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The proline rich domain of p53 is dispensable for MGMT-dependent DNA repair and cell survival following alkylation damage

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In addition to promoting cell death and senescence, p53 also has important cellular survival functions. A mutant p53, lacking a proline-rich domain ($p53^{\Delta P}$), that is deficient in controlling both cell death and cell cycle arrest, was employed to determine the biological means by which p53 mediates survival upon DNA damage. While $p53^{\Delta P}$ and $p53^{-/-}$ cells were equally resistant to many DNA damaging agents, $p53^{\Delta P}$ cells showed an exquisite resistance to high doses of the alkylating agent Diazald (*N*-Methyl-*N*-(*p*-tolylsulfonyl)nitrosamide), as compared to cells completely deficient for p53 function. We determined that $p53^{\Delta P}$ was capable of transcribing the repair gene, MGMT (*O*6-methylguanine-DNA methyltransferase) after irradiation or alkylation damage, resulting in DNA repair and cell survival. Consistent with these observations, $p53^{\Delta P}$ mice show enhanced survival after IR relative to $p53^{-/-}$ mice. Suppression or deletion of MGMT expression in $p53^{\Delta P}$ cells inhibited DNA repair and survival after alkylation damage, whereas MGMT overexpression in p53-deficient cells facilitated DNA repair and conferred survival advantage. This study shows that when cell death and cell cycle arrest pathways are inhibited, p53 can still mediate MGMT-dependent repair, to promote cell survival upon DNA damage.
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P53 is one of the most frequently mutated genes in human tumors, and its proper activity and function is crucial for the maintenance of genomic stability and tumor suppression.[1] Although it remains undisputed that p53 is a potent suppressor of tumorigenesis, the biological means by which p53 prevents cellular transformation remains unclear.[2] To date, no ablation of a p53 target gene fully recapitulates the cancer predisposition of p53 null mice.[3–5] The long-held hypothesis in the field predicted that upon oncogene-induced cellular stress, cell cycle arrest or apoptosis was induced, thus allowing the cell time for efficient repair of the damage, or the elimination of cells that acquired irreparable damage.[6,7] A genetic approach in which three p53 target genes required for cell cycle arrest and apoptosis were deleted, failed to phenocopy the spontaneous tumor incidence seen in $p53^{-/-}$ mice.[8] However, given the requirement for p53 in the transcriptional regulation of hundreds of genes, it seems unlikely that the deletion of individual p53-mediated genes will produce a loss of tumor suppressor activity.[8–10] We used a genetic model in which the proline-rich domain (PRD) of p53 ($p53^{\Delta P}$) was deleted, resulting in an altered transcription function.[11] This mutant allowed us to evaluate the biological role of p53 that a majority of its target functions are ablated.

The PRD, defined by residues 58–98 and residues 62–91 of human and mouse p53 respectively, promotes p53-dependent transcription by an unclear mechanism.[12] Some studies suggest that deletion of this region changes the stability of p53 protein, as a consequence of diminished Pin1 binding resulting in increased susceptibility of p53 to MDM2-dependent degradation. Others suggest that the PRD instead mediates binding to cofactors important for efficient transcription.[13–18] Nevertheless, deletion of the PRD of p53 ($p53^{\Delta P}$ (75–91); and mΔpro (58–88)) resulted in a loss of cell cycle arrest and apoptosis; yet mice lacking this region rarely develop spontaneous tumors seen in $p53^{-/-}$ mice.[11,19–23]

The repair of DNA lesions is crucial to maintain genomic stability and p53 mediates the expression of numerous repair genes involved in nucleotide excision repair, base excision repair, mismatch repair, and non-homologous end joining.[24,25] Mice with mutations or deletions in one of several DNA repair genes are prone to developing tumors.[26] Methylating agents, such as chemotherapies can modify DNA at many different sites, with the most mutagenic lesion being the alkylation of O^6 position of guanine (O^6MeG).[27,28] These lesions are highly mutagenic unless repaired, and fail to block DNA replication.[29–31] As a consequence, following two rounds of DNA replication these lesions manifest as G:C to A:T point mutations and represent a DNA adduct with the potential for tumor formation.[32] In some cases, A:T transitions have been shown to arise in the KRAS oncogene and p53 tumor suppressor gene, thus promoting tumorigenesis.[33–35] P53 induces the transcription of *O*6-methylguanine-DNA methyltransferase (MGMT), an enzyme that repairs O^6MeG, via a one-step suicide reaction, involving the irreversible transfer of

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the methyl groups on the DNA lesion, to an internal cysteine residue in the active center of the alkyltransferase.[36–38] This reaction is stoichiometric involving one molecule of MGMT for each methyl group removed.[39] Following repair, MGMT is tagged by ubiquitin and degraded via the proteasome.[40,41] While MGMT-deficient mice do not develop spontaneous tumors, they are highly vulnerable to oncogenesis induced by treatment with alkylating agents.[42–46] Conversely, mice with elevated MGMT expression levels exhibit resistance to alkylating agents and tumor formation.[47–49] This suggests that MGMT is important in tumor suppression.

We determined that p53 lacking the PRD failed to mediate the expression of key genes involved in apoptosis or cell cycle arrest. While cells harboring $p53^{\Delta P}$ were equally resistant to cell death induced by several DNA damaging agents as compared to $p53^{-/-}$ cells, they surprisingly showed a greater resistance to high doses of the alkylating agent Diazald, than did $p53^{-/-}$ cells. We determined that $p53^{\Delta P}$ was capable of transcribing the repair gene, MGMT and could thereby induce repair alkylating DNA damage, thus promoting $p53^{\Delta P}$ cell survival after alkylation damage.

Results

DNA damage-induced apoptosis and cell cycle arrest requires p53 with a functional proline-rich region. Since the proline-rich region of p53 is described to be essential for the efficient transcription function of p53,[12,50] we first determined whether common target genes involved in apoptosis and cell cycle arrest were induced upon DNA damage in cells expressing p53 lacking a proline-rich region ($p53^{\Delta P}$ (75–91)). H1299 cells, that lack the p53 gene, were reconstituted with wild-type p53 ($p53^{WT}$) or p53 lacking the proline-rich region ($p53^{\Delta P}$) fused to a modified estrogen receptor steroid-binding domain (ERTam) that binds 4-Hydroxytamoxifen (4OHT) specifically. Upon the addition of 4OHT for 5 h, both p21 and PUMA gene expression levels and protein levels were induced in cells expressing $p53^{WT}$, but not $p53^{\Delta P}$ (Figures 1a and c). P21 and PUMA gene expression levels were also induced after 6 h in B cells treated with 5 Gy of irradiation isolated from $p53^{WT}$ mice, but not those from $p53^{\Delta P}$ or $p53^{-/-}$ mice (Figure 1b). E1A/RAS transformed mouse embryonic fibroblasts (MEF) from $p53^{WT}$, $p53^{\Delta P}$, and $p53^{-/-}$ mice were treated with a panel of DNA damage agents and assessed for Annexin V externalization after 18 h by flow cytometry. $P53^{WT}$-expressing MEF were sensitive to all DNA damaging agents tested, but $p53^{\Delta P}$-expressing cells and $p53^{-/-}$ cells were equally resistant to these death-inducing stimuli (Figure 1d). In addition, $p53^{\Delta P}$-expressing, and $p53^{-/-}$ cells exhibited reduced cell cycle arrest upon 5Gy irradiation (IR) compared to $p53^{WT}$-expressing cells (Figure 1e). Even without irradiation, a greater number of $p53^{\Delta P}$ and $p53^{-/-}$ cells were observed to be in S-phase as compared to $p53^{WT}$-expressing cells, and exhibited more rapid growth than $p53^{WT}$ cells (Figure 1f). Irreversible cell cycle arrest was also compromised in $p53^{\Delta P}$-expressing cells, as evidenced by an inability to undergo replicative- and oncogene-induced senescence (Figures 1g and h respectively).

