Sonic Hedgehog signaling impairs ionizing radiation–induced checkpoint activation and induces genomic instability

Jennifer M. Leonard,1 Hong Ye,2 Cynthia Wetmore,1,2 and Larry M. Karnitz3,4,5

1Department of Biochemistry and Molecular Biology, 2Department of Pediatrics and Adolescent Medicine, 3Department of Molecular Pharmacology and Experimental Therapeutics, 4Department of Radiation Oncology, and 5Division of Oncology Research, College of Medicine, Mayo Clinic, Rochester, MN 55905

The Sonic Hedgehog (Shh) pathway plays important roles in embryogenesis, stem cell maintenance, tissue repair, and tumorogenesis. Haploinsufficiency of Patched-1, a gene that encodes a repressor of the Shh pathway, dysregulates the Shh pathway and increases genomic instability and the development of spontaneous and ionizing radiation (IR)–induced tumors by an unknown mechanism. Here we show that Ptc1+/− mice have a defect in the IR-induced activation of the ATR–Chk1 checkpoint signaling pathway. Likewise, transient expression of Gli1, a downstream target of Shh signaling, disrupts Chk1 activation in human cells by preventing the interaction of Chk1 with Claspin, a Chk1 adaptor protein that is required for Chk1 activation. These results suggest that inappropriate Shh pathway activation promotes tumorogenesis by disabling a key signaling pathway that helps maintain genomic stability and inhibits tumorogenesis.

Introduction

The Sonic Hedgehog (Shh; in vertebrates) signaling pathway, which was originally identified in Drosophila melanogaster, is highly conserved and plays critical roles in embryonic development in multiple organisms, including humans (Hooper and Scott, 2005). In addition to its pivotal role in embryogenesis, aberrant activation of Shh signaling is associated with tumorogenesis in many organs including skin, brain, lung, breast, prostate, and the pancreas (Jacob and Lum, 2007). The Shh pathway was first linked to tumor development in patients with basal cell nevus syndrome (BCNS; also known as Gorlin syndrome), who carry germline mutations in one allele of the Patched-1 (PTCH1) gene (Hahn et al., 1996; Johnson et al., 1996). PTCH1 encodes PTCH1, a transmembrane receptor that binds to and represses the activity of Smoothened (SMOH; Hooper and Scott, 2005). In normal cells, Shh signaling is initiated by the binding of the Shh ligand to PTCH, which relieves PTCH-mediated repression of SMOH (Hooper and Scott, 2005). In cells carrying a mutated PTCH1 allele, SMOH signaling is inadequately repressed, leading to unrestrained activation of Gli1, a transcription factor and putative oncogene that is capable of inducing tumorogenesis in skin and brain (Ruiz i Altaba et al., 2007).

In addition to the predisposition for spontaneous tumorogenesis in BCNS patients, these patients are also at highly increased risk of tumor development in areas exposed to ultraviolet or ionizing radiation (IR; Gorlin, 1987). Similar to humans carrying mutation of PTCH1, deletion of one allele of Ptc1 in mice (Ptc1+/−) recapitulates these phenotypes, including an increased rate of spontaneous brain tumorogenesis (Goodrich et al., 1997; Wetmore et al., 2000) and a two- to fivefold increased incidence of medulloblastoma after exposure to IR (Hahn et al., 1998; Pazzaglia et al., 2002, 2006a). These observations suggest that aberrant Shh signaling in mice and humans increases genomic instability and compounds the tumorigenic effects of IR.

