensor Rad50 (component of the MRN complex), generating a positive feedback loop, which allows spreading of the signalling along the broken chromatin and increased activation of Atm (Lee et al., 2010; Mochan et al., 2003). It is tempting to speculate that, as in MEFs (Fernandez-Capetillo et al., 2002), 53BP1 is essential to promote Atm, Atr or DNA-PK activation in Epi cells upon low dose radiation and amplifies a DNA damage signalling which might be otherwise insufficient to induce apoptosis. Thus, the specific focal recruitment of 53BP1 in Epi cells may sensitize them to low dose irradiation. However, how this is achieved and how this affects Atm, Atr and DNA-PK remains to be elucidated.
Rapid activation of p53 controls the apoptotic behaviour in the Epi

Following enhanced 53BP1 recruitment at double-stranded breaks, p53 is phosphorylated on Ser18 remarkably quickly in Epi cells: 10 minutes after 0.5 Gy irradiation, versus 1 hour in extra-embryonic tissues (Fig. 4A). To our knowledge, such a fast kinetic of p53 activation is unique and not reported so far. Thus we demonstrate a specific overall exacerbation of the DNA damage signalling in the Epi cells, both at the level of 53BP1 recruitment on the chromatin and at the level of p53 activation. These two phenomena are very likely functionally linked since 53BP1 is essential to support p53 activation by Atm (Panier and Boulton, 2014; Wang et al., 2002; Ward et al., 2003). We should emphasize however, that alternative Atm-independent mechanisms might account for p53 fast activation since Atm-null embryos were able to undergo apoptosis even more efficiently than wild type (Fig. 3C). In fact, Atr and DNA-PK kinases are also able to phosphorylate human p53 on Ser15 (Lees-Miller et al., 1992; Tibbetts et al., 1999). Accordingly, we found that p53 was equally activated in Atm-null and wild type embryos after 0.5 Gy irradiation (Fig. S7).

In the Epi, p53 activation results in the induction of its transcriptional properties: p21 and Noxa were up-regulated as early as 1 hour after 0.5 Gy irradiation (Fig. 4C). However p53 function in the apoptotic process cannot exclusively depend on its transcriptional activity, since embryos incubated in α-Amanitin were able to undergo an although milder apoptosis (Fig. 4D). Therefore we conclude that p53 must control the mitochondrial apoptotic pathway primarily through a post-transcriptional mechanism, reinforced by the transcriptional induction of p53 targets. Indeed, increasing evidence suggests that p53 first wave of action on apoptosis is exerted via direct interactions with Bax (Chipuk et al., 2004), Bak (Leu et al., 2004), Bcl-2 (Tomita et al., 2006) and Bcl-xL (Mihara et al., 2003), activating pro-apoptotic factors, or via inhibition of anti-apoptotic factors. Such a strategy for p53 action has been reported in human embryonic stem cells (Liu et al., 2013).

Although P-p53 immunoreactivity was reproducibly observed with a significant delay in the ExE compared to the Epi, 1 hour after irradiation it had reached a similar level of intensity in both tissues (Fig. 4A). But even 2 hours after irradiation, p53 activation in the ExE was not associated to apoptosis (Fig. 2A). Thus, exposure to p53 Ser18-phosphorylation for similar periods of time (1 hour) in the Epi and in the adjacent ExE leads to two different cellular outcomes. Similarly, the quick
activation of p53 in the Epi of e5.5 embryos was not followed by an apoptotic behaviour (Fig. 4E). This emphasizes the complexity of p53 activity, which is regulated via numerous post-translational modifications (Carvajal and Manfredi, 2013; Vousden and Prives, 2009). In e6.5 Epi cells, p53 may carry additional modifications, or meet different interacting partners, which would explain its differential effect on apoptosis. Alternatively, this result suggests that p53 activation is necessary but not sufficient for apoptosis induction and confirms the idea that cytoplasmic determinants are also critical for p53-induced apoptosis (Fig. 4D and (Kracikova et al., 2013)).