MGMT mediates repair and long-term survival in response to alkylating damage in cells expressing $p53^{\Delta P}$. We next used clonogenic assays to examine the long-term survival and proliferation of cells treated with DNA damaging agents. We suspected that since $p53^{\Delta P}$ and $p53^{-/-}$ cells are resistant to the apoptotic effects of DNA damage in short-term assays, they would likely exhibit enhanced long-term survival and proliferation, as compared to $p53^{WT}$ cells. Indeed, $p53^{\Delta P}$-expressing and $p53^{-/-}$ cells exhibited better survival than their $p53^{WT}$-expressing counterparts when treated with intermediate doses of UV (5 mJ/cm^2) and STS (3.5 μM) (Figure 2a, Supplementary Figure 1A). $P53^{\Delta P}$-expressing cells exhibited a slightly enhanced sensitivity to intermediate doses of IR (12 Gy) as compared with $p53^{-/-}$ cells, but higher doses (14 Gy) resulted in equally diminished survival in both cell types (Figure 2a, Supplementary Figure 1A). P53 status was not important for long-term survival after treatment with Doxorubicin (0.75 μM and 1.0 μM) and Etoposide (25 μM and 50 μM).

Significantly however, $p53^{\Delta P}$-expressing cells were highly resistant to the high dose of alkylating agent, Diazald (250 μM), surviving and proliferating better than either $p53^{WT}$-expressing or $p53^{-/-}$ cells (Figure 2a, Supplementary Figure 1A) (highlighted by red circle). To evaluate this observation further, we performed a time-course of high dose (250 μM) Diazald-treatment and analyzed proliferation and survival by clonogenic assay (Figure 2b). Compared with $p53^{WT}$ and $p53^{-/-}$ cells, $p53^{\Delta P}$-expressing cells showed a superior capacity to survive, even when treated for 8 h with a high dose of Diazald (Figure 2b). Only after a 24 h exposure to 250 μM Diazald, did cells expressing $p53^{\Delta P}$ succumb to DNA damage and fail to survive (Figure 2b).

Alkylating damage induces specific types of DNA adducts at the N- and O-atoms in DNA bases. Although O^6-methylguanine (O^6MeG) adducts are less common, they are stable and will persist unless specifically repaired by the DNA repair protein MGMT.[32] We determined that MGMT gene expression was elevated following either IR (5 Gy) or Diazald-treatment of $p53^{WT}$ and $p53^{\Delta P}$ immune cells, but not in $p53^{-/-}$ cells (Figure 3a). $P53^{\Delta P}$ cells expressed higher levels of MGMT after IR at 6–8 h *in vitro* and *ex vivo* compared with $p53^{WT}$ cells. Diazald treatment of thymocytes for 18 h showed approximately equal expression of MGMT in $p53^{WT}$ and $p53^{\Delta P}$ cells (Figure 3a). P53-dependent transcription of MGMT is consistent with the identification of a consensus binding sequence for p53 within the MGMT promoter. CHIP assays confirmed direct binding of p53.[51,52] We tested whether the difference in the levels of MGMT observed in $p53^{WT}$ and $p53^{\Delta P}$ after IR may be due to a difference in the regulation and stability of $p53^{\Delta P}$ protein after damage. However, less $p53^{\Delta P}$ is stabilized after damage, compared with $p53^{WT}$, with a concomitant decrease in its negative regulator, MDM2, suggesting that there is not an increased turnover of $p53^{\Delta P}$ protein (Supplementary Figure 2A). The difference in MGMT expression levels after IR between $p53^{WT}$- and $p53^{\Delta P}$-cells may be due to the differences in cell survival between the cells.

Since the PRD is crucial for the efficient transcription function of p53, we were interested to determine if additional repair genes could also be induced upon IR treatment in

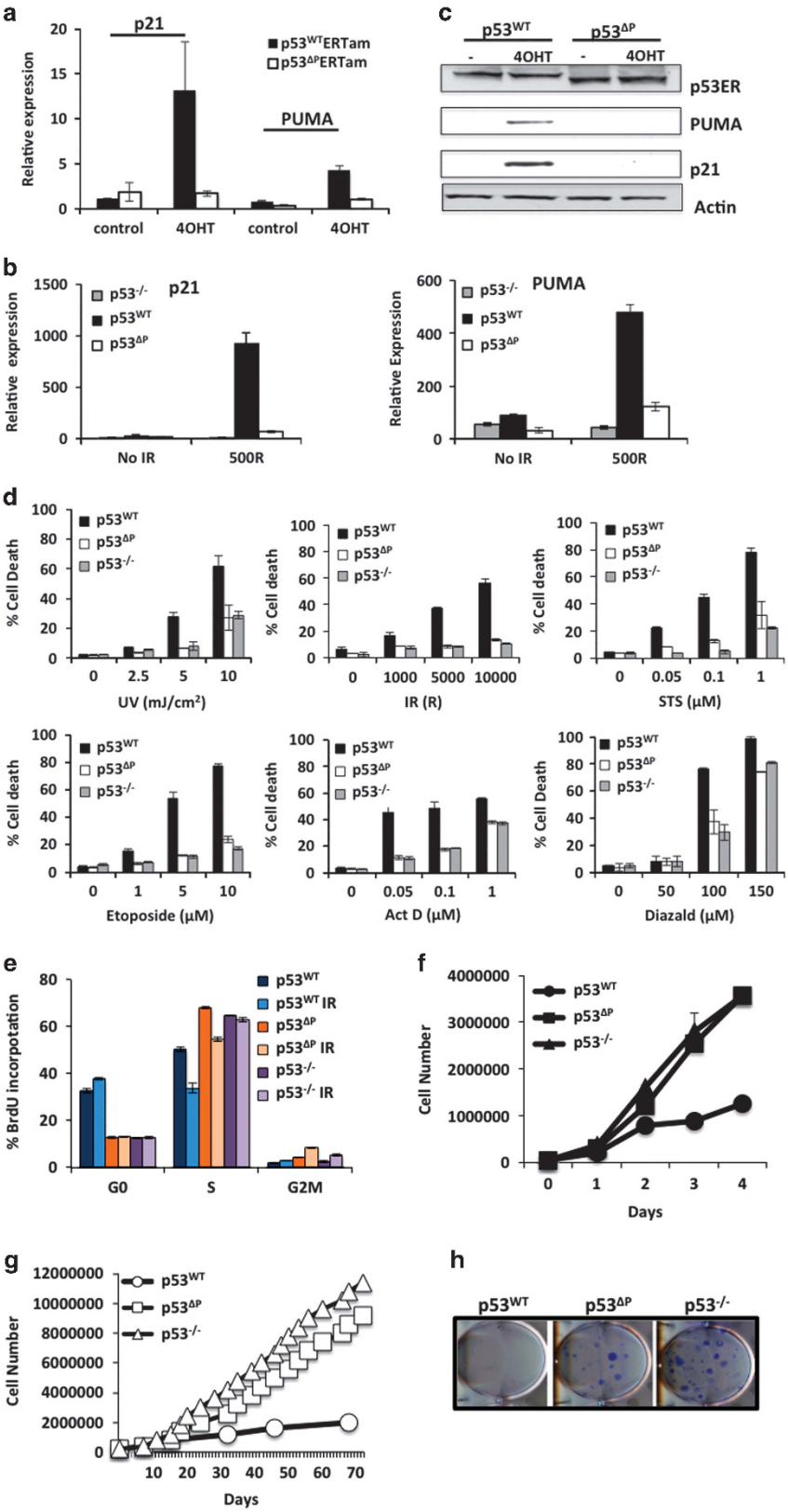

Figure 1 P53$^{\Delta P}$ cells do not express p21 or PUMA and are resistant to apoptosis, cell cycle arrest, and senescence. p21 and PUMA gene expression levels in (**a**) H1299 cells expressing p53^{WT}ERTam or p53$^{\Delta P}$ERTam constructs 5 h after 4OHT treatment, and (**b**) primary B cells 6 h post irradiation. (**c**) p21 and PUMA protein levels in H1299 cells expressing p53^{WT}ERTam or p53$^{\Delta P}$ERTam constructs 8 h post 4OHT. (**d**) Cell death at 18 h as measured by flow cytometric analysis of Annexin V staining in p53^{WT}, p53$^{\Delta P}$, and p53$^{-/-}$ MEF cells treated with a variety of apoptotic stimuli. (**e**) Brdu incorporation into MEFs 18 h after 4 Gy IR. Growth of (**f**) E1A/RAS transformed MEF cells over 4 days and (**g**) primary MEF cells over time. (**h**) Colony-forming assay looking at survival of primary MEF cells transduced with RAS oncogene