IR-induced double strand breaks (DSBs) activate the phosphatidylinositol 3-kinase–related kinases ATM and ATR, which regulate apoptosis, cell cycle progression, and DNA repair (Abraham, 2001). After the appearance of a DSB, ATM activates Chk2 and triggers the nucleolytic processing of the DSB into extended regions of single stranded DNA (ssDNA;
Jazayeri et al., 2006). ATR is then activated when the ssDNA is coated by replication protein A (RPA), which recruits ATR (in a complex with ATR-interacting protein ATRIP) and triggers the loading of the Rad9–Hus1–Rad1 (9-1-1) complex. The 9-1-1 complex then induces ATR-mediated phosphorylation and activation of the protein kinase Chk1 (Zou, 2007) in a process that requires Claspin, an adaptor protein that is phosphorylated in an ATR-dependent manner. Once activated, Chk1 prevents cells from exiting G2, regulates DNA repair, stabilizes stalled replication forks, and triggers the S-phase checkpoint (Bartek and Lukas, 2003). The importance of these pathways is underscored by the observations that they play critical roles in maintaining genomic stability (Liu et al., 2000; Weiss et al., 2000; Wang et al., 2003; Lam et al., 2004; Syljuåsen et al., 2004; Durkin et al., 2006; Pandita et al., 2006) and in blocking the development of tumors (Bartkova et al., 2005; Gorgoulis et al., 2005).

Despite the longstanding observation that IR dramatically increases the incidence of tumors in BCNS patients and Ptc1+/− mice, how the Shh pathway influences tumorigenesis has remained elusive. Here we show that Shh pathway signaling attenuates activation of a genotoxic-triggered ATR–Chk1 checkpoint signaling pathway that serves as a barrier to the development of tumors.

Results and discussion

Ptc1+/− mice develop accelerated medulloblastomas after IR

To explore how dysregulated Shh signaling contributes to tumorigenesis, we first established an IR-induced model of tumorigenesis using Ptc1+/− mice. Previous studies demonstrated that irradiation of early postnatal Ptc1+/− mice on CD1/129 background dramatically increased the incidence of medulloblastoma, whereas irradiation at postnatal day (P) 10 or later did not increase the incidence of tumors above background (Hahn et al., 1998; Pazzaglia et al., 2002, 2006b). Because only limiting amounts of tissue are available for study in P3 mice, we asked whether Ptc1+/− mice on a C57Bl/6SJv background developed medulloblastomas when irradiated at P5–6. Fig. 1 A shows that irradiated wild-type mice did not develop medullo-

blastomas over the 30-wk time course. In contrast, Ptc1+/− mice irradiated at P3–4 and P5–6 showed vastly increased rates of tumor incidence. Because irradiation of P3–4 and P5–6 Ptc1+/− mice led to comparable incidences of medulloblastoma, we used P5–6 animals for the studies that follow.

Ptc1+/− cerebella have a defect in Chk1 but not ATM activation

Defects in checkpoint signaling can lead to increased tumorigenesis, raising the possibility that the accelerated development of medulloblastomas in Ptc1+/− mice results from a defect in checkpoint signaling in developing cerebella. To address this possibility, we analyzed the activation of the ATM–Chk2 and ATR–Chk1 pathways in developing cerebella after irradiation of wild-type and Ptc1+/− mice. Activation of the ATM pathway was assessed by immunoblotting dissected cerebella for phosphorylation of ATM on Ser1981, a site that is autophosphorylated upon ATM activation (Bakkenist and Kastan, 2003). Activation of the ATR pathway was detected by phosphorylation of Chk1 on Ser317 and Ser345, sites that are phosphorylated by ATR and required for Chk1 activation (Zhao and Piwnica-Worms, 2001). IR-induced ATM phosphorylation was equivalent in wild-type and Ptc1+/− cerebella (Fig. 1 B). In contrast, when compared with wild-type mice, IR-induced Chk1 phosphorylation on Ser317 was markedly reduced in the Ptc1+/− mice in two separate experiments (Fig. 1, B and C), as was phosphorylation of Chk1 Ser345 (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200804042/DC1). To rule out that differences in IR-induced Chk1 phosphorylation were the result of cell cycle redistribution in the Ptc1+/− cerebella, we analyzed cell cycle distributions in cells isolated from wild-type and Ptc1+/− mice. No difference was observed (Fig. S2 A). Collectively, these results demonstrate that the early postnatal cerebella of Ptc1+/− mice have a selective defect in IR-induced activation of the ATR–Chk1 signaling pathway, which may contribute to cellular transformation and tumorigenesis.

