Inhibition of Transforming Growth Factor-β1 Signaling Attenuates Ataxia Telangiectasia Mutated Activity in Response to Genotoxic Stress

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Abstract

Ionizing radiation causes DNA damage that elicits a cellular program of damage control coordinated by the kinase activity of ataxia telangiectasia mutated protein (ATM). Transforming growth factor β (TGFβ)-1, which is activated by radiation, is a potent and pleiotropic mediator of physiologic and pathologic processes. Here we show that TGFβ inhibition impedes the canonical cellular DNA damage stress response. Irradiated *Tgfβ1* null murine epithelial cells or human epithelial cells treated with a small-molecule inhibitor of TGFβ type I receptor kinase exhibit decreased phosphorylation of Chk2, Rad17, and p53; reduced γH2AX radiation-induced foci; and increased radiosensitivity compared with TGFβ competent cells. We determined that loss of TGFβ signaling in epithelial cells truncated ATM autophosphorylation and significantly reduced its kinase activity, without affecting protein abundance. Addition of TGFβ restored functional ATM and downstream DNA damage responses. These data reveal a heretofore undetected critical link between the microenvironment and ATM, which directs epithelial cell stress responses, cell fate, and tissue integrity. Thus, *Tgfβ1*, in addition to its role in homoeostatic growth control, plays a complex role in regulating responses to genotoxic stress, the failure of which would contribute to the development of cancer; conversely, inhibiting TGFβ may be used to advantage in cancer therapy. (Cancer Res 2006; 66(22): 10861-9)

Introduction

An orchestrated response to DNA damage in multicellular organisms is important for rapid restoration of homeostasis and long-term prevention of cancer, but how signaling is regulated across tissues is unknown. Transforming growth factor β1 (TGFβ) is rapidly and persistently activated in response to DNA damage (1). TGFβ coordinates responses to a great variety of other stimuli by regulating cell proliferation, differentiation, and apoptosis (reviewed in refs. 2, 3); is involved in many aspects of development and growth regulation; and is known as a classic tumor suppressor (reviewed in ref. 3). We previously showed that epithelial tissues of irradiated *Tgfβ1* null embryos fail to undergo either apoptosis or inhibition of cell cycle, suggesting a surprising requirement for its activity in the responses to DNA damage (4). Neither the point at which *Tgfβ1* affects the genotoxic stress program nor the specific mechanisms of action have been identified.

Independent of its control of proliferation and differentiation, studies by Glick et al. (5) have implicated TGFβ in maintenance of genomic stability. *Tgfβ1* null cells are genomically unstable, cannot repair alkylating damage (6), and fail to apoptose or undergo cell cycle inhibition after ionizing radiation (IR) exposure *in vivo* (4). *Tgfβ1* null keratinocytes exhibit increased frequency of gene amplification as marked by *N*-phosphonoacetyl-L-aspartate (PALA) resistance (5). *Tgfβ1* null keratinocytes transduced with v-ras^{Ha} develop aneuploidy at higher frequencies, have more chromosomal abnormalities than the wild-type controls, and undergo spontaneous malignant transformation more frequently and with shorter latency than wild-type counterparts (7–9). The fact that TGFβ treatment of *Tgfβ1* null cells inhibits PALA resistance, reduces the percentage of aneuploid metaphases, and decreases the number of spontaneous chromosome breaks indicates an ongoing process rather than selection of genomically unstable subpopulation.

IR elicits TGFβ activity both *in vivo* and in cell culture (10–14). Radiation-induced apoptosis and cell cycle arrest in epithelial cells *in vivo* are decreased in a *Tgfβ1* gene dosage–dependent fashion (4). Similarly, the mammary epithelium of *Tgfβ1* heterozygous mice or animals treated with TGFβ neutralizing antibodies fails to undergo an apoptotic response and exhibits diminished phosphorylation of p53 in response to IR. Although these data suggest that TGFβ plays a direct role in the DNA damage response, the mechanism by which TGFβ signaling affects the DNA damage response program has not been identified.

We postulated that TGFβ provides a microenvironment signal to ensure coordinated epithelial fate decisions and restoration of homeostasis. The primary transducer of genotoxic stress caused by IR is the nuclear protein kinase ataxia telangiectasia mutated (ATM; refs. 15–18). ATM is a phosphoinositide 3-kinase–related serine/threonine kinase that mediates DNA damage responses to initiate, recruit, and activate a complex program of checkpoints for cell cycle, apoptosis, and genomic integrity (for reviews, see refs. 19, 20). Mutations in human ATM lead to ataxia-telangiectasia, which is characterized by genomic instability, cellular radiation sensitivity, and increased cancer. ATM is activated in response to double-strand breaks caused by ionizing radiation and, in turn, phosphorylates numerous substrates, thereby modulating cell fate decisions. We show here that both *Tgfβ1* depletion by genetic knockout in mouse cells and inhibition of TGFβ signaling in human cells compromise ATM kinase activity and autophosphorylation,

Note: J. Kirshner and M.F. Jobling contributed equally to this work.

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leading to reduced phosphorylation of critical DNA damage transducers, abrogation of the cell cycle block, and increased radiosensitivity. Linking ATM to TGFβ ensures that cell fate decisions are functionally connected to tissue damage, which is a novel mechanism for maintaining homeostasis, but failure of this control would greatly accelerate neoplastic potential.

Materials and Methods

Cell culture. C57bl/129SV or BALB/c *Tgfβ1*$^{+/+}$ and *Tgfβ1*$^{+/-}$ primary mammary epithelial cells were cultured in serum-free medium as described (21). Serum was removed 24 hours after culture initiation. BALB/c *Tgfβ1* wild-type and null primary keratinocytes cultured from neonates gave rise to two independently derived, spontaneously immortalized keratinocyte cell lines of each genotype (5). These cell lines, H01 and H04 from *Tgfβ1* heterozygote and K01 and K03 from *Tgfβ1* null cultures, were used in these experiments and showed similar responses. Cells were plated in calcium-free EMEM medium containing 8% chelexed fetal bovine serum and 0.2 mmol/L Ca^{2+}, then passaged in serum with 0.05 mmol/L Ca^{2+}, which was changed every 3rd day until confluence. Twelve to eighteen hours before irradiation, the medium was replaced with 8% serum-replacement medium (Knockout SR, Life Technologies, Inc., Carlsbad, California) to remove exogenous sources of TGFβ. MCF10A cells (purchased from American Type Culture Collection, Manassas, Virginia) were cultured under serum-free conditions in MEGM medium (Cambrex, East Rutherford, New Jersey) supplemented with 0.1 μg/mL of cholera toxin (Calbiochem, San Diego, California).