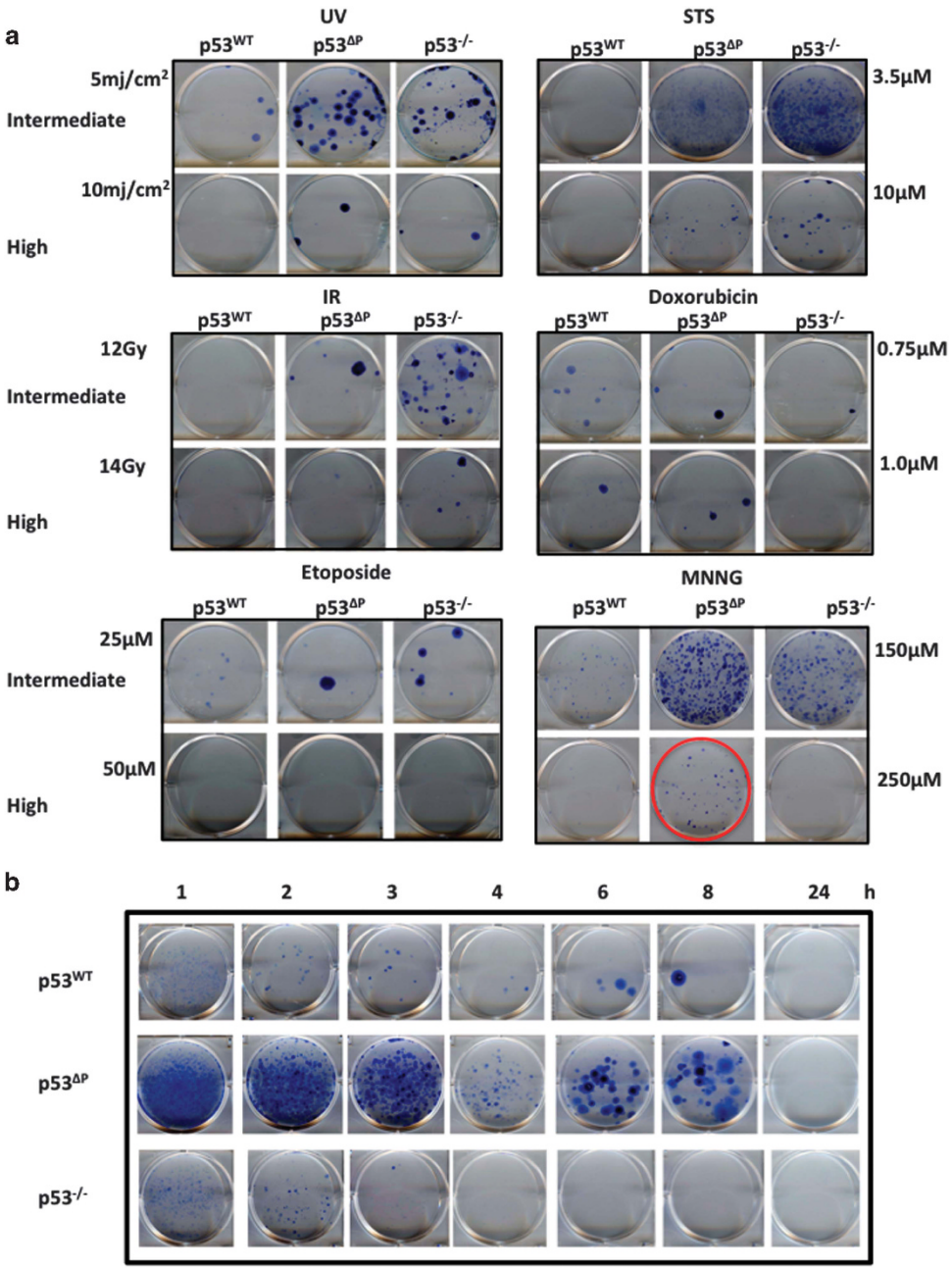

Figure 2 P53$^{\Delta P}$ cells exhibit a differential long-term survival response to alkylating agent Diazald compared with other damaging agents. (**a**) Colony-forming assay examining the survival of p53^{WT}, p53$^{\Delta P}$ and p53$^{-/-}$ MEF cells treated with intermediate or high doses of death-inducing agents. Red circle highlights the increase in survival of p53$^{\Delta P}$ cells after Diazald (250 μM) treatment as compared to p53$^{-/-}$ cells. (**b**) Colony-forming assays examining survival in p53^{WT}, p53$^{\Delta P}$, and p53$^{-/-}$ MEF cells after 250 μM Diazald treatment for increasing time intervals

p53$^{\Delta P}$ cells. No repair genes analyzed were induced in a p53$^{\Delta P}$-manner (Supplementary Figure 1B). To assess repair of Diazald-mediated damage by MGMT, levels of phosphorylated H2AX (γH2AX) were assessed at 3 h and 24 h after Diazald treatment by intracellular staining and subsequent flow cytometry. Briefly, cells were treated for 3 h with Diazald after which they were stained for γH2AX, or washed, and re-suspended in fresh media for an additional 24 h before staining for γH2AX. Compared with untreated cells, Diazald treatment for 3 h induced an equivalent shift in fluorescence (based on mean fluorescence intensity), indicating damaged DNA, in p53^{WT}, p53$^{\Delta P}$ and p53$^{-/-}$ cells (Figure 3b). Twenty-four hours after Diazald treatment, both p53^{WT}- and p53$^{\Delta P}$-expressing cells showed a decrease in the levels of detectable γH2AX staining, comparable to those measured in untreated cells, indicating that repair of Diazald-mediated damage had occurred (Figure 3b). By contrast, p53$^{-/-}$ cells, which are unable to induce MGMT expression, maintained high levels of γH2AX staining after 24 h, consistent with an inability to repair Diazald-imposed DNA damage (Figure 3b).