Gli1 disrupts Chk1 but not Chk2 activation in a model system

To develop a biochemically tractable model system to further investigate how Shh signaling impacts Chk1 activation, we reasoned...
that expression of Gli1, a key mediator of the Shh pathway that is critical for medulloblastoma development (Kimura et al., 2005), would also affect Chk1 activation. Consistent with this hypothesis, Gli1 expression in human embryonic kidney (HEK) 293 cells suppressed IR-induced Chk1 Ser317 (Fig. 2 A) and Ser345 (Figs. S1 B) phosphorylation as well as the Chk1-dependent degradation of Cdc25A (Fig. 2 C). To rule out that this effect on Chk1 phosphorylation was the result of a Gli1-mediated disruption of the cell cycle, we analyzed the cell cycle profile of vector-transfected and Gli1-expressing cells. Gli1 did not disrupt cell cycle progression (Fig. S2 B).

To further characterize the effects of Gli1 on checkpoint signaling, we analyzed IR-induced Chk2 phosphorylation. Similar to what was observed in Ptc1+/− cerebella, Gli1 expression in HEK293 cells did not block the IR-induced phosphorylation of two ATM substrates, Chk2 and SMC1 (Fig. 2 A), thus demonstrating that Gli1 selectively blocks the ATR–Chk1 but not the ATM–Chk2 pathway. Notably, a Gli1 mutant (Gli1ΔZFD) lacking the five zinc finger DNA-binding domains did not activate a Gli1-dependent promoter (not depicted) and did not suppress IR-induced Chk1 phosphorylation (Fig. 2 C), suggesting that Gli1 mediates its effect via transcription.

Although our results demonstrated that ATM phosphorylation of Chk2 and SMC1 was not compromised in Gli1-expressing cells, it was possible that Gli1 prevented ATM from generating the ssDNA intermediates that activate ATR. To address whether Gli1 affected the pathway upstream or downstream of ATR, we treated Gli1-expressing HEK293 cells with hydroxyurea, an agent that blocks DNA replication and induces ATR-mediated Chk1 phosphorylation that, unlike IR, does not require ATM (Feijoo et al., 2001). As was observed with IR, hydroxyurea–induced Chk1 phosphorylation was attenuated by Gli1 expression (Fig. 2 B). These results further confirm that Gli1 selectively disrupts the ATR–Chk1 signaling pathway in a manner distinct from ATM activation.

Gli1 interrupts the ATR-Chk1 pathway downstream of ATR

We next asked where Gli1 affected the Chk1 activation pathway. One of the first events in the pathway is the recruitment of the 9-1-1 complex to chromatin. As shown in Fig. 2 C, Gli1 did not affect the expression of Hus1, Rad1, and Rad9, the proteins that assemble into the 9-1-1 clamp (Volkmer and Karnitz, 1999), nor did it affect the expression of Rad17, the clamp loader for the 9-1-1 clamp (Zou, 2007). Consistent with these findings, Gli1 did not block the IR-induced binding of Rad9 (Fig. 2 D), a readout for the loading of the 9-1-1 complex at sites of DNA damage (Burtelow et al., 2000; Zou et al., 2002). Further examination of the ATR–Chk1 signaling pathway revealed that Gli1 expression also did not affect the levels of ATR or TopBP1 (Fig. 2 C),
nor did Gli1 block the IR-induced phosphorylation of RPA32 (Fig. 2 C), another ATR substrate (Olson et al., 2006). These results demonstrate that Gli1 does not block the early steps of the ATR signaling pathway or the ability of ATR to phosphorylate RPA32.

The ability of Gli1 to selectively disrupt Chk1 phosphorylation was similar to what has been observed when Claspin, a Chk1 adaptor protein, is depleted from human cells or Xenopus laevis egg extracts (Chini and Chen, 2004). Given these similar effects, we asked how Gli1 expression affected Claspin. Similar to the other checkpoint proteins, Claspin levels were not changed by Gli1 expression (Fig. 2 C) in HEK293 cells or by Ptc1 status in mouse cerebella (Fig. S1 A). To further probe the effect of Gli1 on Claspin, we also assessed the impact of Gli1 expression on the DNA damage-induced interaction between Claspin and Chk1, an interaction that is critical for ATR-mediated Chk1 phosphorylation. Interestingly, we found that the IR-induced binding of Chk1 to Claspin (Fig. 2 E) was abolished by Gli1, suggesting that this transcription factor specifically disrupts the ability of Claspin to inducibly bind Chk1.