**Overexpression of Noxa, Bim and Bak lowers the apoptotic threshold of Epi cells**

The 9-fold higher expression of *Noxa* mRNA in the Epi-containing distal cell population compared to the proximal extra-embryonic annexes (Fig. 5A) agrees with the hypothesis that Epi cells require permissive cytoplasmic context for p53-dependent induction of apoptosis. Unfortunately, no antibody was available to test the levels of the expression of Noxa protein. Interestingly, Gutekunst and colleagues reported an Oct4-mediated Noxa up-regulation in testicular germ cell tumours, rendering them hypersensitive to cisplatin treatment (Gutekunst et al., 2013).

In addition, we showed that Bak and Bim proteins were more abundant in the Epi, while Bcl-xL was over-expressed in the ExE and VE (Fig. 5B-E). These results suggest that Epi cells have a low apoptotic threshold, which must sensitize them to apoptosis. Noteworthy, the timing of the cytoplasmic priming of the Epi cells correlates with the acquisition of hypersensitivity to radiations (Fig. 5F-H).

Although we found that Bax is highly expressed in the embryo (Fig. 5A-B), we were not able to detect its active form in the absence of irradiation (Fig. 1C), as reported in human embryonic stem cells (Dumitru et al., 2012). On the other hand, we did not detect any increase of *Bim* mRNA after exposure to ionizing radiation (Fig. 4C) as suggested by a previous study on mouse embryos (Pernaute et al., 2014). These discrepancies between *in vitro* and *in vivo* assays underline the multiplicity of mechanisms for which cells can opt in survival/death control.

In conclusion, we show that the hypersensitivity of Epi cells relies on the convergence of (1) an amplified DNA damage response and p53 signalling and (2) a high cytoplasmic priming toward apoptosis. Noteworthy, this low apoptotic threshold
may play an equally important role in other types of stresses, such as the lack of nutrients or growth factors, or Myc-driven cell competition (Claveria et al., 2013; Sancho et al., 2013). Thus, because of the high level of alertness of Epi cells at the e5.5-e6.5 transition, it is not surprising that so many mouse mutants are lethal at this specific stage, one representative example being Mdm2⁺⁻ (Jones et al., 1995): the depletion of p53’s inhibitor induces apoptosis after e5.5 in Epi cells, which we now show concomitantly express consistent levels of p53 and are very close to cross the apoptotic threshold. Future research should aim at unravelling the molecular link coupling the increase of proliferative rate, the commitment to differentiation and the tight regulation of pro-apoptotic balance, which occur at peri-gastrulation stage in the Epi.

MATERIAL AND METHODS

Embryos recovery and treatment

C57BL/6 embryos were extracted by standard procedures in M2 medium (Sigma-Aldrich) and cultured in ES-tested fetal calf serum at 37°C, 5% CO₂. X-ray irradiation was performed in a Faxitron RX-650 device. For α-Amanitin treatment, embryos were incubated in 50µg/mL α-Amanitin-containing serum (Sigma-Aldrich) for 30 minutes prior to irradiation and further cultured for 90 minutes in α-Amanitin. For p53 and Atm genotyping, embryos were lysed in a buffer containing 10mM Tris HCl pH 8.4, 50 mM KCl, 2 mM MgCl, 0.45% NP-40, 0.45% tween-20 and 3µg/µL of proteinase K. Primers sequences are detailed in supplemental material. Animal experiments were carried out following the regulations of the Italian law, upon authorization 109/2011-PR of the Italian Ministry of Health.