Unless otherwise noted, confluent, growth-arrested cells were used in experiments. Unless otherwise noted, cells were exposed to a 5-Gy dose of 250-kVp X-ray or ^{60}Co γ-radiation in air at room temperature. Control cells were sham irradiated. In some experiments, cells were treated as indicated with 500 pg/mL recombinant Tgfβ1 (R&D Systems, Minneapolis, MN). In other experiments, TGFβ type I receptor (TβRI) kinase inhibitor (240 nmol/L; Calbiochem) was added to confluent cultures. To relieve the TGFβRI kinase inhibitor block, medium was replaced with fresh medium and cells were cultured for an additional 48 hours before irradiation.

Protein analysis. Primary cells and immortalized cell lines were isolated at indicated time points and lysed in buffer containing 50 mmol/L Tris (pH 7.5), 50 mmol/L glycerophosphate, 150 mmol/L NaCl, 10% glycerol, 1% Tween 20, 1 mmol/L of phenylmethylsulfonyl fluoride, 100 μmol/L DTT, 10 μmol/L NaVa, and 1 mmol/L NaF. Protein samples collected at times indicated post-IR were stored at −80°C before separation using 4% to 15% SDS-PAGE gel. Proteins were transferred to Immobilon P (Millipore, Billerica, Massachusetts) polyvinylidene difluoride membrane and incubated with primary antibodies, washed, incubated with goat anti-mouse Alexa 680 (Molecular Probes, Carlsbad, California) or goat anti-rabbit Dye800 (Rockland, Gilbertsville, PA) secondary antibodies, and subsequently washed at room temperature. Membranes were scanned on the Odyssey Infrared Imaging System (LiCor). Target proteins were normalized to β-actin for loading and to the irradiated wild-type response for genotype comparisons; mean and SE were determined from three or more independent experiments.

Antibodies. Antibodies to p53 serine 18, p53 serine 23, Rad17 serine 645, and total Rad17 were purchased from Cell Signaling Technology (Beverly, MA). Total p53 was detected with monoclonal G59-12 antibody purchased from PharMingen, San Diego, California. ATM was immunoprecipitated with anti-ATM antibodies (exon 53) from Bethyl (Montgomery, TX) and immunoblotted with ATM 2C1 antibodies from Genetex (San Antonio, TX). β-Actin monoclonal clone EC-15 was from Sigma (St. Louis, MO). Monoclonal antibody clone 10H11.E12 and rabbit polyclonal antibodies to phosphorylated serine 1981 of ATM were purchased from Rockland Antibodies. Sheep anti–phospho-serine 1981 ATM antibody was produced using keyhole limpet hemocyanin-phospho-serine 1981 ATM peptide and affinity purified. Monoclonal γH2AX antibody was from Upstate Cell Signaling (Lake Placid, NY) and affinity-purified rabbit anti-53BP1 antibody (BL181) was purchased from Bethyl Labs.

ATM kinase assay. The ATM kinase assay was done on fresh cell extracts with the glutathione *S*-transferase (GST)-p53$_{1-44}$ substrate as described in ref. 22. Ataxia telangiectasia (A-T) human fibroblasts, purchased from Coriell Institute and cultured as recommended by supplier, were included as negative controls.

Immunofluorescence. Immunostaining to detect indicated target protein foci was done and imaged using cells cultured on LabTek eight-well chamber slides as reported (23). After treatment, cells were fixed with 2% parafomaldehyde for 5 minutes at room temperature followed by 100% methanol for 20 minutes at −20°C. Negative controls were incubated without primary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/mL). Representative false-color images are shown. In some experiments, nuclear fluorescence of 50 to 150 cells from four random fields were quantified by defining the DAPI-stained nucleus as the region of interest and integrating the mean intensity per nucleus. The mean intensity ± SE was determined for each experimental condition.

Flow cytometry. Asynchronously growing cells were pulsed for 30 minutes with 10 μmol/L bromodeoxyuridine (Sigma) at the indicated time after irradiation. Trypsinized cells were fixed in 70% ethanol for 24 hours and stained with 50 μg/mL propidium iodide (Molecular Probes) and analyzed on Beckton Dickinson, San Diego, California FACScan to determine cell cycle distribution.

Colony assay. MCF10A cells were grown to confluence as described above, treated with 240 nmol/L of TβRI kinase inhibitor for 48 hours before irradiation using 250-kVp X-ray (0.61 Gy/min). Cells were trypsinized 3 hours later. Cells were plated in triplicate at three dilutions into six-well plates and colonies were allowed to grow before fixing and staining. Colonies containing >50 cells were counted. To determine percent survival, colony-forming efficiency was determined, averaged, and normalized to those of the nonirradiated control. The mean survival ± SE was calculated for three replicate plates. The data shown are representative of three experiments.

Results

TGFβ dependence of the radiation response is cell intrinsic. We have shown that p53 phosphorylation and apoptosis are significantly decreased in irradiated *Tgfβ1* heterozygote compared with wild-type mouse mammary gland (4). To determine whether chronic TGFβ depletion *in vivo* had fundamentally (i.e., irreversibly) altered the radiation response of *Tgfβ1* heterozygote epithelial cells, or if it actually mediates the execution of the radiation response, we examined primary cultures of murine mammary epithelial cells. Irradiated *Tgfβ1* heterozygote mammary epithelial cell cultures showed significantly reduced phosphorylation of p53 compared with irradiated wild-type cells; p53 phosphorylation at serine 18 was reduced by 54% and at serine 23 by 63% relative to wild-type cell cultures (Fig. 1*A*). Total mammary epithelial cell protein extracts were immunoblotted to confirm that p53 levels were similar between genotypes. IR-induced phosphorylations of p53 increase stability and transcriptional activity to induce downstream effector genes that mediate cell cycle delay and apoptosis, as well as initiating apoptosis directly (24, 25). Consistent with decreased p53 phosphorylation, caspase-3 cleavage, which is a marker of apoptosis, peaked at 2 hours post-IR in wild-type mammary epithelial cells, but was not detected in *Tgfβ1* heterozygote up to 4 hours post-IR (Fig. 1*B*). We then asked whether this phenotype was reverted by supplementation of the serum-free culture with TGFβ (500 pg/mL). TGFβ treatment restored the ability of irradiated heterozygote mammary epithelial cells to phosphorylate p53 (Fig. 1*A*) and induce caspase cleavage (Fig. 1*C*). These data indicated that the TGFβ dependence of appropriate signaling and cell fate decisions are cell intrinsic and are compromised when TGFβ is limited.