The Chk1-dependent activation of the S-phase checkpoint is disrupted by Gli1
To show the functional significance of Gli1-mediated disruption of Chk1 signaling, we examined activation of the S-phase checkpoint, a Chk1-dependent transient inhibition of DNA synthesis that is detected as a reduction in [3H]thymidine incorporation into replicating DNA after IR treatment (Chen and Sanchez, 2004). As a positive control for these experiments, we used caffeine, an ATM/ATR inhibitor that disrupts the S-phase checkpoint in irradiated cells (Sarkaria et al., 1999). Consistent with reduced Chk1 phosphorylation in Gli1-expressing HEK293 cells, Gli1-expressing cells were as defective as caffeine-treated cells in activation of the S-phase checkpoint (Fig. 3 A).

Shh pathway activation sensitizes cells to IR
Chk1 facilitates the survival of irradiated cells, in part, by activating the S-phase checkpoint, preventing the progression from G2 to M phase, and activating DNA repair (Chen and Sanchez, 2004). Therefore, to further show that the reduced Chk1 phosphorylation seen in cells with an activated Shh pathway affects cellular responses, we examined cell survival after IR treatment using two cell systems. Irradiated mouse fibroblasts isolated from Ptc1+/− embryos demonstrated reduced survival in clonogenic assays compared with wild-type fibroblasts (Fig. 3 B). Similarly, transient expression of full-length Gli1 but not the zinc finger mutant Gli1ΔZFD decreased clonogenic survival in irradiated HEK293 cells (Fig. 3 C). Collectively, these results demonstrate that hyperactivation of the Shh pathway in mouse brains and HEK293 cells diminishes Chk1 phosphorylation and disrupts downstream cellular events that are dependent on Chk1.

Gli1 expression enhances IR-induced chromosome aberrations
Cells from BCNS patients show increased rates of IR-induced chromosome aberrations after radiation (Featherstone et al., 1983; el-Zein et al., 1995; Shafei-Benaissa et al., 1995, 1998). Similarly,
Chk1 pathway is particularly important during these phases of the cell cycle, perhaps because ATR and Chk1 participate in the repair of DSBs by homologous recombination, a repair pathway that is particularly important during S and G2 (Petermann and Caldecott, 2006). Consistent with previously published findings, which showed that various components of the Chk1 pathway contribute to genomic stability (Liu et al., 2000; Weiss et al., 2000; Wang et al., 2003; Lam et al., 2004; Syljuasen et al., 2004; Durkin et al., 2006; Pandita et al., 2006), the present results demonstrate that hyperactivation of the Shh pathway contributes to genomic instability by attenuating Chk1 activation.