Whole mount immunofluorescence

Embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature (RT) and washed in PBS/0.1% Tween-20. After permeabilization in PBS/1% Triton X-100 for 30 minutes at RT, embryos were blocked in PBS/0.1% Triton X-100 /3% bovine serum albumin for 2 hours at RT. They were then incubated with primary antibodies diluted in blocking buffer for 1 hour at RT. The references of the antibodies are detailed in supplemental material. Embryos were washed and incubated with
secondary antibodies diluted in PBS/0.1% Triton X-100: Cy3-conjugated donkey anti-rabbit (Jackson Labs, 1:400) and Alexa Fluor 647-conjugated donkey anti-goat (Invitrogen, 1:400). In experiments using the Mito-ID Red dye, Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen, 1:400) was used. After washes, embryos were counterstained with dapi (0.5μg/mL) and eventually with Mito-ID Red (Enzo Life sciences, 1/2000). For active-Bax immunostaining, biotin-coupled primary antibody was detected with FITC-coupled streptavidin (Sigma-Aldrich, 100μg/mL). Confocal images were captured using a Leica TCS SP2 microscope. For the quantification of the immunofluorescence in γH2AX and 53BP1 foci, Z-stack images were acquired every 0.5μm. Using Fiji software, images were cropped at the border of the cells of interest on every confocal section and a maximum Z projection was performed. The amount of fluorescence per cell was calculated as the sum of (the area/foci x the mean intensity/foci) for each focus analysed by Fiji.

**RNA expression analysis**

Embryos were dissected at the embryonic/extra-embryonic border. For each condition, a minimum of 7 isolated embryonic or extra-embryonic poles were pooled and RNAs were extracted using RNeasy kit (QIAGEN). The retro-transcription was performed on 250ng of RNA with the Superscript III (Invitrogen). Samples, along with primers and LightCycler 480 SYBR Green I Master (Roche), were run in duplicates on a LightCycler 480 (Roche) machine. The results were normalized to Gapdh gene expression. Primers sequences are detailed in supplemental material.

**Immunoblotting**

Embryonic and extra-embryonic poles were pooled and protein extracts were obtained by lysis in Laemmli buffer in the presence of proteases inhibitors. References of the primary antibodies are detailed in supplemental material. Protein levels were quantified in ImageJ and normalized to Pcna.

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**Authors contribution.** Experiments were designed and performed by AL under the supervision of FB. This manuscript has been prepared by AL and FB.
REFERENCES


**Figure 1. Irradiation of e6.5 embryos induces cell-cycle arrest and apoptosis**

(A) Representative immunostaining of P-H3 and Oct4 in e6.5 embryos 10 minutes and 1 hour after 5 Gy irradiation (5 Gy IRR) and controls (No IRR), counterstained with dapi. The plot presents the quantification of the mitotic indexes found in each condition at 10 minutes (No IRR: n=5, 5 Gy: n=5), 30 minutes (No IRR: n=5, 5 Gy: n=5) and 1 hour (No IRR: n=4, 5 Gy: n=3). *** p<0.005 and ** p<0.05, Student’s t test.

(B) Representative immunostaining of Cl Casp-3 in e6.5 embryos 30 minutes (n=4), 1 (n=8), 1.5 (n=6) and 2 hours (n=5) after 5 Gy irradiation (5 Gy IRR) and controls (No IRR) (30 min and 1h: n=3, 1h30 and 2h: n=5) (left panel). Representative immunostaining of Cl Casp-3, 1.5 (n=5) and 2 hours (n=6) after 0.5 Gy irradiation in e6.5 embryos (0.5 Gy IRR) and controls (No IRR) (right panel). Embryos were counterstained with dapi. Images of the fourth columns are 5 fold magnifications of the regions highlighted by the squares. Arrows indicate apoptotic cells. The apoptotic indexes measured in each condition are represented in the plot. *** p<0.005 and ** p<0.05, Student’s t test.
Figure 2. Radiation hypersensitivity of peri-gastrulation embryos is tissue- and stage-specific

(A-C). Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE; red double arrow: posterior embryonic PrS (PosEmPrS), green double arrow: anterior embryonic PrS (AntEmPrS); empty double arrow: extra-embryonic PrS (ExPrS).

(A) Representative immunostaining of Cl Casp-3, activated Bax (Act-Bax) and Oct4 in an e6.5 embryo 2 hours after 0.5 Gy irradiation, counterstained with dapi (n=6). Images on the lower panel are magnifications of the regions highlighted by the squares.