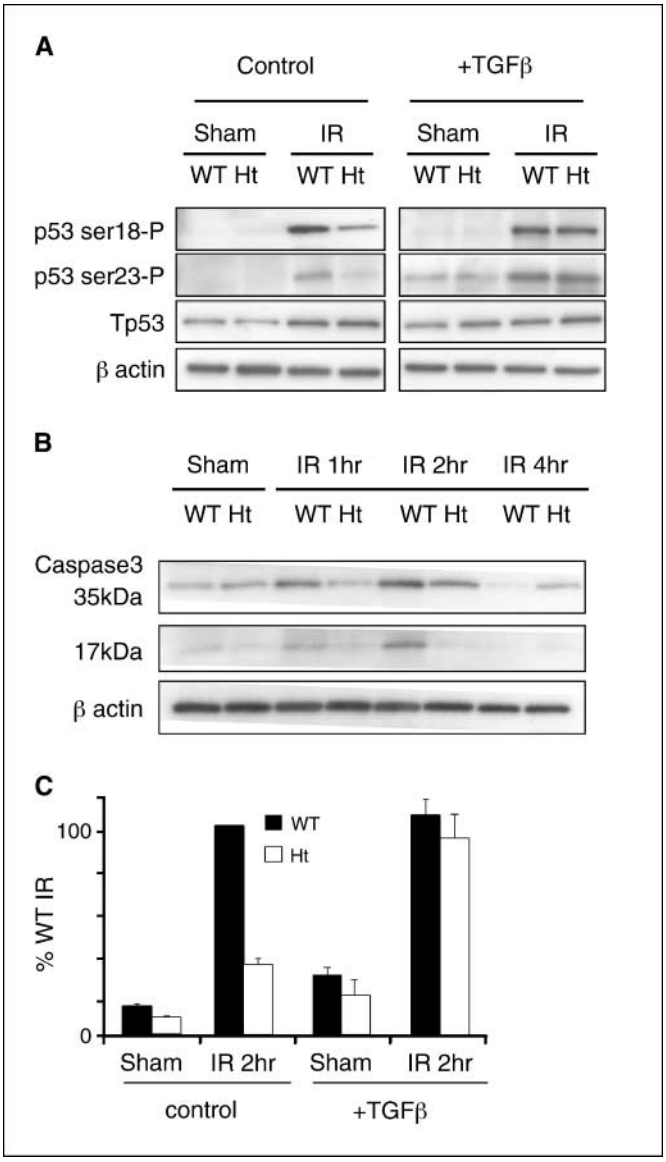

Figure 1. p53 is hypophosphorylated and caspase cleavage is compromised in irradiated *Tgfβ1* heterozygote primary mammary epithelial cells; both are restored by TGFβ treatment. *A,* representative immunoblots of p53 phosphorylation at serine 18 and serine 23 and total p53 in sham and irradiated wild-type (*WT*) and heterozygote (*HT*) mammary epithelial cells in serum-free culture treated with or without TGFβ before IR. β-Actin is shown as protein loading control. *B,* representative immunoblots of total caspase-3 and cleavage product in wild-type and heterozygote mammary epithelial cells following IR. β-Actin is shown as protein loading control. *C,* densitometric analysis of 17-kDa cleavage fragment normalized to total caspase-3 shows restoration of caspase cleavage by TGFβ treatment before IR.

IR-induced phosphorylation of genotoxic stress response proteins and subsequent cell cycle arrest are severely compromised in *Tgfβ1* null keratinocytes. To further characterize the nature and extent of the molecular defects in epithelial response to IR, we used two heterozygote and two null *Tgfβ1* keratinocyte cell lines that spontaneously immortalized from primary cultures (5). *Tgfβ1* heterozygote cells are competent to produce some TGFβ, albeit greatly reduced compared with wild-type (26), whereas null cell lines depend on, and are responsive to, exogenous sources of TGFβ such as serum. Therefore, serum was eliminated by growing cell cultures to confluence before changing the medium to a serum-free formulation, which also ensured that cell cycle distribution was consistent during subsequent experiments.

The prototypic DNA damage response induced by IR is mobilized by the highly cytotoxic double-strand break (27). The mechanism that allows this rapid dissemination of the damage alarm is based on a signal transduction pathway that begins with sensor/activator proteins that sense the damage or possibly the chromatin alterations that follow damage induction. These proteins play a major role in the activation of the transducers, which further convey the signal to multiple downstream effectors (28). Thus, we examined the abundance and phosphorylation status of p53, Chk2, and Rad17 as a function of time post-IR. Unirradiated cells of either genotype showed similar levels of total p53, Chk2, and Rad17 protein (Fig. 2*A*). Irradiation of *Tgfβ1* heterozygote cell lines induced prolonged phosphorylation of p53 serine 18 and Rad17 serine 645 that were maximal at 4 hours, whereas phosphorylation of p53 serine 23 and Chk2 threonine 68 were maximally induced at 1 hour post-IR and undetectable at later time points. In comparison, null genotype keratinocytes were hypophosphorylated in response to IR. Phosphorylation of p53 serine 18 was 30% in *Tgfβ1* null cells relative to heterozygote cells post-IR at 15 minutes and considerably reduced at later time points. p53 serine 23 phosphorylation was undetectable at any time in *Tgfβ1* null keratinocyte cells. p53 serine 23 is phosphorylated by Chk2 (29), which requires phosphorylation at threonine 68 for its kinase activity (30). Compared with heterozygote cells, Chk2 threonine 68 phosphorylation was also significantly reduced in null keratinocyte cells. Phosphorylation of Rad17 at serine 645, which is necessary for the DNA damage–induced activation of cell cycle checkpoints (31), was markedly decreased in *Tgfβ1* null cells relative to heterozygote cell lines.

A hallmark of the DNA damage response is the activation of cell cycle checkpoints, which temporarily halt the cell cycle until the damage is repaired (32). Reduced phosphorylation of Rad17, Chk2, and p53 in response to DNA damage should compromise cell cycle checkpoints in cycling cells (reviewed in ref. 33). Because TGFβ regulates cell cycle, and radiation response is a function of cell cycle phase, we used cells grown to fed-confluence so that cell cycle differences were not a factor. However, experiments using asynchronous cultures showed that molecular responses were also compromised in proliferating *Tgfβ1* null cells (data not shown). Therefore, we examined cell cycle distribution in response to IR in exponentially growing cells. As expected, the percent of S-phase cells in *Tgfβ1* heterozygote cells at 5 hours post-irradiation was reduced from 32% to 22% ($P < 0.005$, Student's t test) and the percent of cells in G_2 increased nearly 3-fold. In contrast, irradiated *Tgfβ1* null cells did not undergo a significant change in cell cycle distribution. The lack of cell cycle arrest in this *Tgfβ1* null cell line is comparable to the absence of DNA synthesis block observed in epithelial tissues of irradiated *Tgfβ1* null embryos (4).