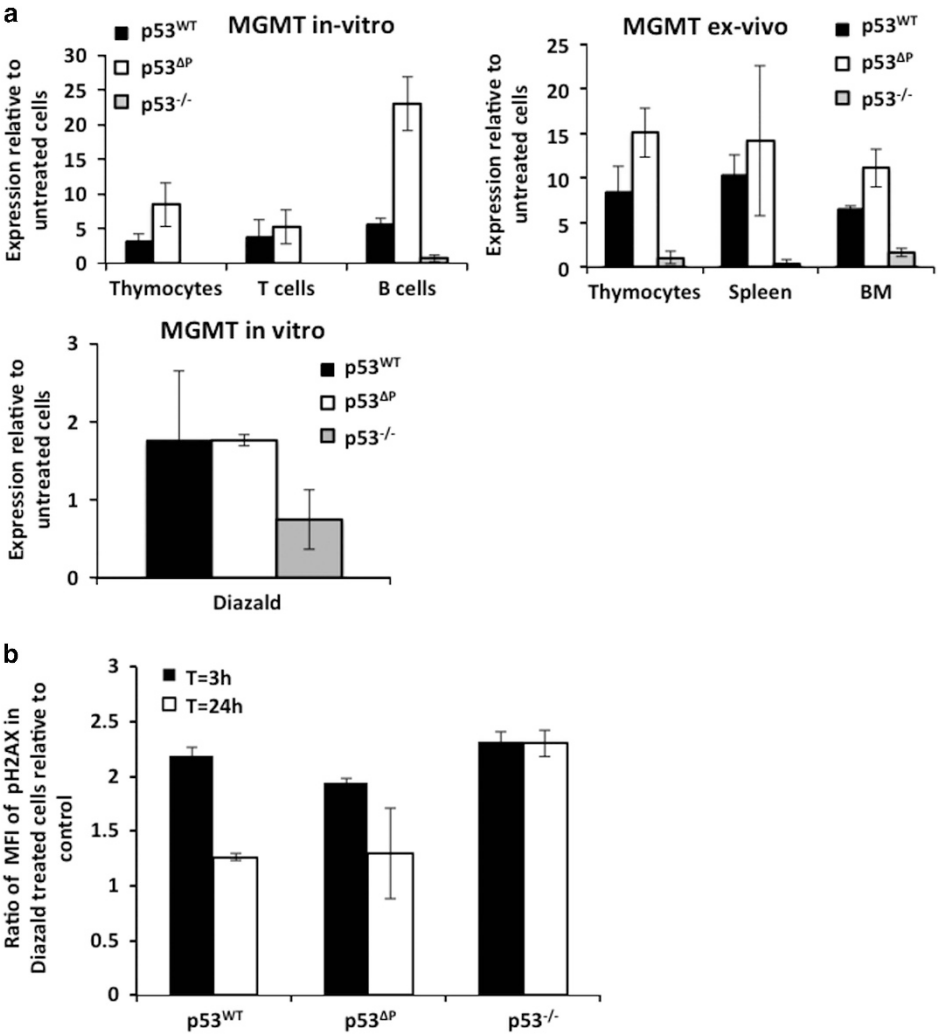

Figure 3 The repair gene MGMT is induced in $p53^{\Delta P}$ cells upon alkylating damage and mediates DNA repair and cell survival in cells. (**a**) MGMT expression levels 6–8 h IR (5 Gy) in irradiated immune cells (*in vitro*) or immune cells isolated from irradiated mice (*ex vivo*). MGMT expression levels in thymocytes treated with 50 μM Diazald for 18 h. (**b**) Intracellular pH2AX levels measured by flow cytometry in $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ MEF cell treated with 100 μM Diazald at 3 h to assess damage, or at 24 h to assess repair

DNA damage engages the DNA damage response pathway to engage p53, and elucidate an appropriate biological response to prevent the perpetuation of potentially tumorigenic mutations.[25] We therefore suggest that following DNA damage $p53^{\Delta P}$ can induce expression of the enzyme MGMT to mediate DNA repair and promote survival.

The role of MGMT in tumorigenesis following alkylation damage has already been characterized in p53-sufficient and -deficient mice.[53] To further explore this idea, we asked whether DNA damage-mediated MGMT induction promotes survival of mice after exposure to 4 Gy IR at p7. Survival after IR was examined in $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ mice, and as expected, $p53^{-/-}$ mice all died at roughly 3 months of age as a consequence of tumor formation (Figure 4a). However, mice lacking the PRD ($p53^{\Delta P}$) showed significant survival after IR relative to $p53^{-/-}$ mice, although with a slightly diminished survival when compared with $p53^{WT}$ mice (Figure 4a). Without MMR or apoptosis induction in $p53^{\Delta P}$ cells, any un-repaired Diazald-mediated damage would be propagated to daughter cells, resulting in genomic instability and aneuploidy.[54] We therefore examined the incidence of aneuploidy in $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ MEF 72 h after 4 Gy IR, as assessed by flow cytometric analysis of $>4n$ cell cycle distribution after propidium iodide staining (Figure 4b). Compared with $p53^{WT}$ cells, $p53^{\Delta P}$ and $p53^{-/-}$ cells displayed more aneuploidy and increased genomic instability. We observed a further increase in the incidence of aneuploidy in $p53^{\Delta P}$ MEF lacking MGMT at 72 h following exposure to IR. This suggests that MGMT mediates some control of genomic stability in $p53^{\Delta P}$ cells. This however was not reflected *in vivo*, since there was no difference in the survival of mice lacking MGMT after IR at p7 (Figure 4a).

MGMT silencing results in diminished repair and long-term survival in response to alkylating damage in cells expressing $p53^{\Delta P}$. We hypothesized that cells expressing $p53^{\Delta P}$ show superior survival after Diazald treatment because MGMT-mediated DNA repair is present and

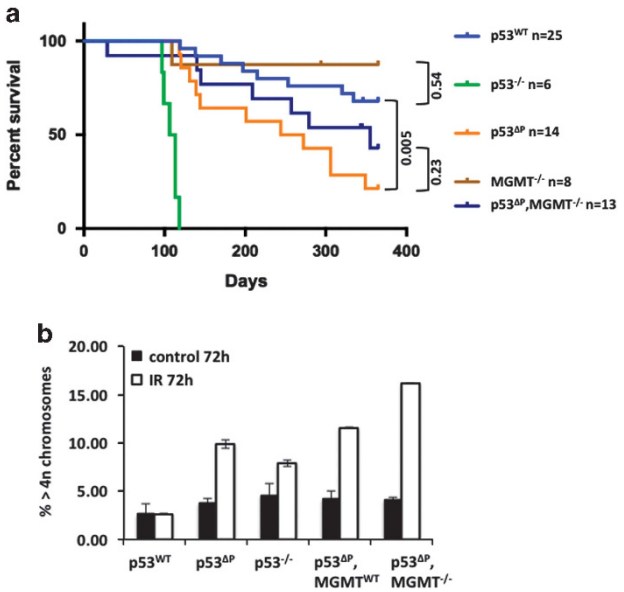

Figure 4 MGMT-dependent repair does not promote survival in mice treated with IR. (**a**) Survival curve of mice irradiated with 4 Gy on day 7. Gehan–Breslow–Wilcoxon test used for statistical analysis. (**b**) Quantification of the percentage of MEF cells with aneuploidy (> 4n chromosomes) 72 h after 4 Gy IR as assessed by flow cytometric analysis of cell cycle distribution after propidium iodide staining