(B) Representative immunostaining of Cl Casp-3 (upper panel) and P-H3 (lower panel) in e5.5 embryos 2 hours after 0.5 Gy irradiation (0.5 Gy IRR) (n=4) and controls (No IRR) (n=3), counterstained with dapi. The plots represent respectively the number of apoptotic cells and the mitotic indexes quantified in each condition in the whole embryos (Whole), Epi, or extra-embryonic tissues (ExEmb). *** p<0.005, Student's t test.
(C) Representative immunostaining of Cleaved Casp-3 and T-Brachyury (T) in an e7.5 embryo 2 hours after 0.5 Gy irradiation (0.5 Gy IRR) (n=5) and control (No IRR) (n=5), counterstained with dapi. Images on the right panel are 4-fold magnifications of the regions highlighted by the squares. The dashed line marks the border between the Epi and PrS.
Figure 3. The DNA damage response is amplified in Epi cells at the level of 53BP1

(A-D) Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.

(A) Representative zoom images of P-S/TQ and Oct4 immunostaining in an e6.5 embryo after 0.5 Gy irradiation (0.5 Gy IRR) (n=5) and non-irradiated control (No IRR) (n=5), counterstained with dapi.

(B) Representative zoom images of \(\gamma\)H2AX and Oct4 immunostaining 10 minutes after 0.5 Gy irradiation in an e6.5 embryo (n=5) and control (n=3), counterstained with dapi. On the right panel, representative Maximum Z projections of \(\gamma\)H2AX performed on cropped images are shown. The plot represents the relative amounts of

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fluorescence per cell measured in the epiblast (Emb) versus extra-embryonic tissues (ExEmb) (26 cells per tissue). *** p<0.005, Student's t test.

(C) Representative immunostaining of Cl Casp-3 and Oct4 in 0.5 Gy-irradiated e6.5 WT (Atm^{+/+} or +/-) (n=7) and Atm^{-/-} embryos (n=2), counterstained with dapi.

(D) Representative zoom images of 53BP1 and Oct4 immunostaining 10 minutes after 0.5 Gy irradiation in an e6.5 embryo (n=6) and control (n=4), counterstained with dapi. On the right panel, representative Maximum Z projections of 53BP1 performed on cropped images are shown. The plot represents the relative amounts of fluorescence per cell measured in the epiblast (Emb) versus extra-embryonic tissues (ExEmb) (26 cells per tissue). *** p<0.005, Student's t test.
Figure 4. Rapid activation of p53 in pluripotent Epi cells

(A) Representative images of P-p53 and Oct4 immunostaining in 0.5 Gy-irradiated e6.5 embryos (0.5 Gy IRR) and controls (No IRR) 10 (0.5 Gy IRR: n=3, No IRR: n=4), 30 (0.5 Gy IRR: n=3, No IRR: n=5) or 60 minutes (0.5 Gy IRR: n=5, No IRR: n=5) after treatment, counterstained with dapi. Images on the right panel are 5-fold magnifications of the regions highlighted by the squares. Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.
(B) Representative immunostaining of Ct Casp-3 in 5 Gy-irradiated e6.5 WT (p53^{+/+} or ^{+/-}) (n=8) and p53^{-/-} (n=3) embryos, counterstained with dapi.

(C) Transcriptional induction of p21, Noxa, Bax, Mdm2 and Bim in e7.5 distal (Emb) and proximal (ExEmb) cell populations, calculated for each tissue as the ratio of the expression measured 1 hour after 0.5 Gy irradiation versus non-irradiated control (n=3).

(D) Representative immunostaining of Ct Casp-3 in irradiated e6.5 embryos (0.5 Gy IRR) and controls (No IRR), in the absence or presence of 50µg/mL α-Amanitin, counterstained with dapi. The plot presents the quantification of the apoptotic indexes measured in each condition.

(E) Representative P-p53 and Oct4 immunostaining 10 minutes after 0.5 Gy irradiation (0.5 Gy IRR) in e5.5 embryos (n=4) and controls (No IRR) (n=4). Embryos were counterstained with dapi. Images on the right panel are 2.5-fold magnifications of the regions highlighted by the squares. Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.
Figure 5. The cells of the epiblast are primed for apoptosis

(A) qRT-PCR study of Bax, Bak, Bim, Noxa, Bad, Bid, Bcl-xL and Mcl-1 in e7.5 distal (Emb) and proximal (ExEmb) cell populations (n=3). *** p<0.005, Student's t test.