Nuclear γH2AX radiation-induced foci (RIF) are an early event elicited by DNA double-strand breaks (34). γH2AX RIF formed readily in *Tgfβ1* heterozygote cells, but were significantly reduced in irradiated *Tgfβ1* null cells (Fig. 2*B*). In contrast, 53BP1, which binds to epitopes in methylated lysine 79 of histone H3 (35), formed RIF in both irradiated *Tgfβ1* genotypes (Fig. 2*C*). 53BP1 RIF confirm the presence of DNA damage caused by IR. Together the reduced phosphorylation of p53, Rad17, Chk2, and H2AX suggests that the necessary kinase is compromised.

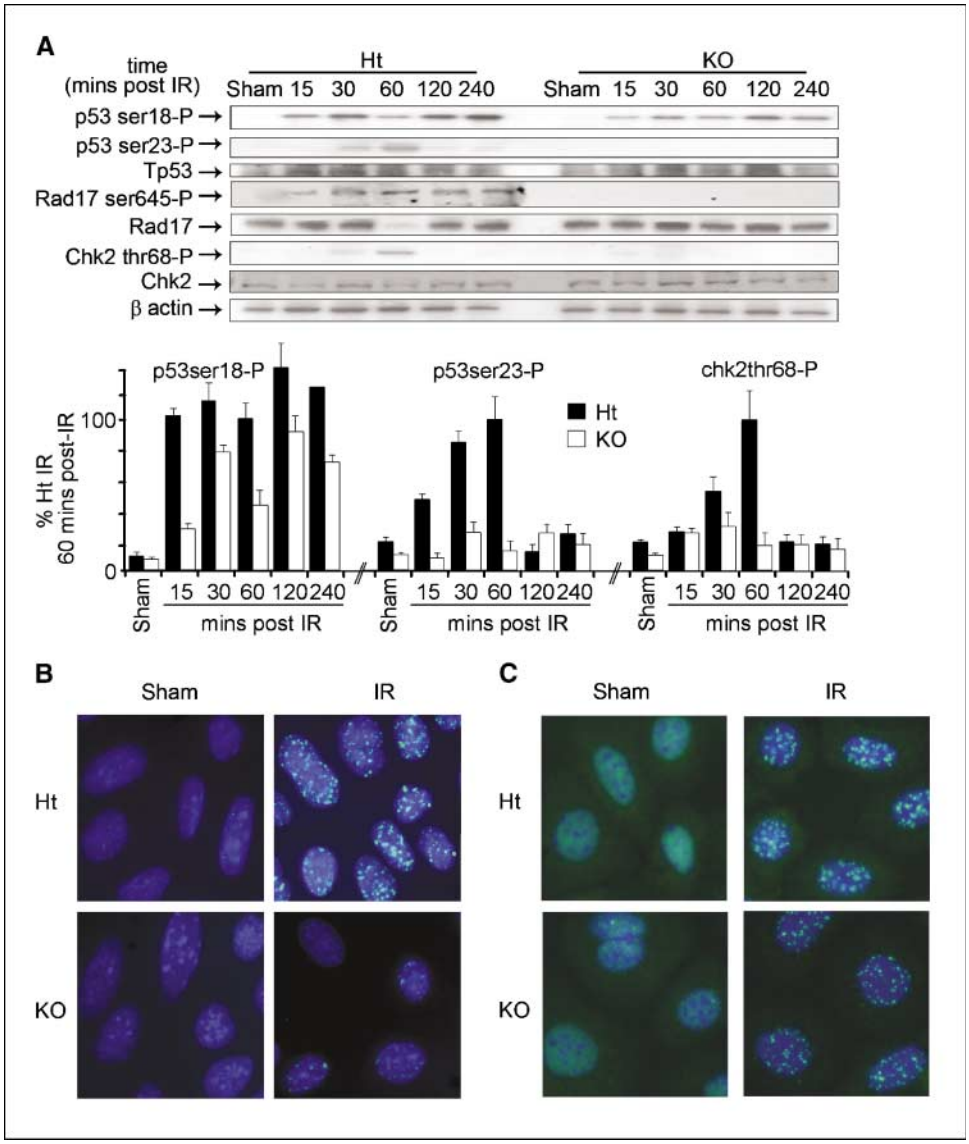

Figure 2. IR-induced phosphorylation of DNA damage response proteins is compromised in *Tgfβ1* null keratinocyte cell lines. *A,* immunoblots of p53 phosphorylation at serine 18 and serine 23, Rad17 serine 645 phosphorylation, and Chk2 threonine 68 of sham and irradiated *Tgfβ1* null and heterozygote keratinocyte cell lines as a function of time after IR. Total proteins and β-actin are shown. *B,* immunofluorescence detection of phosphorylated γH2AX (*green*) RIF formation in DAPI-stained nuclei (*blue*) of sham and irradiated *Tgfβ1* null and heterozygote keratinocytes. γH2AX RIF formation at 30 minutes post-IR (2 Gy) is evident in heterozygote keratinocytes but is barely detectable in irradiated null keratinocytes. *C,* immunolocalization of 53BP1 (*green*) in DAPI-stained nuclei (*blue*). Sham-irradiated *Tgfβ1* heterozygote and null keratinocytes showed diffuse nuclear immunoreactivity. 53BP1 formed distinct RIF at 30 minutes post-IR (2 Gy) throughout the nuclei of both *Tgfβ1* heterozygote and null keratinocyte cells.

Atm kinase activity and autophosphorylation are markedly reduced in *Tgfβ1* null cells. ATM is a serine/threonine protein kinase required for the rapid response to IR-induced DNA double-strand breaks (36). ATM can directly phosphorylate p53 serine 18, Rad17 serine 645, Chk2 threonine 68, and H2AX in response to IR and, thus, is a candidate for the defective DNA damage response of *Tgfβ1* null cells. To test this hypothesis, Atm kinase activity was measured in *Tgfβ1* null and heterozygote keratinocyte cell lines before and 1 hour post-IR using a p53 GST-substrate *in vitro* kinase assay (Fig. 3*A*). The level of substrate phosphorylation by Atm immunoprecipitated from irradiated *Tgfβ1* null keratinocytes was 30% that of *Tgfβ1* heterozygote keratinocytes (Fig. 3*B*). We determined that Atm protein levels were unaffected by *Tgfβ1* gene status as measured by immunoblotting total Atm protein in cell extracts of null versus heterozygote keratinocyte cell lines (Fig. 3*C*), null versus wild-type primary keratinocytes, or heterozygote versus wild-type primary mammary epithelial cells (not shown). These data indicate that Atm activity, rather than abundance, is affected by TGFβ depletion.