functional, despite the fact that these cells are unable to undergo cell cycle arrest and apoptosis after DNA damage (Supplementary Figure 2B). Since $p53^{\Delta P}$ cells and $p53^{WT}$ cells exhibit no differential expression of MGMT protein, either basally or following DNA damage, the enhanced survival observed in $p53^{\Delta P}$ cells is most likely due to their defect in apoptosis and/or cell cycle arrest (Figure 5a). Therefore $p53^{WT}$-expressing cells show reduced survival as compared to $p53^{\Delta P}$ cells, because they are able to undergo cell cycle arrest and apoptosis in response to alkylating damage (Supplementary Figure 2B). A lack of p53 ensures that apoptosis, cell cycle arrest, and MGMT-mediated repair are not engaged upon alkylating DNA damage. Although in short-term assays $p53^{-/-}$ cells are protected from apoptosis (Figure 1d), their inability to survive and proliferate long term suggests that alternative non-apoptotic cell death mechanisms are likely engaged (Figure 2b). It is possible that necrosis or mitotic catastrophe and subsequent death are engaged in these cells upon high dose Diazald treatment. We therefore hypothesized that silencing MGMT in $p53^{\Delta P}$-expressing cells results in less survival after Diazald treatment, similar to that seen with $p53^{-/-}$ cells. A lentiviral expression system was used to silence MGMT in $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ transformed MEF and the efficiency of silencing was confirmed in all cell lines (Figure 5a, Supplementary Figure 2C). $P53^{\Delta P}$ and $p53^{-/-}$ cells remained resistant to apoptosis induced by UV, IR and Diazald as compared to $p53^{WT}$-expressing cells, regardless of whether MGMT was silenced or not (Figure 5b). However, when MGMT was silenced in $p53^{\Delta P}$-expressing cells, Diazald-mediated damage could no longer be repaired (Figure 6a). Importantly, we also saw that these cells could no longer survive and proliferate as compared to MGMT-expressing cells when treated with high dose Diazald (Figure 6e). We also confirmed this result in MEFs isolated from $p53^{\Delta P}$, $MGMT^{-/-}$ mice (Figure 6e). In fact, these MGMT-silenced cells showed similar levels of survival and proliferation as compared to $p53^{-/-}$ cells (Figure 6e). Since $p53^{-/-}$ cells do not express MGMT, it was not surprising that no difference in repair or survival after Diazald treatment was detected when MGMT was silenced or not (Figures 6a and e). Diazald-treatment of $p53^{WT}$ cells in which MGMT was silenced showed less survival and proliferation in long-term assays (Figure 6e). We noted that Diazald-induced damage appeared to be repaired by 24 h in $p53^{WT}$ cells in which MGMT was silenced (Figure 6a). Since $p53^{WT}$-expressing cells can undergo cell cycle arrest and apoptosis following the failure to repair damaged DNA (Supplementary Figure 2B), we examined cell death in parallel to repair at the 24-h endpoint. Briefly, cells were treated for 3 h with Diazald, washed and cultured for an additional 18 h. We observed twice as much apoptosis in $p53^{WT}$ cells lacking MGMT than any other cell line (Figure 6b). We suggest that the apparent repair of DNA damage in $p53^{WT}$ cells lacking MGMT is attributed to a small percentage of cells that failed to efficiently silence MGMT, maintained their repair function, and therefore escaped deletion by apoptosis or cell cycle arrest. To test this possibility, we overexpressed the anti-apoptotic protein BCLxL in all cell lines sufficient or deficient for MGMT (Supplementary Figure 3B). Diazald-induced cell death was completely inhibited in $p53^{WT}$ cells sufficient or deficient for MGMT in which BCLxL was overexpressed (Figure 6c). When Diazald-mediated apoptosis was inhibited by BCLxL expression, a population of cells clearly unable to repair alkylating damage was now visible in $p53^{WT}$ cells lacking MGMT (Figure 6d). $P53^{-/-}$ cells were unable to repair damage in an MGMT-mediated manner, whereas $p53^{\Delta P}$-expressing cells did so, unless MGMT was silenced (Figure 6a). Interestingly, despite BCLxL protection from Diazald-mediated cell death in $p53^{WT}$-expressing cells, this did not confer significant long-term survival (Supplementary Figure 3C). This may be due to cells undergoing permanent cell cycle arrest and/or alternative forms of cell death. To test this we silenced p21 levels in $p53^{WT}$ cells overexpressing BCLxL, rendering these cells unable to engage cell cycle arrest or apoptosis (Supplementary Figure 3B). These cells showed slightly better survival relative to $p53^{WT}$ cells after alkylation, but did not phenocopy the survival seen in $p53^{\Delta P}$ cells (Supplementary Figure 3C). This suggests that the response in $p53^{\Delta P}$ differs from that of $p53^{WT}$ in additional ways to mediate survival after alkylation damage (Supplementary Figure 2B).

MGMT expression in $p53^{-/-}$ cells results in repair and survival in response to alkylating DNA damage. MGMT was overexpressed in $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ cells (Supplementary Fig 3A), which were then subjected to Diazald treatment. We confirmed that MGMT expression did not alter sensitivity to Diazald-induced cell death in any of the cell lines (Figure 7a). Importantly however, $p53^{-/-}$ cells expressing MGMT were now capable of repairing Diazald-induced DNA damage to a similar extent as that seen in $p53^{\Delta P}$-expressing cells (Figure 7b). Clonogenic assays

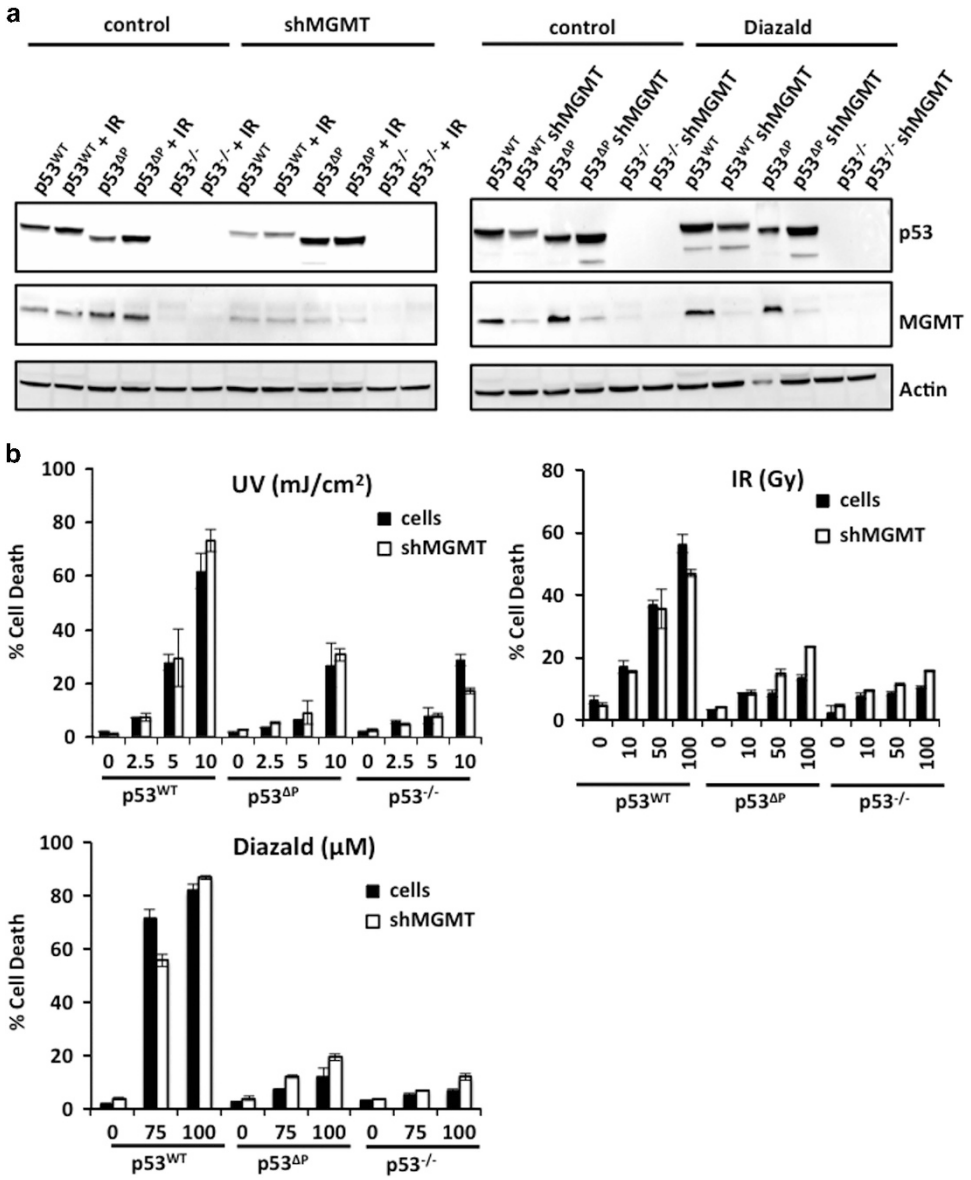

Figure 5 Loss of MGMT in cells does not change their apoptotic response to death inducing stimuli regardless of p53 status. (**a**) MGMT and p53 protein levels in $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ MEF cells, deficient or sufficient for MGMT, 18 h after IR (5 Gy) or Diazald (75 μM) treatment. (**b**) Cell death as measured flow cytometric analysis of Annexin V staining in MEF cells with or without MGMT expression treated with different doses of UV, IR or Diazald for 18 h

confirmed that $p53^{-/-}$ cells expressing MGMT showed increased survival and proliferation in response to Diazald treatment (Figures 6c and d). Overexpression did not fully mimic the survival seen in $p53^{\Delta P}$ cells, probably because MGMT-mediated repair is stoichiometric such that survival is limited to the absolute amount of MGMT molecules per cell.[39]

Discussion

While some studies utilizing $p53^{\Delta P}$ mice suggested that cell cycle arrest is constrained but apoptosis remains functional, others suggest that the opposite is true.[11,22] In the study described herein, we found that $p53^{\Delta P}$ cannot induce the expression of p21 and PUMA, and cells lacking the PRD are correspondingly deficient for apoptosis, cell cycle arrest, and senescence in response to DNA damage to a similar extent as seen in cells fully deficient for p53 function. Importantly however, unlike $p53^{-/-}$ mice that develop spontaneous tumors, $p53^{\Delta P}$ mice rarely do, although some eventually succumbed to tumors with a median survival of approximately 400 days.[22,55] We propose that biological functions of p53 are maintained in $p53^{\Delta P}$, and must be engaged for tumor suppression.