(B) Representative immunoblot of Bax, Bak, Bim, Bcl-xL, Oct4 and Pcna in e7.5 distal (Emb) and proximal (ExEmb) cell populations. The plot presents the average of the quantifications measured in 3 independent experiments. *** p<0.005, Student's t test.

(C-H) Representative immunostainings of Oct4 and Bak (C and F) (n=4), Oct4 and Bim (D and G) (n=5 at e6.5, n=4 at e5.5) and Oct4 and Bcl-xl (E and H) (n=4) in e6.5 (C-E) and e5.5 embryos (F-H), counterstained with dapi. Images on the lower panels are magnifications of the regions highlighted by the squares. Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.
Figure 6. Model of the cellular determinants driving the hypersensitivity of the epiblast

The apoptotic threshold is depicted as a balance, whose equilibrium is impacted by the ratio between pro- and anti-apoptotic factors. After e5.5, in the epiblast, the pro-apoptotic factors Bak, Bim and Noxa (in grey) are more abundant than in extra-embryonic tissues (ExEmb), while anti-apoptotic Bcl-xL (in white) is less expressed, priming the cells for apoptosis. Upon induction of DNA damage, increased signaling in epiblast cells leads to a quick phosphorylation of p53. The activation of p53 is an additional weight, which is sufficient to cross the apoptotic threshold and trigger apoptosis in the primed epiblast cells.
Figure S1. The Epi of e6.5-e7.5 embryos is highly proliferative and shows replicative stress

(A-D) Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE; filled double arrow: embryonic PrS (EmPrS); empty double arrow: extra-embryonic PrS (ExPrS). * p<0.1, ** p<0.05, *** p<0.005, Student’s t test.

(A) Representative immunostaining of P-H3 and Oct4 in an e6.5 embryo (n=6), counterstained with dapi. The plot presents the quantification of the mitotic indexes measured in the whole embryo (Whole), Epi, VE, and ExE.

(B) Representative immunostaining of P-H3 and T-Brachyury (T-Bra) in an e7.5 embryo (n=3), counterstained with dapi. The first plot presents the quantification of the mitotic indexes found in the whole embryo (Whole), Epi, PrS, VE and ExE. The second plot presents the quantification of the mitotic indexes found in the EmPrS and in the ExPrS.

(C) Representative immunostaining of γH2AX and Oct4 in an e6.5 embryo, counterstained with dapi (n=4). The images on the right panel are 5-fold magnification of the areas highlighted by the squares.

(D) Representative immunostaining of 53BP1 and Oct4 in an e6.5 embryo, counterstained with dapi (n=6). The images on the right panel are 5-fold magnification of the areas highlighted by the squares.
Figure S2. 5 Gy ionizing radiations induce Epi-specific Caspase-3 cleavage and Bax activation in e6.5 embryos
Representative immunostaining of Cl Casp-3, active Bax (act-Bax), and Oct4 in an e6.5 embryo 1.5 hours after 5 Gy irradiation (n=3), counterstained with dapi. Images on the lower panel are 5-fold magnifications of the regions highlighted by the squares. Arrow: Epi; filled arrowhead: VE; empty arrowhead: extra-embryonic ectoderm (ExE).
Figure S3. Endogenous and 5Gy irradiation induced P-S/TQ immunoreactivity in the e6.5 epiblast

(A) Representative immunostaining of P-S/TQ and Oct4 in an e6.5 embryo 10 minutes after 5 Gy irradiation (5 Gy IRR) (n=3) and in a non-irradiated control (No IRR) (n=3), counterstained with dapi. Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.