Following radiation exposure, the Atm dimer undergoes autophosphorylation (22, 37, 38). Bakkenkist and Kastan (37) showed that ATM autophosphorylation at serine 1981 (1987 in mouse) is involved in the dissociation of inactive dimer or higher order multimers and the initiation of kinase activity and correlates with its activity. Atm serine 1981 phosphorylation was clearly evident in *Tgfβ1* heterozygote cells from 15 minutes through 4 hours post-IR. In contrast, *Tgfβ1* null keratinocytes showed minimal ability to undergo Atm autophosphorylation at serine 1981 immediately following irradiation, and did not recover up to 4 hours post-IR (Fig. 3*C*). Because Bakkenkist and Kastan showed that ATM autophosphorylation occurs within 2 minutes of DNA damage, these data, in conjunction with compromised substrate phosphorylation and failure of cell cycle arrest, suggest that Atm activation in these cells is absent rather than delayed.

ATM is also involved in the response to UV irradiation (39). UV causes the formation of cyclobutane pyrimidine dimers and formation of single stranded breaks as the cell attempts to repair the damage. Both UV and IR elicit p53 phosphorylation. UV-irradiated *Tgfβ1* heterozygote cells showed prominent p53 serine

18 phosphorylation at 1 and 3 hours post UV irradiation, but *Tgfβ1* null cells did not exhibit detectable p53 phosphorylation (Fig. 3*D*). Likewise, Atm 1981P was readily observed at 1 and 3 hours post UV irradiation in *Tgfβ1* heterozygote cells, although absent in *Tgfβ1* null keratinocytes. Furthermore, asynchronous cultures of *Tgfβ1* heterozygote cells underwent cell cycle arrest in G_2 after UV (15 J/m^2), whereas *Tgfβ1* null cells did not undergo a significant change in cell cycle distribution (data not shown). Thus, *Tgfβ1* depletion broadly compromises the genotoxic stress response to physical damage caused by IR and UV.

A fraction of activated ATM binds to the double-strand break sites (37, 40). Many ATM substrates are phosphorylated by the chromatin-bound fraction of this kinase (41), such as Chk2, which occurs at DNA double-strand breaks (reviewed in ref. 32). On IR exposure, *Tgfβ1* heterozygote cells exhibited formation of Atm serine 1981P nuclear RIF (Fig. 4*A*). Consistent with the biochemical data, neither diffuse nor punctate phosphorylated serine 1981 Atm was detectable in *Tgfβ1* null cells. The average fluorescence intensity of Atm serine 1981P did not change in irradiated null cells [53.5 ± 9.1 (SE) versus 51.9 ± 11.6 (SE) irradiated] whereas the fluorescence relative to sham-irradiated heterozygote cells increased 4.6-fold at 1 hour [mean fluorescence intensity 35.31 ± 6.4 (SE) versus 164.3 ± 22.4 (SE) irradiated]. The difference between genotypes did not alter up to 4 hours post-IR (data not shown). According to current models (18), recruitment of both the ATM monomer and the ATM substrates is mediated by several proteins, including the MRN complex, MDC1, and 53BP1. As shown in Fig. 2*C*, 53BP1 RIF formation is intact. We found Nbs-1 and Mre-11 protein levels to be unaffected by immunoblotting (data not shown). Finally, *Tgfβ1* heterozygote mammary epithelium irradiated *in vivo* exhibited reduced phosphorylated ATM immunoreactivity compared with tissue from wild-type mice (Fig. 4*B*), consistent with our previous observation of reduced p53 phosphorylation. Together, the localization and biochemical data indicate that Atm activation and autophosphorylation fail to respond to IR-induced DNA damage in TGFβ compromised murine epithelial cells.

These data suggest Atm damage responses are a function of TGFβ availability; if so, addition of TGFβ should be sufficient to restore the program. TGFβ treatment for 4 hours or more did not induce Atm serine 1981 in unirradiated cells but restored Atm serine 1981 autophosphorylation in response to IR as determined by immunoblotting (Fig. 5*A*). IR-induced Atm serine 1981 RIF were also restored, indicating fully functional activation (Fig. 5*B*). Restitution of autophosphorylation correlated with function as shown by restoration of p53 serine 18 phosphorylation (Fig. 4*A*) and nuclear γH2AX after IR (Fig. 5*C*). Thus, treatment with TGFβ is sufficient to restore Atm autophosphorylation and activity in the *Tgfβ1* null keratinocytes, indicating that TGFβ is an essential regulator of this pathway.

TβR1 kinase inhibitor abrogates genotoxic stress responses in human epithelial cells. To test whether the DNA damage response of human epithelial cells is mediated by TGFβ, MCF10A human mammary epithelial cells were treated with a small-molecule inhibitor of TβRI kinase (42). Control experiments showed that TβRI kinase inhibitor treatment released MCF10A cells from TGFβ growth inhibition and blocked phosphorylation of Smad2 (data not shown). When growth-arrested MCF10A cells were irradiated following a 48-hour exposure to the TβRI kinase inhibitor, phosphorylated p53, Chk2, and Rad17 were significantly reduced compared with irradiated cells treated with vehicle (Fig. 6*A*). Consistent with this, ATM serine 1981 phosphorylation was decreased by >50%. Releasing the cells from the inhibitor by refeeding with fresh media for 48 hours before IR exposure restored the DNA damage–induced phosphorylations. Furthermore, TβRI kinase inhibitor blocked the induction of ATM serine