We observed that $p53^{\Delta P}$ cells exhibited a differential long-term survival response to the alkylating agent Diazald, as compared to other DNA damaging agents. We determined that MGMT levels were induced in a p53-dependent manner in response to IR and Diazald, resulting in repair and survival

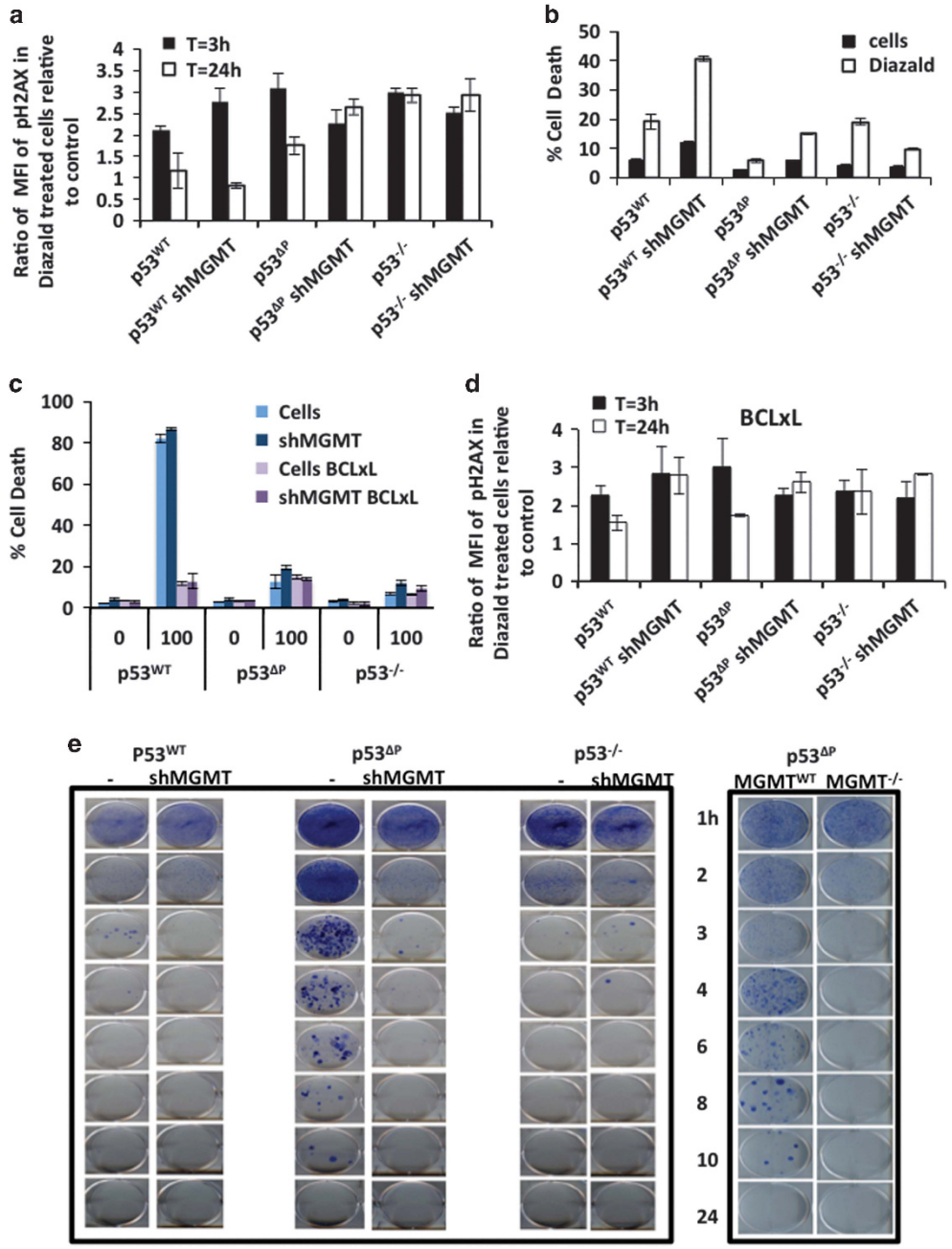

Figure 6 Loss of MGMT in cells inhibits repair and survival of $p53^{\Delta P}$ cells. Intracellular pH2AX levels measured by flow cytometry in $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ MEF cells (**a**) sufficient or deficient for MGMT or, (**d**) expressing BCLxL, treated with 100 μM Diazald for 3 h to assess damage or 24 h to assess repair. (**b**) Cell death as measured flow cytometric analysis of Annexin V staining in MEF cells treated for 3 h with 100 μM Diazald, washed and assessed for Annexin V staining 24 h later. (**c**) Cell death as measured flow cytometric analysis of Annexin V staining in MEF cells expressing BCLxL and treated with 100 μM Diazald for 24 h. (**e**) Colony-forming assays examining survival in *Left*: $p53^{WT}$, $p53^{\Delta P}$ and $p53^{-/-}$ MEF cells sufficient or deficient for MGMT; *Right*: MEF cells from $p53^{\Delta P}$, $MGMT^{-/-}$ mice after 250 μM Diazald treatment for increasing time intervals

of cells exposed to DNA damage. Importantly, our results corroborated those using genetic mouse models in which p53 showed an altered biological function.[8,52] Cells from mice deficient for p21, PUMA, and NOXA displayed γ-irradiation-induced MGMT expression (among other repair genes), and could clear DNA lesions more rapidly than that observed in p53-deficient cells.[8] Additionally, gene expression profiles of oncogene-transduced HRAS-transduced MEF from the TAD mutant knockin ($p53^{L25Q,\ W26S}$) mouse showed the induction of repair genes, including MGMT, and identified a p53 consensus-binding site in the MGMT promoter.[52] These studies suggested that MGMT is induced in a p53-dependent manner after DNA-damage or in response to oncogenes. Our studies show that in the absence of apoptosis, cell cycle arrest, and senescence, $p53^{\Delta P}$ can mediate cell survival through MGMT-mediated DNA repair.