(B) Representative immunostaining of P-S/TQ in a non-irradiated and not cultured e6.5 embryo, counterstained with dapi (n=4). Images on the right panel are 2-fold magnifications of the regions highlighted by the squares. Filled arrowhead: P-S/TQ-negative VE cell; empty arrowhead: P-S/TQ-negative ExE cell; *: P-S/TQ-positive mitotic chromosomes in Epi cells.
Figure S4. Focal recruitment of 53BP1 upon irradiation is amplified in Epi cells

(A-B) Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.

(A) Representative zoom images of 53BP1 and Oct4 immunostainings performed 30 minutes (upper panel) (n=5) and 1 hour (lower panel) (n=3) after 0.5 Gy irradiation in e6.5 embryos (0.5 Gy IRR) and controls (No IRR) (30 min: n=6, 1h: n=4), counterstained with dapi.

B. Representative zoom images of 53BP1 and Oct4 immunostainings performed 30 minutes after 5 Gy irradiation in e6.5 embryos (5 Gy IRR) (n=5) and controls (No IRR) (n=6), counterstained with dapi.
Figure S5. Untreated e7.5 dissected embryos do not show differential expression of epigenetic remodelers

(A) Schematic representation of an e7.5 embryo. The dashed line indicates the embryonic/extra-embryonic border where embryos were dissected in order to obtain Oct4+ and Oct4− enriched cell populations. ExE: Extra-embryonic Ectoderm, ExM: Extra-embryonic Mesoderm, Epi: Epiblast, PrS: Primitive Streak, EmVE: Embryonic Visceral Endoderm, ExVE: Extra-embryonic Visceral Endoderm.

(B) Relative expression of Oct4 mRNA in distal (Emb) and proximal (ExEmb) cell populations of non-irradiated e7.5 embryos at time 0 (No IRR) or 1 hour (No IRR + 1h) and 0.5 Gy-irradiated embryos kept in culture for 1 hour (IRR + 1h). These results show the clear distinction between the two cell populations (n=3).

(C) Immunoblot analysis of Oct4 and Pcna expression in protein extracts of e7.5 distal (Emb) and proximal (ExEmb) cell populations. The plot presents the average of the quantifications measured in 2 independent experiments. These results show the clear distinction between the two cell populations (n=3).

(D) Relative expression of Rnf8, Rnf168, Tip60, Hdac1, Hdac2, Nsd, Nsd2 and UbcM4 mRNAs in whole e7.5 embryos (Whole), distal (Emb) and proximal (ExEmb) cell populations (n=3). ** p<0.05, *** p<0.005, Student’s t test.
Figure S6. Phospho-p53 immunoreactivity after 5 Gy irradiation is heterogeneous in e6.5 embryos in spite of the ubiquitous expression of p53

(A) Time course of Phospho-p53 (Ser18) (P-p53) immunostaining after 5 Gy irradiation (5 Gy IRR) in e6.5 embryos and controls (No IRR) at 10 minutes (5 gy IRR: n=5, No IRR: n=4), 30 minutes (5 gy IRR: n=5, No IRR: n=5) and 1 hour (5 gy IRR: n=4, No IRR: n=5). Embryos were counterstained with dapi. Images on the right panel are 5-fold magnifications of the regions highlighted by the squares. Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.

(B) Representative immunostainings of p53 (tot p53) and Oct4 in a non-irradiated e6.5 embryo (n=3), counterstained with dapi. Images on the lower panel are 2.5-fold magnifications of the regions highlighted by the squares. Arrow: Epi; filled arrowhead: VE; empty arrowhead: ExE.
**Figure S7. Atm-independent p53 phosphorylation (Ser18) upon irradiation**

Immunostaining of P-p53 in e6.5 WT (Atm$^+$/+ or +/−) (n=4) and Atm$^{-}$/− embryo (n=1) 2 hours after 0.5 Gy irradiation, counterstained with dapi.
Supplementary Materials and Methods