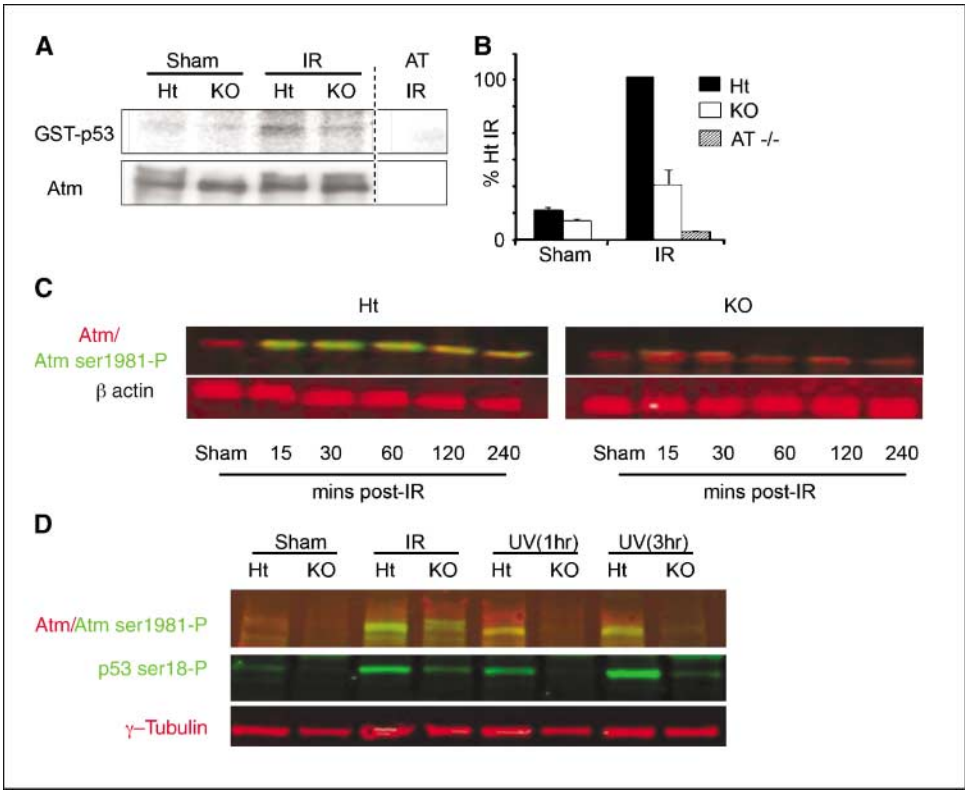

Figure 3. Atm kinase activity and autophosphorylation are markedly reduced in *Tgfβ1* null cells. *A,* representative kinase assay of immortalized *Tgfβ1* null compared with heterozygote keratinocyte cell lines. Irradiated A-T fibroblasts were included as a negative control. Atm immunoblotting after immunoprecipitation shows similar Atm loading for each genotype and treatment. Kinase activity was dramatically reduced in null versus heterozygote cells. *B,* quantitation by densitometry of the kinase activity normalized to Atm protein immunoprecipitation of *Tgfβ1* null and heterozygote cell lines. *Columns,* mean (*n* = 3 experiments); *bars,* SE. *C,* dual immunoblot with infrared antibodies shows Atm serine 1981 autophosphorylation (*green/yellow*) and total Atm (*red*) as a function of time post-IR. Heterozygote keratinocytes show rapid and persistent Atm autophosphorylation that is lacking in null keratinocyte cells. β-Actin is shown as protein loading control. *D,* dual immunoblot analysis of *Tgfβ1* heterozygote and null keratinocyte cell extracts shows that phosphorylation of p53 serine 18 and Atm serine 1981 are diminished in *Tgfβ1* null cells at 1 hour post UV and do not recover by 3 hours. γ-Tubulin is shown as protein loading control.

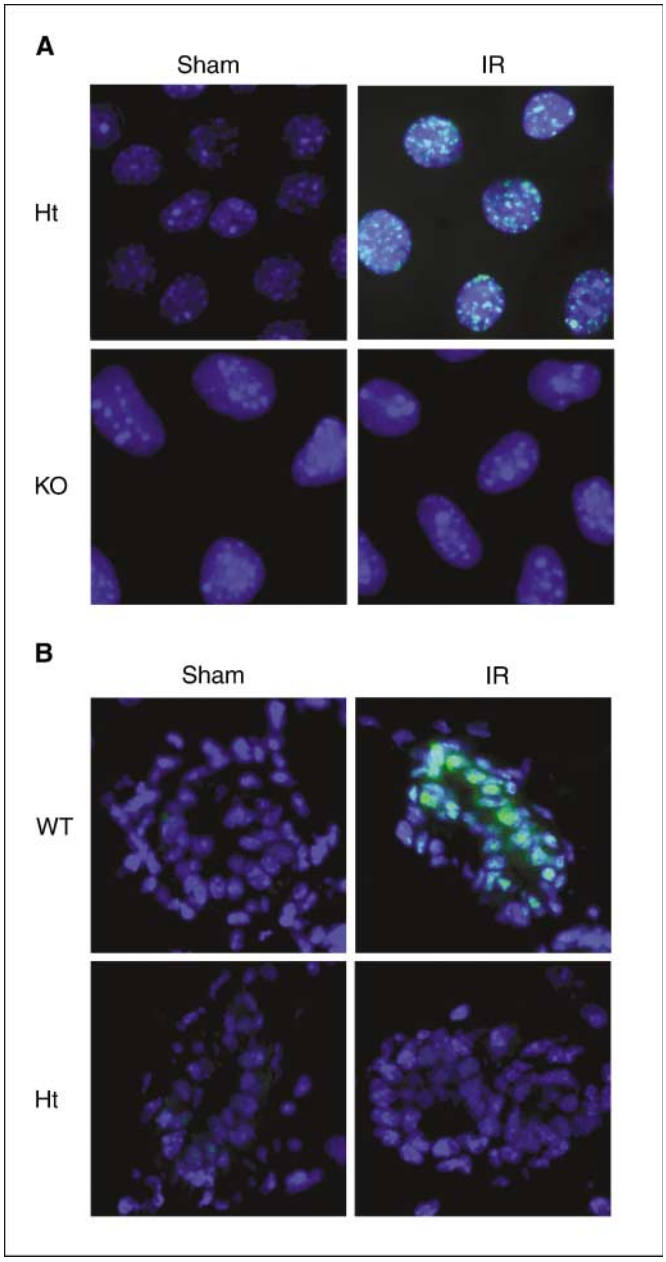

Figure 4. Reduced localization of nuclear Atm serine 1981 *in vitro* and *in vivo*. *A,* immunolocalization of phospho-specific antibodies to Atm serine 1981 (*green*) and DAPI-stained nuclei (*blue*). Sham-irradiated *Tgfβ1* heterozygote and null keratinocytes showed little immunoreactivity. Irradiated A-T cells were negative (not shown). At 30 minutes post-IR (2 Gy), immunolocalized phosphorylated Atm serine 1981 was evident as distinct RIF throughout the nuclei of *Tgfβ1* heterozygote keratinocyte cells but was absent from null cells. *B,* immunolocalization of phospho-specific sheep anti-Atm serine 1981 (*green*) and DAPI-stained nuclei (*blue*) in *Tgfβ1* wild-type and heterozygote mammary gland. Sham-irradiated tissue showed little immunoreactivity in either genotype. At 1 hour post-IR (5 Gy), prominent Atm serine 1981 immunostaining of nuclei was evident in the wild-type mice. Significantly less Atm serine 1981 immunostaining was present in epithelium of *Tgfβ1* heterozygote mouse mammary gland.

1981 (Fig. 6*B*) and γH2AX RIF in irradiated MCF10A cells (Fig. 6*C*). As in murine cells, 53BP1 RIF were unaffected (data not shown).