In the present study, we first demonstrate that there is a selective reduction in Chk1 activation in the cerebella of irradiated $\text{Ptc1}^{+/-}$/H11002 mice. We then show that expression of Gli1 in HEK293 cells phenocopies the reduced IR-induced Chk1 phosphorylation that was observed in irradiated $\text{Ptc1}^{+/-}$ mouse cerebella. Finally, we demonstrate that Gli1 disrupts the ATR–Chk1 pathway at the level of the IR-inducible interaction of Claspin and Chk1, and we show that this disruption in Chk1 activation leads to S-phase checkpoint defects and increases the accumulation of IR-induced chromosome aberrations. These results therefore suggest that Shh pathway activation may contribute to disabling the ATR–Chk1 pathway at the level of Rad17, Rad9, ATR, or Chk1 leads to genomic instability (Weiss et al., 2000; Liu et al., 2000; Wang et al., 2003; Lam et al., 2004; Syljuasen et al., 2004; Durkin et al., 2006; Pandita et al., 2006). Because Shh signaling disrupts IR-induced Chk1 phosphorylation, we reasoned that Shh pathway activation might also affect the accumulation of IR-induced chromosome aberrations. To test this hypothesis, we expressed exogenous Gli1 in HEK293 cells. As a control, we also depleted Chk1 with siRNA. 24 h after irradiation, we analyzed the chromosomal aberrations by scoring for chromosome type, chromatid type, and other (dicentrics, rings, anaphase bridges, and pulverized chromosomes) aberrations (Fig. 4A). Overall, there were large increases in all types of IR-induced aberrations in cells expressing Gli1 or depleted of Chk1 compared with control irradiated cells. Notably, however, when broken down by aberration type, both Gli1 expression as well as Chk1 depletion skewed the distribution of chromatid- versus chromosome-type aberrations so that chromatid-type aberrations became the dominant type of lesion when Chk1 signaling was attenuated by Gli1 expression or Chk1 depletion. Because chromatid breaks occur when cells are irradiated during S and G2, the results agree with previous studies showing that the ATR–Chk1 pathway is particularly important during these phases of the cell cycle, perhaps because ATR and Chk1 participate in the repair of DSBs by homologous recombination, a repair pathway that is particularly important during S and G2 (Petermann and Caldecott, 2006). Consistent with previously published findings, which showed that various components of the Chk1 pathway contribute to genomic stability (Liu et al., 2000; Weiss et al., 2000; Wang et al., 2003; Lam et al., 2004; Syljuasen et al., 2004; Durkin et al., 2006; Pandita et al., 2006), the present results demonstrate that hyperactivation of the Shh pathway contributes to genomic instability by attenuating Chk1 activation.

In the present study, we first demonstrate that there is a selective reduction in Chk1 activation in the cerebella of irradiated $\text{Ptc1}^{+/-}$ mice. We then show that expression of Gli1 in HEK293 cells phenocopies the reduced IR-induced Chk1 phosphorylation that was observed in irradiated $\text{Ptc1}^{+/-}$ mouse cerebella. Finally, we demonstrate that Gli1 disrupts the ATR–Chk1 pathway at the level of the IR-inducible interaction of Claspin and Chk1, and we show that this disruption in Chk1 activation leads to S-phase checkpoint defects and increases the accumulation of IR-induced chromosome aberrations. These results therefore suggest that Shh pathway activation may contribute to...
Gli1 or Gli1 Δ ZFD (EGFP-Gli1 Δ ZFD) expression vectors. Drug-resistant cells stably expressing EGFP-Gli1 were derived by Lipofectamine 2000 using a 225-V, 20-ms pulse. Luciferase (control) and Chk1 siRNAs were (Cell Signaling Technology); Cdc25A (Thermo Fisher Scientific); RPA32, were electroporated as described previously (Volkmer and Karnitz, 1999). HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented, allowed to adhere, and irradiated with a Shepard 137 Cs-irradiator. HEK293 or mouse embryonic fibroblast (MEF) cells were plated in 6-well dishes, allowed to adhere, and irradiated with a Shepard 137 Cs background.

The incidence of IR-induced medulloblastoma was assessed after irradiation of wild-type and Ptc1+/− mice with 3 Gy IR. Animals displaying atomic, poor grooming, or severe malaise were killed, and brains were examined for medulloblastomas. Each brain was removed and examined for the presence of tumor. All mice that died during the period of observation had a grossly apparent brain tumor.

Cell culture and transfection

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For plasmid transfections, cells were electroporated as described previously (Volkmer and Karnitz, 1999) using a 225-V, 20-ms pulse. Luciferase (control) and Chk1 siRNAs were described previously (Volkmer and Karnitz, 2000) and transfected by electroporation using the same conditions used for plasmid transfections. HEK293 cells stably expressing EGFP-Gli1 were derived by Lipofectamine 2000 (Invitrogen) transfection with empty vector (pEGFP-C3) or the Gli1 (EGFP-Gli1) or Gli1 Δ ZFD (EGFP-Gli1 Δ ZFD) expression vectors. Drug-resistant cells were selected in 500 μg/ml G418; >95% of the G418-resistant population was positive by GFP expression.