Primary antibodies used for immunofluorescence or immunoblotting

Goat anti-Oct4 (Santa Cruz #sc-8628, 1:100); goat anti-T (Santa Cruz #sc-17745, 1:100); rabbit anti-phospho-histone H3 (Millipore #06-570, 1:1000); rabbit anti-cleaved Caspase-3 (Cell Signaling #9661, 1:200); rabbit anti-phospho-S/TQ (Cell Signaling #2851, 1:100); rabbit anti-gH2AX (Cell signaling #9718, 1:100); rabbit anti-53BP1 (Novus Biologicals #NB-100-304, 1:100); rabbit anti-phospho-p53 (Ser15) (Cell Signaling #9284, 1/100); mouse biotin-conjugated anti-active Bax (Abcam #ab79459, 1/50); rabbit anti-Bak (Cell Signaling #12105, 1/100); rabbit anti-Bim (Cell Signaling #2933, 1/50); rabbit anti-Bcl-xL (Cell Signaling #2764, 1/100); rabbit anti-p53 (Abcam #ab131442, 1/200).

Table S1. Primers

Primer sequences used for p53 and Atm genotyping

p53wt/ko-F GGATGGTGGTATACTCAGAGC;
p53wt-R AGCGTGGTGGTACCTTATGAGC;
p53ko-R GCTATCAGGACATAGCGTTGG;
Atmwt/ko-F GTAGTAACTATTAGTTTCGTGCAAATAG;
Atmwt-R CACTAGGGTGTACTAGTGGAGGATC;
Atmko-R ACGTAAACTCCTCTTCAGACCT.

Primer sequences used for RT-PCR

Gapdh-F CCCACTCTTCCACCTTCTCGATG;
Gapdh-R GTCCACCACCTCTGGTGGTCTGA;
Oct4-F TGGGCGTTCTCTTCTTGGAAGA; Oct4-R GGGGTGCTGGCTCCTCCCCAC;
Noxa-F CAGATGCCCTGGGAAGTCG; Noxa-R TGAGCACAAGCTGCCTCCCAA;
Bax-F GGTCGCCAGGTAGAGGAGGA; Bax-R GTGAGCGGCTTCTTGCTCT;
Mdm2-F AGGCGACGACGTCTCATAG;
Mdm2-R CCTCCCTCAAAAGGAGTCTGTGATCG;
Bim-F CCCGGGAGATACGGATTGCAC; Bim-R GCCTGCGGCCTAATCTTTGCC; p21-F AACATCTCAGGGGCCGAAA; p21-R TGCGCTTGGAGGTAGAAA;
Bak-F GGAATGCTACGAGACTCCTCC; Bak-R CCAGCTATGCGCCACTCTTTAA;
Bad-F GGAGCAACATCCATCGACGAG; Bad-R TACGAACTGTGGCGACTCC;
Bid-F GTGAGGAACTTGGTGATAGAAGC;
Bid-R CAGGCCAAGGTTTTCCAT;
Bcl-xL-F TGACCACCTAGGCTTTGGGA;
Bcl-xL-R GCTGCATTGTTCCCGTAGA;
Mcl-1-F GGTATTTAAGCTAGGGTCATTGAA;
Mcl1-R TACAGCGGAGGCATCAG.
Rnf168-F CAGAAAATCAGATCCAGTCACA;
Rnf168-R CAGGCTTCAACTTAGGTGACAA;
Tip60-F GCAACCAGGACAACGAAGAT;
Tip60-R CCATTCCAGACGTTTGT;
Hdac1-F TGGTCTCTACCGAAAAATGGAG;
Hdac1-R TCATCCTCTTGTTACTTGGCT;
Hdac2-F CAGGAGATGGTGGAGCAG;
Hdac2-R CCCAATTGACAGCCATATCA;
Setd8-F CGGAGATGGTGGAGCAG;
Setd8-R GCAGAGCACTTGTTCGGACT;
Nsd1-F GGGTAAAAGTTGGTCGCTACA;
Nsd1-R AGAGGGAACAGCCTGAGGAT;
Nsd2-F CAGGAGCAACAGGAGGAGAC;
Nsd2-R GCATCTGGATGTCAGCTACAA;
UbcM4-F GAAGGGGCGAGGTCTGCTG;
UbcM4-R TCACCAGTGCTATGAGGGACT.