A-T cells are very radiosensitive and deletion of ATM leads to radiation hypersensitivity as measured by clonogenic survival (43). We determined that clonogenic survival following a graded IR dose response was significantly decreased by TGFβ inhibition relative to cells irradiated without inhibitor treatment (Fig. 6*D*). The survival of MCF10A cells irradiated with 2 Gy following treatment with TβRI kinase inhibitor decreased by 35% compared with vehicle-treated controls [36.1 ± 1.9 (SE) versus 56.0 ± 2.0 (SE); n = 3 experiments]. *Tgfβ1* null keratinocytes were also more radiosensitive as measured by clonogenic survival (data not shown).

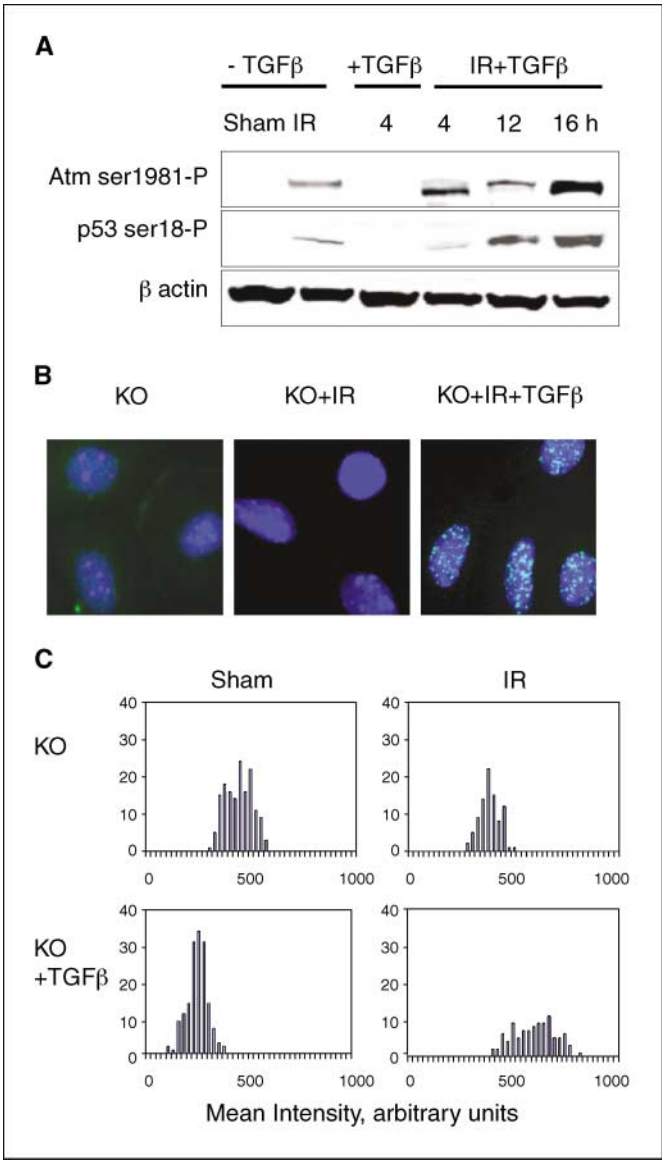

Figure 5. TGFβ treatment restores ATM autophosphorylation, localization, and function in *Tgfβ1* null keratinocytes. *A,* immunoblot of Atm serine 1981 phosphorylation, p53 serine 18, and β-actin loading control for null keratinocytes. Treatment with TGFβ for 4 hours before IR (5Gy) significantly increased ATM serine 1981 autophosphorylation and p53 serine 18 phosphorylation. *B,* immunolocalization of phospho-specific antibodies to Atm serine 1981 (*green*) and DAPI-stained nuclei (*blue*) of null keratinocyte cells 30 minutes after irradiation (2 Gy). RIF formation was restored in TGFβ-treated null keratinocyte cells. *C,* histograms of the mean intensity per nucleus of γH2AX immunoreactivity of *Tgfβ1* null cells cultured in the presence or absence of *Tgfβ1* before being irradiated. γH2AX immunoreactivity was not induced by TGFβ treatment alone. *Tgfβ1* exposure before irradiation restored γH2AX in the majority of irradiated *Tgfβ1* null cells.