Clonogenic analysis

HEK293 or mouse embryonic fibroblast (MEF) cells were plated in 6-well dishes, allowed to adhere, and irradiated with a Shepard 137 Cs-irradiator. After 14 d, the plates were stained with crystal violet and colonies containing >25 cells were counted.

5-phase checkpoint and chromosome analyses

The intra-S-phase checkpoint was analyzed as described previously (Roos-Snelling et al., 1999). Rad9 chromatin binding was performed as described previously (Burtelow et al., 2000). The Claspin-Chk1 interaction was analyzed in HEK293 cells that transiently expressed S- and flagged-tagged Claspin as described previously (Chini and Chen, 2006).

HEK293 cell cycle analysis

HEK293 cells were transfected with empty vector or the EGFP-Gli1 expression vector. We also cotransfected an EGFP–histone H2B expression vector because fusion of EGFP to histone H2B anchors the EGFP in cells. This allowed us to analyze the cell cycle of the transfected cells and maintain the EGFP signal. 24 h after transfection, the cells were permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100, 50 μg/ml propidium iodide, and 10 μg/ml heat-treated RNase A; incubated 30 min at 30°C; and analyzed for DNA content by flow cytometry.

Cerebellar granule cell cycle analysis

Cerebella were isolated and the cells were dissociated as described previously (Wang et al., 2000). The dissociated cells were prepared for cell cycle analysis using the Cycletest Plus kit (Becton Dickinson) and analyzed by flow cytometry to detect DNA content.

Online supplemental material

Fig. S1 shows that IR-induced Chk1 phosphorylation on Ser245 is disrupted in Ptc1+/− cerebella and in HEK293 cells expressing Gli1. Additionally, Fig. S1 shows that Claspin levels are not altered in Ptc1+/− cerebella. Fig. S2 demonstrates that cells from Ptc1+/− cerebella do not have an altered cell cycle compared with wild-type cells and that expression of EGFP-Gli1 does not affect cell cycle distribution in HEK293 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200804042/DC1.

We gratefully acknowledge Scott H. Kaufmann for insightful discussions, Junjie Chen for providing the SFB:Claspin expression vector and the Rad51 antibody, the Mayo Clinic Flow Cytometry/Optical Morphology Resource and the Cytogenetics Shared Resource for technical assistance, and Pam Becker for manuscript preparation.

This work was supported by CA084321 (L.M. Karnitz), the Mayo Clinic Foundation (L.M. Karnitz and C. Wetmore), the Sontag Foundation, the Bernard and Edith Watermann Foundation for Cancer Genetics (C. Wetmore), and a Bonner Predoctoral Fellowship (J.M. Leonard).

Submitted: 8 April 2008
Accepted: 1 October 2008

References

Abraham, R.T. 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. 15:2177–2196.
Babu, J.R., K.B. Jeganathan, D.J. Baker, X. Wu, N. Kang-Decker, and J.M. van Deursen. 2003. Rael is an essential mitotic checkpoint regulator that cooperates with Bub3 to prevent chromosome missegregation. J. Cell Biol. 160:341–353.
Bakkenist, C.J., and M.B. Kastan. 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature. 421:499–506.
Bartek, J., and J. Lukas. 2003. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell. 3:421–429.
Bartkova, J., Z. Horejsi, K. Kodl, A. Kramer, F. Tort, K. Ziegler, P. Friedburg, M. Sehested, J.M. Nesland, C. Lukas, et al. 2005. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 434:864–870.
Burtelow, M.A., S.H. Kaufmann, and L.M. Karnitz. 2000. Retention of the hRad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage. J. Biol. Chem. 275:26343–26348.
Chen, Y., and S. Sanchez. 2004. Chk1 in the DNA damage response; conserved roles from yeasts to mammals. DNA Repair (Amst). 3:326–344.
Chen, C.C., and J. Chen. 2004. Claspin, a regulator of Chk1 in DNA replication stress pathway. DNA Repair (Amst). 3:1033–1037.
Chen, C.C., and J. Chen. 2006. Repeated phosphopeptide motifs in human Claspin are phosphorylated by Chk1 and mediate Claspin function. J. Biol. Chem. 281:33276–33282.