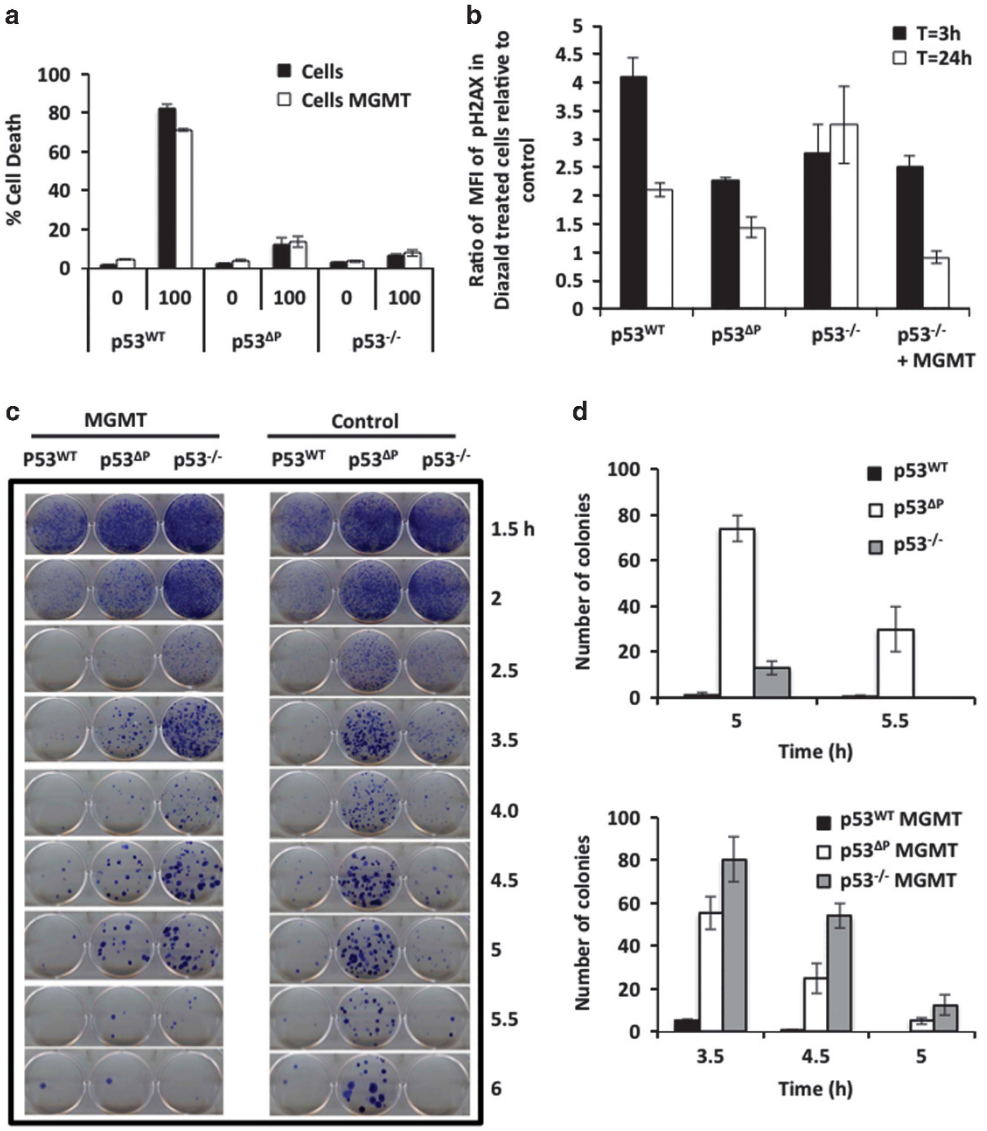

Figure 7 MGMT overexpression facilitates repair and some survival advantage in $p53^{-/-}$ cells. (**a**) Cell death at 18 h as measured by flow cytometric analysis of Annexin V staining in MEF cells overexpressing MGMT and treated with 100 μM Diazald. (**b**) Intracellular pH2AX levels measured by flow cytometry in MEF cells overexpressing MGMT and treated with 100 μM Diazald for 3 h to assess damage, or at 24 h to assess repair. (**c**) Colony-forming assays examining survival in $p53^{WT}$, $p53^{\Delta P}$ or $p53^{-/-}$ MEF cells with or without MGMT overexpression after 250 μM Diazald for increasing time intervals. (**d**) Quantification of colony-forming assay in $p53^{WT}$, $p53^{\Delta P}$, or $p53^{-/-}$ MEF cells

$P53^{-/-}$ mice have an increased incidence of MNU-induced thymic lymphomas compared to MNU-treated wild-type mice, and overexpression of MGMT in the thymus of $p53^{+/-}$ mice significantly reduced this lymphoma incidence.[53] We found that whole body ionizing radiation (4 Gy) induced p53-mediated MGMT expression, facilitating DNA repair. While $p53^{-/-}$ mice succumbed to tumors by 3 months, this was dramatically reduced in $p53^{\Delta P}$ mice after whole body irradiation. Other studies have shown some differences in the survival between $p53^{WT}$ and $p53^{\Delta P}$ mice after whole body irradiation, which we also observed.[22,55] It is feasible that the accumulating aneuploidy seen in $p53^{\Delta P}$ cells after irradiation may contribute to the difference in survival seen in $p53^{\Delta P}$ mice compared with $p53^{WT}$ mice. Indeed, MEF lacking MGMT show increased aneuploidy after irradiation.[56] Both MEF cells and mice lacking the PRD, exhibit elevated aneuploidy in comparison to WT cells, under basal and irradiated conditions.[21,55]

Although MGMT-deficient mice fail to develop spontaneous tumors, they do exhibit an increased sensitivity to the genotoxic effects of methylating agents, and are highly vulnerable to tumors induced by alkylating agents as compared to WT mice.[42–46] We saw that contrary to our expectation, irradiated $p53^{\Delta P}$ mice lacking MGMT had a median survival of 250 days, failing to phenocopy $p53^{-/-}$ mice. As $p53^{\Delta P}$, $MGMT^{-/-}$ MEF showed higher levels of aneuploidy after irradiation than with $p53^{\Delta P}$ MEF, the extent to which aneuploidy may contribute to tumor induction remains unclear. Mice with defective mitotic checkpoint genes exhibit chromosomal changes in the absence of structural abnormalities, and despite the

presence of aneuploidy, many of these mice are not predisposed to tumor formation.[57] A p53 mutant mouse ($p53^{3K}$ KI) exhibits aneuploidy and genomic instability without spontaneous tumor formation.[58,59] Therefore, chromosomal aneuploidy may be a consequence rather than a cause of deregulated cell growth.

We found that suppression or deletion of MGMT expression in $p53^{\Delta P}$ cells inhibited DNA repair and survival after alkylation damage and that its overexpression in p53-deficient cells facilitated DNA repair and conferred a survival advantage to the cells.

While p53-induced MGMT repair of DNA damage may be beneficial to prevent genomic instability and potential transformation of cells, its expression in established tumors may be detrimental to patient treatment and survival. Interestingly, many different types of cancers show promoter hypermethylation of MGMT, resulting in diminished MGMT expression.[60] The epigenetic silencing of the *MGMT* gene compromises DNA repair mechanisms and increases chemosensitivity, and has been shown to be a strong predictor of survival.[60–62] While MGMT mediated repair of DNA damage is crucial for survival of normal cells, a lack of MGMT induction in established tumors lacking p53 may actually be advantageous for overall patient survival.

Materials and Methods

Plasmids and retroviral transduction. The following previously published plasmids were used in this study to generate stable cell lines: pBabePuro.p53ER^{tam} and pBabePuro.p53$^{\Delta PP}$ER^{tam63} and LZRS-HA-BclxL.[64] Mouse Mgmt cDNA (Thermo Scientific, Waltham, MA, USA) was cloned into the retroviral expression vector, LZRS-Zeo, and fully sequenced. To generate stable cell lines, Phoenix virus producer cells were transfected with the appropriate plasmid by using Lipofectamine 2000 for 48 h. Target cells were infected with filtered virus containing the culture medium from packaging cells supplemented with 5 μg/ml polybrene. Stable transductants were selected after adding 200 μg/ml Zeocin (pLZRS vectors) (Invitrogen, Waltham, MA, USA), or 1 μg/ml puromycin (pBabe vectors) (Sigma-Aldrich, St. Louis, MO, USA). To generate knockdown of MGMT in MEF cells, we used SMARTchoice mouse MGMT-GFP shRNA (Thermo Scientific), and selected for GFP-positive clones. We used SMARTpool ON-TARGETplus Cdkn1a siRNA (Dharmacon, Lafayette, LA, USA), to silence p21.