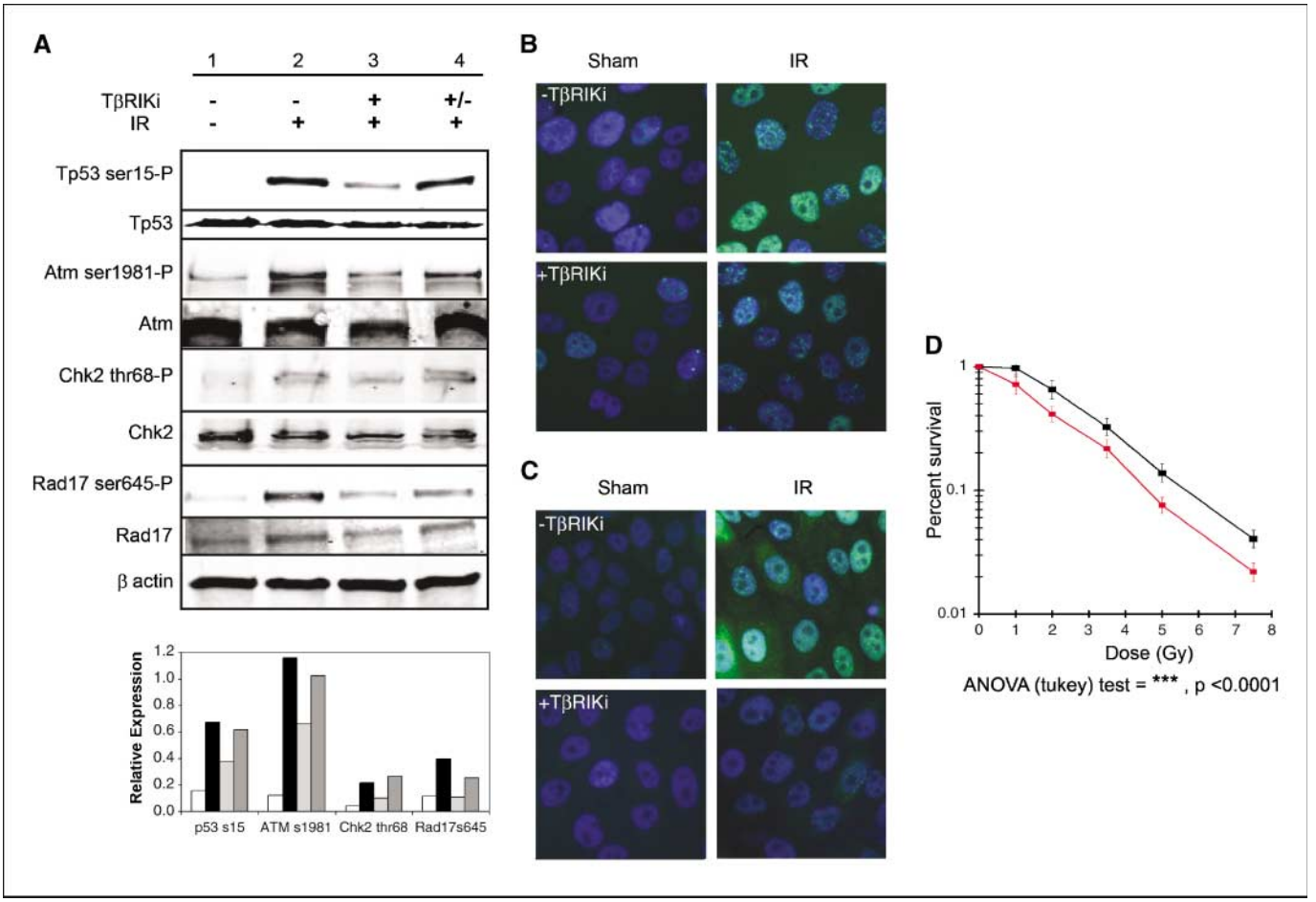

Figure 6. TβR1 kinase small-molecule inhibitor decreases the DNA damage response in human epithelial cells. *A,* confluent MCF10A human mammary epithelial cells cultured in serum-free medium for 96 hours were treated as follows: Control (*white column*); irradiated (*black column*); treated for the final 48 hours with TβR1 kinase inhibitor and irradiated (*light gray column*); and treated for 48 hours with TβR1 kinase inhibitor and refed with fresh medium without inhibitor and irradiated (*dark gray column*). Cultures were harvested 1 hour after irradiation (5 Gy) for immunoblotting. Quantitation of phosphorylation-specific antibodies using the LiCor Odyssey was normalized to the respective total protein. TβR1 kinase inhibitor treatment did not change the abundance or phosphorylation status of proteins in the absence of irradiation (not shown). *B* and *C,* RIF formation in MCF10A cells treated for 24 hours with and without 240 nmol/L TβRI kinase inhibitor and sham or irradiated (2 Gy). Forty minutes post-IR, cells were fixed and stained with phospho-specific antibodies to either γH2AX (*B*; *green fluorescence*) or phosphorylated ATM serine 1981 (*C*; *green fluorescence*). Nuclei are DAPI stained (*blue*). Treatment with TβRI kinase inhibitor impedes formation of nuclear RIF of both γH2AX and phosphorylated ATM serine 1981. The formation of 53BP1 nuclear RIF was unaffected (not shown). *D,* colony-forming efficiency of irradiated MCF10A control cells (*black*) and treated for 48 hours with 240 nmol/L TβRI kinase inhibitor (*red*). Inhibition of TGFβ decreased clonogenic survival compared with controls as a function of radiation dose ($P < 0.0001$, ANOVA).

Discussion

TGFβ is a key extracellular player for initiating and integrating multiple cellular responses to tissue response to IR and other types of damage (44). Our studies show that the activation of the ATM-mediated genotoxic stress program in mouse and human epithelial cells is severely compromised by loss of *Tgfβ1* signaling. Rather than affecting kinase or substrate abundance, our data point to modulation of ATM kinase activation by one or more TGFβ transcriptional targets. Decreased Atm activity and serine 1981 autophosphorylation suggests that inhibition of TGFβ signaling affects its ability to initiate the damage response. This conclusion is supported by compromised substrate phosphorylation (i.e., p53, Chk2, and Rad17) as well as by the absence of γH2AX foci in irradiated *Tgfβ1* heterozygote cells and human cells treated with small-molecule inhibitor of the type I receptor kinase. The abrogated genotoxic stress signaling by Atm in cultured cells and the compromised autophosphorylation observed in irradiated *Tgfβ1* heterozygote mammary gland (Fig. 5) provide a mechanism to explain our previous observation that both apoptosis and cell cycle arrest are absent in the skin or liver of irradiated *Tgfβ1* null embryos (4).

Our study reveals a novel functional link between TGFβ signaling and the ATM-mediated molecular cascades that dictate epithelial cell fate. Notably, TGFβ treatment of null keratinocytes before irradiation was essential for restoration of the DNA damage response, indicating that TGFβ signaling primes cells to respond to DNA damage either by assisting in the recruitment of ATM to the site of DNA damage or by facilitating ATM activation. This would suggest that an additional signal is required for these processes in epithelial cells or that one of the proteins normally involved in ATM activation is missing or defective. Alternatively, TGFβ may directly or indirectly suppress an inhibitory function of the activation process, although, at this time, there is no known inhibitor of ATM activation. In the absence of TGFβ production or signaling, this

inhibitory function seems dominant in both human and mouse epithelial cells; this possibility remains to be explored.

An interesting question raised by these studies is why normal epithelial cells should require an extracellular factor to respond to DNA damage. The coupling of intracellular response mediated by ATM and extracellular signaling by TGFβ would ensure an integrated tissue response to damage and restoration of homeostasis, which is a novel mechanism for preventing cancer (45). Unlike fibroblasts and lymphoid cells, epithelial cells function, in large part, as an integrated unit, which, if breached, makes the organism susceptible to a wide range of pathologies. By ensuring that extracellular and intracellular signaling in the response to DNA damage are intrinsically and reciprocally intertwined, then organisms can maintain homeostatic coordination of cellular events within an epithelium.

Importantly, these studies suggest an additional mechanism by which early escape from TGFβ signaling could contribute to the development of cancer. Preneoplastic lesions exhibit high levels of DNA damage response proteins, which is postulated to increase the potential for genomic instability (46). Our studies suggest that escape from *Tgfβ1*, in addition to releasing epithelial cells from growth control, would compromise responses to genotoxic stress, thus priming cells to become unstable. Consistent with this, keratinocytes from *Tgfβ1* null mice exhibit a 100- to 1,000-fold greater genomic instability measured by gene amplification than wild-type cells (5). TGFβ has been considered a canonical tumor suppressor of epithelial tissues (reviewed in refs. 3, 47, 48); our studies indicate that TGFβ acts to maintain homeostasis in an even more comprehensive manner than previously recognized. Furthermore, current development of TGFβ inhibitors for use in cancer therapy, potentially in combination with DNA damaging agents, may well provide therapeutic advantage, as is shown by increased radiosensitivity in our model systems.

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