Cell lines and cell culture. All cell lines were maintained at 37 °C/5% v/v CO_2 in a humidified incubator. H1299 cells ATCC expressing pBabePuro.p53ER^{tam} or pBabePuro.p53$^{\Delta PP}$ER^{tam} were cultured in Dulbecco's modified essential medium (DMEM) (GIBCO, Waltham, MA, USA) supplemented with 10% dextran/charcoal-treated fetal bovine serum (dcFBS), L-glutamine, and penicillin. Primary MEF cells were transformed with H-RasG12V and 12S E1A (pWZL-Hygro-12S-E1A and pBabe-Puro-H-RasG12V) and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine (GIBCO), and 100 U/ml penicillin–streptomycin (GIBCO), 1 mM sodium pyruvate (GIBCO), nonessential amino acids (GIBCO), and 55 μM β-mercaptoethanol (GIBCO).

Flow cytometry. For Annexin V staining, detached and adherent cells were harvested, washed, and re-suspended in Annexin V binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) with Annexin V-APC or Annexin V-FITC (1/200; Caltag Laboratories, Waltham, MA, USA). For intracellular pH2AX staining, cells were treated with 100 μM Diazald (Sigma-Aldrich) for 3 h to assess damage, or treated with 100 μM Diazald for 3 h, washed twice with PBS and re-suspended in cell culture media (DMEM (GIBCO) supplemented with 10% FBS, 2 mM L-glutamine (GIBCO), to assess repair. Cells at 3 and 24 h were fixed and permeabilized using a cytofix/cytoperm kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA), and stained with Alexa Fluor-647 or Alexa Fluor-488 mouse anti-H2AX (pS139) antibody (1/200; BD Biosciences). Experiments were quantitated by flow cytometry, using FACScan and FACsCalibur systems (BD Biosciences) and FlowJo Collectors' Edition software (Tree Star, Ashland, USA). Flow cytometry data are represented as mean $\pm$ S.D. of three independent experiments, unless otherwise stated.

Cell growth, senescence and cell cycle analysis. Transformed MEF cells were plated at 5e4 and growth rate determined by viable cell count through trypan-blue dye exclusion. Cell cycle analysis was performed 24 h after 4 Gy y-irradiation using the BD Pharmingen BrdU Flow Kit staining procedure, and analyzed by flow cytometry and cell cycle analysis. Replicative senescence was examined in primary MEF after serial passaging. All primary MEF were plated at 2e5 and the number of days taken to reach 90% confluence was noted. Cells were subsequently split at a ratio of 1:4, which represents a population doubling of 2. Total cell number was then calculated based on the number of days taken to reach 90% confluence. Oncogene-induced senescence was examined in primary MEF cells plated at 1e3 and transduced with H-RasG12V. Puromycin-resistant clones were stained with methylene blue. To determine aneuplodidy, cells were treated with 4 Gy y-irradiation and 72 h later cells were lysed and stained using a hypotonic buffer (0.1% Triton-100, 0.1% sodium citrate, 50 μg/ml PI) for 30 min on ice to determine the fraction of nuclei with $>$4N DNA content by flow cytometry and cell cycle analysis.

Apoptosis and survival assays. Cells were treated for 18–24 h with the following DNA damaging agents: Actinomycin D (ActD; Sigma-Aldrich), Staurosporine (STS; Sigma-Aldrich), Etoposide (Sigma-Aldrich), Diazald (Sigma-Aldrich), y-irradiation, and UV irradiation (using a Stratagene UV cross-linker). Clonogenic survival was assessed after methylene blue staining of cells treated with y-irradiation, and UV irradiation, or after treatment with very high doses of DNA damaging agents; Staurosporine (3.5 and 10 μM), Etoposide (25 and 50 μM), Doxorubicin (Sigma-Aldrich, 0.75 and 1 μM) and Diazald (150 and 250 μM) for 3 h, washed and suspended in media.

Irradiation tumor model. Mice were treated with 4 Gy γ-irradiation at p7 and monitored for clinical symptoms. All experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals, and the St. Jude Institutional Animal Care and Use Committee approved all animal procedures.

Western blotting. Cells were lysed in cell lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, complete protease inhibitors cocktail (Roche, Indianapolis, IN, USA), and 0.5% Nonidet P-40). Protein concentration in cell lysates was measured by the BCA assay (Pierce, Waltham, MA, USA) and systematically normalized before western blotting. Lysates were resolved by SDS-PAGE. The proteins were transferred to supported Hybond C nitrocellulose (Amersham Bioscience, Pittsburgh, PA, USA) and immunodetected using appropriate primary and peroxidase-coupled secondary antibodies (Amersham Bioscience). Proteins were visualized by enhanced chemiluminescence (Amersham Bioscience).

The following antibodies were used for western blotting: anti-hp53 (Do7 clone, BD Pharmingen, San Jose, CA, USA) anti-mp53 (1C12, Cell Signaling, Danvers, MA, USA), anti-p21 (clone SXM30, BD Pharmingen), anti-PUMA/bbc3 (N-terminal, Sigma-Aldrich), anti-MGMT (Clone 300008, RnD systems, Minneapolis, MN, USA), anti-$BCLxL_{s/L}$ (D3, Santa Cruz, Dallas, TX, USA) and anti-actin (clone C4, Santa Cruz, St. Louis, MO, USA).

qRT-PCR. Cells were treated with 4 Gy γ-irradiation or with 100 μM 4-Hydroxytamoxifen (Sigma-Aldrich) and RNA for qRT-PCR was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Reverse-transcription reactions were preformed with M-MLV reverse transcriptase (Invitrogen) following the manufacturer's protocol and using random hexamers. Real-time PCR was performed with SYBR Green and a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The following primers were used: m18S-S 5′-ATGGTAGTCGCCGTGCCTAC-3′, m18S-AS 5′-CCGGAATCGAACCCTGATT-3′; mp21-S 5′-CCGTTGTCTCTTCGGTCCC-3′, mp21-AS 5′-CATGAGCGCATCGC AATC-3′; mPUMA-S 5′-AGCAGCACTTAGAGTCGCC-3′, mPUMA-AS 5′-CCTGGG TAAGGGGAGGAGT-3′; mMGMT-S 5′-CTGCATGGGATACGGTTGCT-3′, mMGMT-AS 5′-GTTCACGGAAATAGGCTTCCAG-3′; hGAPDH-S 5′-TCATTTCCTGGTATGAC AACG-3′, hGAPDH-AS 5′-ATGTGGGCCATGAGGT-3′; hp21-S 5′-GCGATGGAA CTTCGACTTTG-3, hp21-AS 5′-CAGGTCCACATGGTCTTCCT-3′; hPUMA-S 5′-CTCAACGCACAGTACGAG-3′; hPUMA-AS 5′-GTCCCATGATGAGATTGTACAG -3′; mGADD45a-S 5′-CCGAAAGGATGGACACGGTG-3′, mGADD45a-AS 5′-TTATC GGGGTCTACGTTGAGC-3′; mMLH1-S 5′-GTTTTACTCCATTCGGAAGCAGT-3′, mMLH1-AS 5′-TGTGAGCGGAAGGCTTTATAGAT-3′; mPOLK-S 5′-AGCTCAAATTA

CCAGCCAGCA-3′, mPOLK-AS 5′-GGTTGTCCCTCATTTCCACAG-3′; mXPC-S 5′-TCCAGGGGACCCCACAAAT-3′, mXPC-AS 5′-GCTTTTTGGGTGTTTCTTT GCC-3′; mPMS2-S 5′-GAGCAAACCGAAGGCGTGA-3′, mPMS2-AS 5′-GC GGTGCTTAAACTGAGTACC-3′; mMSH2-S 5′-GTGCAGCCTAAGGAGACGC-3′, mMSH2-AS 5′-CTGGGTCTTGAACACCTCGC-3′; mRRM2B-S 5′-GAGCCACTC CTAAGAAAGAGTTC-3′, mRRM2B-AS 5′-GAGGGAGGTCCTTTGACAAGT-3′.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on *Cell Death and Differentiation* website (http://www.nature.com/cdd)